Isolation of Saprolegnia and the Influence of Root Ethanolic Extract of *Ruta graveolens* on *Saprolegnia. Spp* Growth

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Abstract—**Aquatic fungi of the genus saprolegnia often cause serious damage to fresh water fish such as rainbow trout. Malachite green is quite an effective antifungal agent but it is teratogenic, mutagenic and carcinogenic. Other antifungal agents like hydrogen peroxide, formalin, sodium chloride, have some negative points, so an alternative antifungal agent is needed to be effective and safe, not synthetic but natural substance like plant metabolits. Ruta graveolens is an herbal plant which is traditionally used as an energizer and an anti-bleeding to heal injuries. Many of Ruta species contain various natural components that are anti-fungal and phytotoxic. In this experimental research antifungal effect of ethanolic extract of Ruta graveolens root on saprolegnia, in vitro by diffusion tests (disk and well) and dilution tests (MIC and MFC nomination) was studied. It was shown that root ethanolic extract of Ruta graveolens had antifugnal effects and prevented from saprolegnia growth .MIC of ethanolic extracts was determined to be 25×10^3 µg/ml.**

Index Terms—***Saprolegnia*, Ruta graveolens, Antifungal effect**

I. Introduction

Fungal infections of fish by Oomycetes, commonly known as water molds, are widespread in fresh water and represent the most important fungal group affecting wild and cultured fish. The Saprolegniacea, in particular members of the genus saprolegnia, are responsible for significant infections involving both living and dead fish and eggs especially in aquaculture facilities. Infections due to saprolegnia species were reported from many commercial fish hatcheries especially in salmonid species and channel catfish [1].Characterization of fish pathogenic Saprolegnia is useful for the furtherance of epidemiological studies of the source of infection, disease transmission, disease spreading and control of the disease. Some morphological and physiological studies have made it possible to identify different species and subgroups of Saprolegnia isolates. Variation in esterase isoenzyme pattern, difference in radial growth rate and different biochemical characteristics have been used for determination of distinct species and groups of Saprolegnia isolates [1]. Saprolegniasis is one of the main types of fungal diseases in fresh water fishes such as rainbow trout, which causes considerable economic problems in the fish farming industry, infecting both fish and fish eggs [2]. Saprolegniasis in fish usually starts as a cotton wool like, white to dark gray or brownish growth over the head region or dorsal fin of fish and then spread all over the body [3]. In the past, this problem was solved with the extremely effective fungicide, malachite green. But recently it has proved that malachite green is carcinogenic, mutagenic and teratogenic. Carcinogenic substances are agents capable of causing cancer. Mutagenic substances can cause changes in amount or chemical structure of DNA resulting in changes in the characteristics of an organism or an individual cell. Teratogenicity means the cabability of a substance to cause malformations during embryonic development [4], [5]. Meinertz et al concluded that undetectable residues of malachite green would still remain in fish grown from eggs which had been exposed to the chemical so that they cloud reach market size [6]. And, therefore, malachite green has been banned in the United States and some other countries. It has been banned in USA since 1991, in Italy since 1994 and in Iran since 2003[7]. Other antifungal agents are effective but have some negative points. For example, formalin is potentially harmful to the user's health and remains in the environment. Hydrogen peroxide, the undiluted solution is strongly corrosive and combustible and the effective concentration of which is as high as 1000 mg/L, is not permitted in the USA. Sodium chloride, in spite of its safety may be limited in its applicability due to the high cost of acquiring effective concentration of which is as high as 30,000 mg/L [8], [9]. Zaki et al reported that potassium permanganate is a strong antifungal agent to prevent saprolegniasis in fish [3]. Furthermore, an alternative antifungal agent is needed to be safe, not synthetic chemical, but natural substances like plant metabolites. Ruta graveolens, commonly known as Rue, which belongs to the family Rutaceae. It is an herbaceous perennial, which is originally native to the Mediterranean region, but it is believed that it traditioneiiy belongs to north of Iran. It is now cultivated in many parts of the world. It has blue- green foliage and yellowflowers. It is used as an energizer and antibleeding to heal injuries [10], [11]. Ruta graveolens contains many secondary metabolites such as furocoumarins, furoquinolines and acridone alkaloids, mainly present in the leaves, especially before blooming. The volatile rue oil has a pungent smell and bitter taste, and possesses antibacterial activity against Micrococcus pyogenes var aureus and Escherichia coli. It is used as anthelmintic, antispasmodic, antiepileptic, rubefacient and emmenagogue in veterinary medicine and,

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when obtained from steam distillation of fresh material to encourage the onset of menstruation. In addition it is utilized as a scent in perfumes and soaps as it is rich in methyl-nonyl-ketone which is used in the preparation of methyl-n-nonyl-acetaldehyde, widely used in synthetic perfumes [12]. Many of Ruta species contain various natural components that are anti- fungal and phytotoxic [13]. The ethyl acetate extract of Ruta graveolens leaves have anti-fungal effects [14]. Thus, the present investigation was carried out to evaluate antifungal effects of ethanolic extract from *Ruta graveolens* roots on Saprolegnia.spp.

