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The Effect of Aquatic and Alcoholic Extracts of *Citrullus colocynthis* on Growth of the *Saprolegnia parasitica*

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Abstract: *Saprolegnia parasitica* is an opportunistic pathogenic fungus that attaches to the dead eggs and damaged tissues, grows and leads to Saprolegniosis in fish. Since chemical compounds that are used in prevention and treatment of Saprolegniosis infection causes cancer and is harmful to human's health, in this research, for alternative materials that affects *S. parasitica*, the aquatic and alcoholic extracts of *Citrullus colocynthis* were tested on this fungus. *S. parasitica* has developed completely in aquatic and ethanolic extract of *C. colocynthis* by disk diffusion method, but it showed good sensitivity against methanolic extract. This plant has a very little sensitivity against aquatic and ethanolic extract by well method. The methanolic extract of *C. colo*cynthis showed more sensitivity on *S. parasitica* and its MFC and MIC were determined 25×10^3, 625×10 mg/ml respectively. Thus, we can hope that in future the *C. colocynthis* extract can be used as a good substitution of Malachite green to treat Saprolegniosis in freshwater fish (especially rainbow trout salmon).

Key words: *Saprolegnia parasitica* % *Citrullus colocynthis* % Antifungal

INTRODUCTION

Fish breeding, particularly the cellar fish, is being done due to rapid increasing population and the need to supply the protein requirement of human communities in most parts of the country. On the other hand, salmon trout is one of the most important species to produce protein in aquaculture industry and one of the most important goals of fishery is to increase production efficiently. Meanwhile one major obstacle in production is that the fungal disease appears among fish and their eggs. Saprolegniosis family, especially *Saprolegnia* genus member, is a factor of important infections that causes economical damages and deaths to fishes, especially salmon trout and their eggs in aquaculture industry [1]. In India, 1980, Srivastava identified 21 fungi of *S. ceous* family from 3 geniuses of *Aphanomyces, Achlya* and *S. parasitica* among 19 species of fish [2]. According to a high value of trout and their eggs, the direct economic loss is significant which is caused by the indirect losses made by use of materials such as Malachite green that is used to treat fungal eggs despite their carcinogenic and malformation nature. This loss may lead to environmental problems and polluted chemical and pharmaceuticals that can enter the nature cycle. Although other several treatments (except Malachite green) aroused, such as using potassium permanganate, hydrogen peroxide, formalin and sodium chloride, according to their chemical nature each of them has special abuse aspects. Since both Malachite green and Formalin have carcinogenic natures and Formalin has environmental effects, stability in environment and harmful effects on people who work with it, they are not used. FDA has banned the use of Malachite green and Formalin for free eggs of fish [1, 3, 4].

On the other hand, with promotion of global trends of green aquaculture and development of organic aqua systems in which maximum natural materials, minimum pollutant and chemical ones are used, with attention to the variety of medical plants, we used Iran's local plants to treat fungal diseases. The *C. colocynthis* is one of the old plants that are used in Iran's traditional medicine and different nations to treat many diseases. *C. colocynthisis*, a worldwide plant, that looks like watermelon from pumpkin family with rich oil and protein. *C. colocynthis* flesh contains Alkaloid compounds, Saponins,

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Glycosides, Colocynthis, Elaterin, Elatericin B, Dyhydri- Elatericin B, a glycoside phto-citrullos, fixed oil, ether-soluble resin, chloroform, gum, pectin acid (pectin), Albuminoids, calcium phosphate, magnesium, Lignin and water. Its fruit is used as a strong laxative to treat refractory edema, amenorrhea, imbalance of brain, stop menstruation in severe cases, jaundice, gut cramping, nerves pain fever, snake-bite, corns, anti-parasite (worms), open varicose veins, epilepsy, unilateral headaches, inflammation, leprosy, ophthalmic, muscle pain in hands and feet. Being anti-viral, anti-bacterial and anti-cancer effects are its other important features. Recent research shows that consumption of *C. colocynthis* with radioactive radiation (ray) has barrier effects on growth of cancerous tumors such as larynx cancer [5-8]. In 2008, Peter [9] had studied anti-bacterial effects *C. colocynthis* on seven bacteria's: *Bacillus subtilis, Escherichia coli, klebsiella pneumonia, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella typhi* and *Staphylococcus areus*. Atool, *et al*. [10] in 2003 studied immune rate assessment of *C. colocynthis* on diabetic rats. The result indicated that *C. colocynthis* in different doses is safe as an anti-diabetes [10]. In this study, anti-fungal effects of *C. colocynthis* on *S. parasitica* were investigated.

MATERIALS AND METHODS

After collecting and washing the plant, it was put it in a shade and warm atmosphere to dry and then grind it so that the extraction could be done easier and better. The extract was prepared in percolation method by using water, Ethanol 80% and Methanol 80% [11-12-13].

