



Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: [www.sciencedirect.com](http://www.sciencedirect.com)

Original article

# Anti-saprolegnia potency of some plant extracts against *Saprolegnia diclina*, the causative agent of saprolegniasis

Ashraf Abdel-Fattah Mostafa<sup>a,b,\*</sup>, Abdulaziz Abdulrahman Al-Askar<sup>a</sup>, Mohamed Taha Yassin<sup>a</sup><sup>a</sup> Botany and Microbiology Dept., College of Science, King Saud University, P.O. 2455, Riyadh 11451, Saudi Arabia<sup>b</sup> National Institute of Oceanography and Fisheries, Al-Kanater Fish Research Station, Egypt

## ARTICLE INFO

### Article history:

Received 30 January 2020

Revised 31 March 2020

Accepted 3 April 2020

Available online 13 April 2020

### Keywords:

Saprolegniasis

Plant extracts

Alternative fungicides

*Punica granatum**Thymus vulgaris*

## ABSTRACT

Saprolegniasis of fresh water fishes caused by *Saprolegnia diclina* often results in serious economic losses to fish hatcheries. Despite the proven efficiency of malachite green as a potential fungicide in prevention and control of fish saprolegniasis, there is a strong debate about its safety aspects in use since it was documented to be responsible for many carcinogenic and teratogenic attributes. Bioactivity of four ethanolic plant extracts were assessed to attain a natural alternative to the traditional fungicide currently used in saprolegniasis control. Ethanolic extracts of *Punica granatum* and *Thymus vulgaris* exhibited a potential efficacy in suppressing mycelial growth of *S. diclina* at concentration of 0.5 mg/ml while extracts of *Nigella sativa* and *Zingiber officinale* were not effective respectively. The extract of pomegranate showed the highest antifungal potency with minimum inhibitory concentration (MIC) of 200 ppm while thyme extract was less effective and recorded MIC of 400 ppm against *S. diclina*. The acute fish toxicity of the plant extracts indicated the low toxicity of *P. granatum* and *T. vulgaris* extracts as no fish mortalities were detected at aquaria containing 200, 400 and 800 ppm of plant extracts respectively. Considering the low toxicity of these plant extracts, it may be concluded that 200 and 400 ppm of pomegranate and thyme extracts which suppressed the mycelial growth of the *S. diclina* could be safely used for saprolegniasis control.

Both of pomegranate and thyme extracts which proved to possess a potential antifungal activity can be considered as a natural alternative fungicides to control saprolegniasis avoiding carcinogenic malachite green application.

© 2020 The Author(s). Published by Elsevier B.V. on behalf of King Saud University. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

## 1. Introduction

Saprolegniasis of fresh water fishes induced by *Saprolegnia parasitica* and *S. diclina* was regarded as the most serious fungal disease threaten fish industry causing a high fish mortality and huge economic losses to fish hatcheries (Thoen et al., 2011; Van Den Berg et al., 2013; Songe et al., 2016). Saprolegniasis was controlled with application of commonly available fungicides as malachite green, formaldehyde, hydrogen peroxide and copper oxysulfate (Barnes et al., 2001; Mitchell et al., 2009; Straus et al., 2009; Earle and

Hintz, 2014). Despite the proven efficiency of these chemical fungicides in the prevention and controlling of mycotic fish diseases, their excessive application has resulted in the accumulation of residual toxicity in fish flesh (Sudova et al., 2007). Moreover, the overuse of these fungicides altered water biological balance by decimating beneficial hydrophytes and contaminated the environment (Battaglin and Fairchild, 2002). Also, these fungicides were proven to be responsible for many carcinogenic and teratogenic attributes (Corcoran et al., 2010). In an attempt to modify these conditions, efforts will be exerted to expose the natural sources of antimicrobial agents. These bioactive agent must be effective, inoffensive for fish, safe for human health and don't pose any environmental problems (Madhuri et al., 2012). The search has been extended to plant extracts which possess fungicidal properties, easily degradable by natural microbes and doesn't contributed with any environmental or health risk. Fungicidal activities of plant extracts have been investigated on controlling saprolegniasis of fish and their eggs by several investigators. For example; (Mori et al., 2002; Ghasemi Pirbalouti et al., 2009; Shin, et al., 2017) tested suppression of aquatic fungi as *Saprolegnia* species by some plant extracts; (Rai et al., 2002)

\* Corresponding author at: Botany and Microbiology Dept., College of Science, King Saud University, P.O. 2455, Riyadh 11451, Saudi Arabia.

E-mail address: [ashraf812@yahoo.com](mailto:ashraf812@yahoo.com) (A.A.-F. Mostafa).

Peer review under responsibility of King Saud University.