II. Materials and Methods

A. Preparing Extract

Root of Ruta graveolens were collected, washed with clean water for several times, and then dried in shade. Dried roots of Ruta graveolens were reduced to a fine powder with a mechanical grinder an then its extract was obtained by percolation method using 80% ethanol and a rotary evaporator.0.5 g of dried ethanolic extracts was resolved in 45^{occ} of sterile distilled water, so the resulting solution dilution would be 1/10, that in, any 1cc of the solution contains 10^5 microgram of the blend.

B. Isolation of Saprolegnia

Water samples were collected from rainbow trout fish culture farms in Mazandaran, a Province in northern Iran. The water samples were cultured on Glucose peptone agar contain peniciline G and streptomycine, which were incubated at 25^{oc} for 4-5 days. After the incubation period, the developing colonies were examined.Saprolegnia was characterized by external, cotton- like appearance. It was shown that under the microscope through using lactophenol cotton blue (LCB), Saprolegnia had filamentous mycelium, hyphae was hyaline, broad and coenocytic.

C. Preparing Fungal Suspension

The colony of Saprolegnia was added to sterile distilled water and then Tween20 was added to it. It would help to separate spore from mycelium and the isolated spores were used for susceptibility tests.

D. Susceptibility Test of Saprolegnia to the Root Ethanolic Extracts by Disk Diffusion

40, 50, 60 and 70 microliter of ethanolic extract were poured separately on standard disks; they were put in an oven at 45^{oc} to drain. Saprolegnia was cultured at Glucose pepton agar medium without antibiotics. Drained disks were added to the medium. After incubation at $25\text{-}30^{oc}$ for a few days, the presence of inhibiting halo of growth was studied. This test repeated for three times and the mean of inhibiting halo diameter was determined after the third time repeat.

E. Susceptibility Test of Saprolegnia to the Root Ethanolic Extracts by Wells

In the Glucose peptone agar medium (without antibiotics), four wells were made and 80, 90, 100 and 110 microliter of extracts were poured in the wells. Then, the spores of Saprolegnia were cultured in the medium, after incubation at $25\text{-}30^{oc}$ for a few days, The presence of inhibiting halo of growth was studied. This test was repeated for three times and the mean of inhibiting halo diameter was determined after the third time.

F. MIC and MFC Determination of Root Ethanolic Extracrs on Saprolegnia

The broth macrodilution method was performed to determine MIC. Minimum inhibitory concentration(MIC) refers to the lowest concentration of antimicrobial agent that inhibits fungal growth or multiplication and MFC refers to the lowest concentration of antifungal agent that allows less than 0.1% of the original inoculums to survive. The broth macrodilution method was performed using 11 sterile tubes that in each tube was poured 1cc tripticase soy broth. Then 1000 microliter of ethanolic extract was poured to the first tube and respectively 1000 microliter from the first tube was taken out and poured in to the second tube. This action was continued up to 10th tube, 1000 microliter of 10th tube was discarded, since the 11th tube is blank tube (Table 3). In the next step, 20 microliter of the suspension, which contains the spore of fungi, was added to each tube. To prepare microbial suspension, it was necessary to point the transmittance of spectrophotometer to 90% with the wave 20 length of 520nm, therefore in 1cc of the suspension there would be 10^6 fungal spores. After few days incubation at $25\text{-}30^{oc}$, turbidity in tubes was compared with that of the blank tube and MIC was determined. In order to determine MFC, 10 microliter of non-turbid tubes was subcultured in Glucose pepton agar plates and was incubated at 25-30°c for 24-48h. Then CFU on plates was determined; the lowest concentration of extracts that allows the survival of less than 0.1% of the original fungal inoculums would survive was reported as MFC [15], [16].

III. Results

A. Diffusion by Disk

Saprolegnia was susceptible to ethanolic extract of roots and therefore an inhibiting halo of growth was observed (Fig. 1). With 70 microliter of ethanolic extract the created halo was 17mm in diameters (Table I).

B. Diffusion by Wells

Saprolegnia was susceptible to ethanolic extract of roots (Fig. 2). With 110 microliter of ethanolic extract, the created halo was 30mm in diameters (Table II).

C. MIC and MFC Determination

Saprolegnia was susceptible to ethanolic extracts of roots and MIC was determined to be $25\times10^3\mu g/ml$ and also MFC was determined to be $25\times10^3\ \mu g/ml$.

IV. Discussion

Fungal infections are uncommon when water quality is good. Uneaten food and a general decline in water condition will promote fungi and lead to higher rates of infection. Saprolegnia generally invades fish that have been stressed or otherwise have a weakened immune system, because immunosupression provides a mechanism that causes the

transformation of normally non-pathogenic organisms, including Saprolegnia, to become pathogenic. Malnutrition among cultured fishes has been continues to be a primary cause of Saprolegniasis. There is an annual mortality rate of 50% in Coho salmon (Oncorhynchus kisutch Walbaum) due to S. parasitica [2].