Aquatic Extraction: 25 grams of plant with 250cc water and heat was extracted in percolation method and then it was placed in oven at 45°C to completely remove the water and dry the extract [14].

Ethanolic and Methanolic Extraction: 25grams of plant with 250cc ethanol 80% and separately methanol 80% was extracted the same as previous method.

Preparation of Soluble Extract: Half grams of variety of dried extracts and four and a half cc of sterile distilled water were placed in a sterile tube. 1/10 solution was obtained that has 10^5 micrograms of effective material per cc.

Determining the Fungal Sensitivity to Extract by Using Disks: 40,50,60 and 708 amounts of dilution that were prepared from each extract, were placed on standard disks, after drying in oven at 45°C and marking, they were put in sabouraud dextrose agar culture of certain distances which were prepared by fungal suspension of uniform culture. Presence and absence of growth inhibition zone around them were determined after 48-72 hours incubation at 25-30°Cfor the fungus [15-18].

Determining the Fungal Sensitivity to Extract by Using Wells: At first four wells were created with equal and certain distance in sabouraud dextrose agar culture and then 80, 90, 100, 1108 amount of extract were added; after for 3 to 4 hours until the extract was completely penetrated around well, the fungal suspension of uniform culture was used. Growth and lake of growth around well were studied after 48-72 hours incubation at 25-30°C [19].

Determining the MIC of Extract on Fungus: Eleven sterilized tubes containing 1cc sabouraund dextrose broth culture were used; one cc1/10 dilution was entered to the first tube, so the amount of effective material in first tube was 5×10^4 mc/ml. Then, 1/2 dilution of that was prepared in next tubes so that by sterile sampler tap 10008 was taken from first tube and entered to the second tube. The amount of effective material in second tube proved to be 25×10^3 mc/ml. To prepare related serials, you should consider that after 1 cc content of ninth tubes entered to tenth tube, 1 cc content of tenth tube, should be thrown away and nothing is added to eleventh and it is kept as control sample of growth. In the next step, we had to add a fixed amount of experimental fungus to the fixed amount of 508 to all related tubes. These tubes were incubated for 48-72 hours at 25-30°C, then darkness of control sample tube with eleventh tube and finally the results were analyzed [15, 20-21].

Determining the MFC of Extract on Fungus: To determine the minimum fungal concentration (MFC), 108 of MIC and other tubes without darkness was taken were cultured and in glucose-peptone agar atmosphere, after the required time, CFU was determined in plates, thus, the clones were counted and multiplied by 100. So, we could get the number of the germs in plates. The least bacterial concentration that was less than one thousandth of raw number was considered as MFC [11- 22].

Table 1: The mean of growth inhibition zone diameter (mm) against different amounts of aquatic, ethanolic and methanolic extract *C. colocynthis* by disk method and well method

Methods	Cons.	Aquatic extract	Etanolic extract	Metanolic extract
Disc	40 8	+	+	11.13
	50 8	+	+	13.33
	60 8	+	+	14.16
	70 8	+	+	16.13
Well	80 8	+	+	12.13
	90 8	+	+	13.16
	1008	+	+	14.16
	110 8	+	+	15.26

Table 2: Determine MIC and MFC of *C. colocynthis* extract on *S. parasitica*

Methanol extract		Ethanol extract		Extract	
MFC	MIC	MFC	MIC	MFC	MIC
25×10^5	625×10^1	+	+	+	+

*MIC and MFC are according to mg/ml and the results determine approximately the mean
of three times examination.

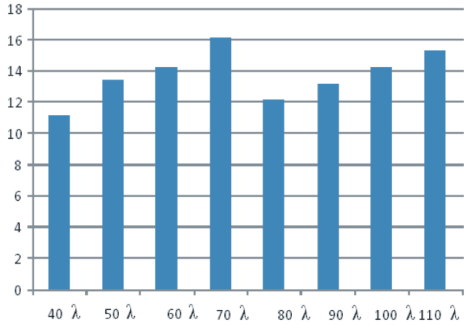

Fig. 1: Comparison between the disk and well of zone diameter of methanol extract of the *C.colocynthis*

RESULTS

The Results of Aquatic, Ethanolic and Methanolic Extraction of *C. Colocynthis* on *S. Parasitica* Disk Diffusion Method: *S. parasitica* was grown completely against aquatic, ethanol and methanolic extract of *C. colocynthis* in disk diffusion method. But it showed a good sensitivity against methanolic extract of *C. colocynthis*. The mean of growth inhibition zone diameter containing 40, 50, 60 and 708 methanolic extract by disk reached 11, 13, 14 and 16 mm, respectively (Table 1).

