2011 International Conference on Biotechnology and Environment Management
IPCBEE vol.18 (2011) © (2011)IACSIT Press, Singapoore

Survey the Antifungal Effect of Root Ethanolic extract of Ruta graveolens on *Saprolegnia. Spp*

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Abstract. Aquatic fungi of the Genus *saprolegnia* often cause serious damage to fresh water fishsuch 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.In this experimental research studied antifungal effect of ethanolic extract of *Ruta graveolens* root on *saprolegnia*, in vitro by diffusion test(disk and well) and dilution test (MIC and MFC nomination).
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 25×10^3µg/mL

Keywords: Saprolegina, *Ruta graveolens,* Antifungal effect

1. Introduction

One of the main types of fungal diseases in fresh water fishes such as rainbow trout is *saprolegniasis*, which caused by species of the genus *saprolegnia*. It causes considerable economic problems in the fish farming industry, infecting both fish and fish eggs (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(16,13). 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(15). 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(16). 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 concentrations <which is as high as 30,000 mg/L (11, 12).
Zaki.M et al reported that potassium permanganate is a strong antifungal agent to prevent *saprolegniasis* in fish (22).
Furthermore, an alternative antifungal agent needs 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 yellow flowers. It is used as an energizer and antibleeding to heal injuries (19, 20). Many of Ruta species contain various natural components that are anti- fungal and phytotoxic (14)]. The ethyl acetate extract of *Ruta graveolens* leaves have anti-fungal effects (18).
Thus, the present investigation was carried out to evaluate antifungal effects of ethanolic extract from *Ruta graveolens* roots on *Saprolegnia*.sp.

2. Materials and Methods

2.1. Preparing extract:

Root of *Ruta graveolens* were collected, washed with clean water for several times, and then dried in shade. Dried roots of R. *Ruta graveolens* were reduced to a fine powder with a mechanical grinder and then its extract was obtained by percolation method using 80% ethanol and a rotary evaporator.0.5 gr of dried ethanolic extracts was resolved in 4.5cc of sterile distilled water, so the resulting solution dilution would be 1/10, that in, any 1cc of the solution contains 10^5 microgram (1/10g= 100mg= 10^5µg=10^8ng) of the blend.

2.2. 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°c for 4-5 days. After the incubation period, the developing colonies were examined.*Saprolegnia* was characterized by an external, cotton- like appearance. It was shown that under the microscope through using lactophenol cotton blue(LCB),*Saprolegnia* had filamentous mycellium , hyphae was hyaline, broad and coenocytic.

2.3. 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.

2.4. Susceptibility test of Saprolegnia to the root ethanolic extracts by disk diffusion:

40, 50, 60 and 70µl of ethanolic extract were poured separately on standard disks; they were put in an oven at 45°c to drain. *Saprolegnia* was cultured at Glucose pepton agar medium without antibiotics. Drained disks were added to the medium. After incubation at 25-30°c for afew 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.

2.5. 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 110µl of extracts were poured in the wells. Then, the spores of *Saprolegnia* were cultured in the medium, after incubation at 25-30°c for afew 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.

2.6. 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 inoculum to survive. The broth macrodilution method was performed using 11 sterile tubes that in each tube was poured 1cc tripticase soy broth. Then 1000 µl of ethanolic extract was poured to the first tube and respectively 1000 µl from the first tube was taken out and poured in to the second tube. This action was continued up to 10^{th} tube, 1000 µl of 10^{th} tube was discarded, since the 11^{th} tube is blank tube (Table 3). In the next step, 20 µl of the suspension, which contain 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

length of 520nm , Therefore in 1cc of the suspension there would be 10^6 fungal spore. After few days incobation at 25-30°c, turbidity in tubes was compared with that of the blank tube and MIC was determained.

In order to determine MFC, 10 µl 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 inoculum would survive, was reported as MFC [4, 5, 9].

3. Results

3.1. Diffusion by Disk

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

3.2. Diffusion by wells

Saprolegnia was susceptible to ethanolic extract of roots **(Figure 2).**with 110 µl of ethanolic extract, the created halo was 30mm the diameters **(Table 2**).

3.3. MIC and MFC determination

Saprolegnia was susceptible to ethanolic extracts of roots and MIC was determined to be 25×10^3µg/mL and also MFC was determined to be 25×10^3 µg/ml

4. Discussion

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 acetat extract of *Ruta graveolens* leaves has been demonstrated (Oliva 2003). 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 (aderotimi. 2006).

In the study of Meepagala et al, (2005), the ethylacetate extract from Roots of *R.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 *R. graveolens* roots which showed fungicidal activity. Numbers are millimeter-based. the Mean±(SD)diameter of halo was determined the third time repeating the experiment. Rutacridone epoxide also showed significantly higher fungicidal activity than commercial fungicides ,captan and benomyl.

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 Rota's Roots have fungicidal activity against *Saprolegnia.*

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 mycellia growth was 68 mg/L with 60- min exposure and MIC for Zoospore germination was 42.5mg/L with 5- min exposure. 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 all(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. In the study of El-Sheekh(2008), methanol extracts of *Anabaena wisconsinense* and *Oscillatoria curviceps* had antifungal effects against *A.niger* and *Saprolegnia parasitica*.

In the a study by Endler et all (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.

Mousavi et all(2009), 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.

Due to the antifungal effect of *Ruta graveolens* roots against *Saprolegnia*, in future extracts of *R. 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 1: Diameter of halo in various amounts of the Ruta graveolens extract in Disk method

Ethanolic extract				kind of extract
70 µl	**60 µl**	**50 µl**	**40 µl**	**(amountλ)**
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 2: Diameter of halo in various amounts of the Ruta graveolens extract in wells method

Ethanolic extract				kind of extract
110 µl	**100 µl**	**90 µl**	**80 µl**	**(amountλ)**
30.33±.57	28.66±.57	26.66±.57	23.33±.57	saprolegnia

Table 3: 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

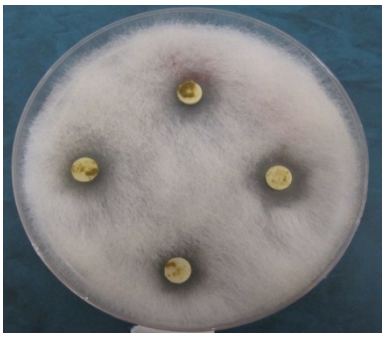

Figure 1. Etanolic extract of *Ruta graveolens* on Saprolegnia in Disk method

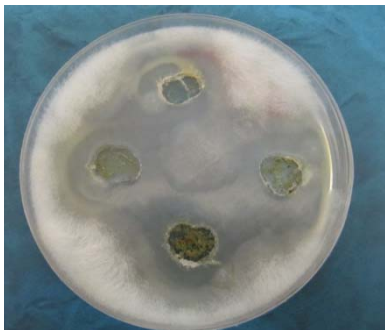

Figure 2. Etanolic extract of *Ruta graveolens* on Saprolegnia in wells method

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