In this study BioCare by using Ruta graveolens root extracts turned out to be the best candidate substance in order to treat Saprolegniasis. Many Ruta species are sources of diverse classes of natural products with biological activities including antifungal, phytotoxic and antidotal activities .Previously, the presence of antifungal agents against some agriculturally important fungi in the ethyl acetate extract of Ruta graveolens leaves has been demonstrated [14]. In Ruta graveolens, the existance of saponin, thnnin, alkaloid and glycosid has been proved. Saponin has soap characterstics and its anti- fungal effect has been tested. Tannin sediments contain microbial proteins [17].

In the study of Meepagala et al, (2005), the ethylacetate extract from Roots of Ruta graveolense had shown fungicidal activity against several agriculturally important pathogenic fungi like colletotichum fragariae, C.gloeosporioides, C.acutatum, Botrytis cineara and Fusarium oxysporium. They found that Rutacridone epoxide was the bioactive constituent from the ethylacetate extract of Ruta graveolens roots which showed fungicidal activity. Rutacridone epoxide also showed significantly higher fungicidal activity than commercial fungicides, captan and benomyl [17]. In our study ethanolic extract from Ruta graveolens roots had good antifungal effects on saprolegnia, and MIC was equal to MFC. We found that, ethanolic extract of Ruta's Roots have fungicidal activity against Saprolegnia. Numbers are millimeter-based. The Mean ± (SD) diameter of halo was determined after three times repeating the experiment. In the study of khomvilai et al(2006), Fungicidal activity of horse radish extract on Saprolegnia parasitica was investigated and they reported the MIC for mycelia growth was 68 mg/L with 60- min exposure and MIC for Zoospore germination was 42.5mg/l with 5- min exposure [9]. In our study MIC was 25×10^3µg/ml and these values were significantly lower than those reported by khomvilai.

In the study by Rohani et al (2006), they reported that zataria multiflora is a new challenge substitution of Malachite Green. The MIC result of zataria essence against Saprolegnia was 0.9 and against fusarium was 1.4 ppm [18]. In the study of El-Sheekh (2008), methanol extracts of Anabaena wisconsinense and Oscillatoria curviceps had antifungal effects against A.niger and Saprolegnia parasitica [19]. In the study by Endler et al (2008), they reported that the accumulation of alkaloids and coumarins but not flavonoids was enhanced in Ruta graveolens suspension cultures upon the addition of fungal elicitor [20]. Mousavi et al reported that the combination of essential oils may be apromising antifungal agent in aquaculture and combination of essential oils had greater antimicrobial activity than their individual components [7]. Due to the antifungal effect of Ruta graveolens roots against Saprolegnia, in future extracts of Ruta graveolens roots will be an effective substitution of Malachite green to treat Saprolegniasis in fresh water fishes like rainbow trout. We suggest in future investigating combination of Ruta graveolens and other plants in order to have greater antifungal activity than their individual components.

TABLE I: DIAMETER OF HALO IN VARIOUS AMOUNTS OF THE RUTA GRAVEOLENS EXTRACT IN DISK METHOD

Ethanolic extract				**kind of extract**
70λ	**60λ**	**50λ**	**40λ**	**(amount(λ**
17.33±1.1	13.66±1.5	12.33±0.57	11.66±0.57	*saprolegnia*

Numbers are millimeter-based. The Mean ±(SD) diameter of halo was determined after three times repeats.
Diameter of each disk was 6 mm.

TABLE II: DIAMETER OF HALO IN VARIOUS AMOUNTS OF THE RUTA GRAVEOLENS EXTRACT IN WELLS METHOD

Ethanolic extract				**kind of extract**
110λ	**100λ**	**90λ**	**80λ**	**(amount(λ**
30.33±.57	28.66±.57	26.66±.57	23.33±.57	*saprolegnia*

Numbers are millimeter-based. The Mean (SD) diameter of halo was determined after three times repeats.

TABLE III: VARIOUS AMOUNTS OF THE EXTRACT IN 11 TUBES MEASURING MIC

tubes	1	2	3	4	5	6	7	8	9	10	11
µg/ml	5×10^4	25×10^3	125×10^2	6250	3125	1562.5	781.25	390.62	195.31	97.65	0

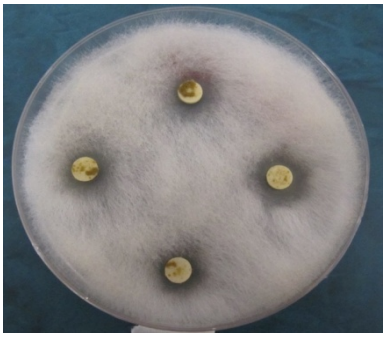

Fig. 1. Etanolic extract of *Ruta graveolens* on *Saprolegnia* in Disk method

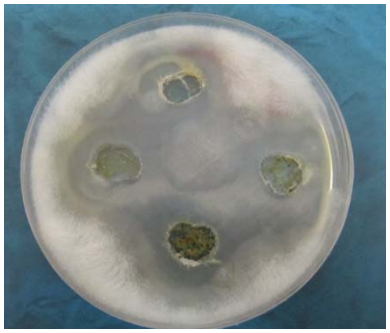

Fig. 2. Etanolic extract of *Ruta graveolens* on *Saprolegnia* in wells method

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