The Results of Aquatic, Ethanolic and Methanolic Extraction of *C. Colocynthis* on *S. Parasitica* by Well Method: *S. parasitica* have a very little sensitivity against aquatic, ethanolic and methanolic extract of *C. colocynthis* in well method. But it showed a good sensitivity against methanolic extract of *C. colocynthis*. *S. parasitica* against well possessing 110, 100, 90 and 808 created the growth inhibition zone with 15, 14, 13 and 12 diameter respectively (Table 1). In both well and disc methanol, zone diameter with increasing concentrations of methanol extracts *C.colocynthis* increases inhibition zone diameter (Fig 1).

The Results of Determining the MIC and MFC of Aquatic, Ethanolic and Methanolic Extract of *C. Colocynthis* on *S. Parasitica*: Methanolic extract showed more sensitivity on *S. parasitica* and its MIC equals with tube number 4 i.e. 625× 10 mc/ml and MFC equals with tube number 3 i.e. 25×10^3. But ethanolic and aquatic extracts had no sensitivity on *S. parasitica* and the fungus grew completely. This shows that methanolic extract of *C. colocynthis* has an inhibitory effect via killing fungus and therefore is fungicidal. On the other hand, fungicidal effects are helpful to treat fungal infections better and faster (Table, 2).

DISCUSSION

Trout (*Oncarhynchus Mykiss*) is reproduced in many aquacultures in Iran and one of the major obstacles in artificial breeding stage is the fungal disease that appears among trout and their eggs. The common treatment is the use of Malachite green. Malachite green, despite the known risk and harms such as teratogenic effects and mutagenicity, is used as an effective fungicidal due to its high efficiency in controlling a fungal infection. But at the same time, America's FDA prohibits the use of it and also its use has been banned in many other countries [4, 1]. The remarkable point in this study is that the antifungal effect of methanol extract of *C. colocynthis* was higher comparing to that of aquatic and ethanolic extract. This can indicate that the effective and active materials in *C. colocynthis* in methanol have a higher dissolving power. Therefore, it seems that in supplementary studies or for medicinal purposes, using methanolic extract of *C. colocynthis* is much more appropriate than the aquatic and ethanolic extract. The plants which contain compound such as Alkaloid, Flavonoid, Tannins, Saponin and Glycoside proved to have higher antifungal effects [6, 23-25]. Usman *et al*. [8] in 2003 had an anti-bacterial test on *C. colocynthis*. They tested the methanolic extract of fruits, roots and stems of *C. colocynthis* on negative and positive gram bacilli. The survey results indicated that fruit and root extract on

some bacterial like staphylococcus against more concentration of extract have a better answer than other positive gram bacteria, whereas these extracts have no effect on negative gram bacilli. Rohani et al. [24] in 2006 announced that *Zataria Multiflora* is an appropriate alternative of Malachite green. This plant is very effective on 25, 50, 100 ppm days. Khomavilaai [3] in 2006 announced that mustard extract has isotiosianat Alyl and can stop the *S. parasitica* growth with concentration of 68 mg/l for 60 minutes in MIC method. Also to stop zoospore germination, the MIC should be 42/5 mg/l; therefore, mustard extract is the effective antifungal factor against Saprolegniosis. Rajamonickam *et al*. [26] in 2010 evaluated the anti-inflammatory activity of *C. colocynthis*. This plant is used in biological activities in traditional treatment system. In the study, the various effects of *C. colocynthis* on inflammations, creative factor of leg edema and pulmonary inflammation on rats have been studied. The survey results indicated that pharmacologists succeeded to confirm the effect of *C. colocynthis* as an anti-inflammation factor. Marzouk *et al*. [22] studied the anti-bacterial activity of a mature fruit and *C. colocynthis* seed against positive and negative-gram bacteria which unripe extract inhibited the growth of all bacteria (*Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi murium, Vibrio parahaemolyticus and Vibrio alginolytiws*) and gram positive (*Enterococcus faecalis, Staphylococcus areus, Staphylococcus epidermidis, Listeria monocytognes and Micrococcus luteus*) and various *Candida spp*. (*C. glabrata, C. albicans, C. parapsilosis and C. kreusei*). In the current study, *S. parasitica* was resistant against aquatic ethanolic extract in disk diffusion and well method, but it showed good sensitivity against methanolic extract. MIC and MFC determined 625×10 and 6×1000 microgram per ml, respectively. To prove that *C. colocynthis* is effective on *S. parasitica*, this extract can be used as suitable alternative of malachite green to treat the *S. parasitica* sis fish (especially rainbow trout). To study for the study of pharmacological effects of *C. colocynthis* and its effective treatment doses, doing more research In vitro and In vivo condition seems to be necessary.

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