Production and hosting by Elsevier

screened antifungal effect of five essential oils against the pathogenic *Saprolegnia ferax* strain isolated from diseased fish; (Tampieri et al., 2003) evaluated fungicidal activities of some selected essential oils on mycelial growth of *S. parasitica*; (Chukanhom et al., 2005) studied antifungal activities of *Alpinia galange* against water molds; Thymoquinone extracted from *Nigella sativa* was potentially effective against *Saprolegnia* spp. isolated from diseased fish (Hussein et al., 2002). Rohani et al. (2006) evaluated the antifungal activities of some essential oils of *Geranium herbarum* against *Saprolegnia* species isolated from diseased rainbow trout; (Ilundu et al., 2009) treated saprolegniasis of *Clarias gariepinus* (fresh water fish) with aqueous extract of *Vernonia amygdalina*; (Madhuri et al., 2012) reported antimycotic activity of some medicinal plants against saprolegniasis of fresh water fishes and (Agbebi et al., 2012) investigated the antifungal potential of *Euphorbia kamerunica* (spurge) against growth of *Saprolegnia* species isolated from diseased eggs of catfish. They concluded that, 25 ml and 50 ml concentration of spurge could be used to control *Saprolegnia* infection in fish hatchery. Furthermore, study by Salehi et al. (2015) demonstrated that *Euclayptus globules*, *Thymus daenensis* and another forty essential oils were effective in suppressing fungal growth of *Saprolegnia parasitica* with MIC of 2.5 and 5 ml respectively. *Thymus vulgaris* and *Zingiber officinales* extracts exhibited a highly antifungal efficacy and showed fungicidal potency against *Rhizoctonia solani*, *Fusarium oxysporium* and *Pythium aphanidermatum* with MIC of 4 mg/ml and MFC of 8 mg/ml (Mostafa et al., 2012). On the other hand, leaves extract of *Thymus vulgaris* suppress completely mycelial growth and degenerate hyphae of *Pythium ultimum* at plant extract concentration of 400 ppm (Ramanathan et al., 2004). However, Xue-Gang et al. (2013) recorded fungitoxicity of ginger (*Z. officinale*) extract against a range of fungi among which certain species of Oomycetes were present. The plant extracts of *Nigella sativa*, *Z. officinale*, *P. granatum* and *T. vulgaris* exhibited a potential antifungal efficiency against a number of phytopathogenic fungi such *Aspergillus flavus*, *Fusarium oxysporium*, *Rhizoctonia solani* and *Pythium ultimum* (Kumar et al., 2008; Pane et al., 2011). Research concerning the efficiency of the above mentioned plant extracts against aquatic fungi (*Saprolegnia* spp.) is scanty. Therefore, the main goal of the present study was to assess antifungal potency of some plant extracts as *Nigella sativa*, *Punica granatum*, *Thymus vulgaris* and *Zingiber officinale* against saprolegniasis caused by *S. diclina* in vitro.

## 2. Materials and methods

### 2.1. Isolation of saprolegnoid strain

The fish pathogenic isolate *Saprolegnia diclina* was isolated from diseased tilapia fish (*Oreochromis niloticus*), purified and identified

by the methods described by Beakes (1994), Johnson et al. (2002). The culture of pathogenic *S. diclina* was maintained on Sabouraud's dextrose agar (SDA) slant supplemented with (0.5 gm/L) of penicillin G and streptomycin. The identified strain, *S. diclina* was kept at 10 °C on SDA and renewed at regular intervals.

### 2.2. Plant extraction preparations

Phyto-materials of four plant species correlated to four botanical families (Table 1) were purchased from local markets of Riyadh, Saudi Arabia. These materials were identified and their identifications were confirmed by herbarium of botany dept. college of science, King Saud university. Phyto-materials were collected, washed with tap water, disinfected by sodium hypochlorite solution (0.5%). Plant materials were rinsed with distilled water to remove chlorine residues and finally dried in shade. The dried material of each plant species was ground into a fine powder using blender to pass 100 mm sieve. Fifty grams of the powdered material was immersed in 200 ml of ethanol with stirring for 48 hrs then filtered through double layers of muslin to discard plant debris. The filtrates were centrifuged at 8000 rpm for 10 min and finally filtered again through Whatman filter paper No.(41) to attain a clear filtrate. The filtrates were dried at 35 °C under reduced pressure. The extract yields were stored in small bottles and refrigerated at 4 °C till used.

### 2.3. Preparation of zoospores suspension

Zoospore suspension of *S. diclina* was prepared by subculture the pathogenic isolate on potato dextrose agar supplemented with 0.2% yeast extract at 18 ± 2 °C for three days. Agar disc from the edge of actively growing *S. diclina* were cut and immersed in sterilized aquarium water flasks supplement with (0.5gm/L) chloromphenicol and (0.1%) tween 20 to eliminate bacterial contamination and facilitate zoospores emigration from fungal mycelium. Flasks were incubated at 18 ± 2 °C for 18 hrs and the agar discs were discarded. Zoospores of *S. diclina* was harvested, diluted and their absorbance were measured at wavelength ( $\lambda$ ) 580  $\mu$ m to 30% using spectrophotometer. The viable count of zoospores at this absorbance was approximately 10<sup>5</sup> zoospores/ml.

### 2.4. Fungicidal analysis of the plant extracts

#### 2.4.1. Antifungal screening test

Agar disc diffusion method was achieved to detect antifungal potency of the plant extracts using potato dextrose agar medium (PDA) supplement with (0.2%) yeast extract and (0.5gm/L) of chloromphenicol to eliminate bacterial contamination. About 10 ml of PDA medium was dispensed into sterile petridishes

**Table 1**  
Ethnobotanical data of employed plant species and their traditional medicine uses.

Ethnobotanical data	Plant species			
Scientific name	<i>Nigella sativa</i>	<i>Punica granatum</i>	<i>Thymus vulgaris</i>	<i>Zingiber officinale</i>
Family	Ranunculaceae	Lythraceae	Lamiaceae	Zingiberaceae
Common name	Black caraway	Pomegranate	Thyme	Ginger
Local name	Black seeds	Romman	Za'ater	Zanjabil
Plant organ used	Seeds	Peels	Leaves	Rhizome
pH of the extract	6.8	4.7	5.3	7.1
Main chemical composition	Linoleic acid, thymoquinone, nigellone, nigilline, melanthin, damascenine, and anethole.	Catechins, galocatechins, prodelphinidins, punicalagins tannins, and anthocyanins	Thymol, linalol carvacrol, cymene, pinene, menthone, and borneol	Zingerone, shogaols, gingerols, cineol, zingiberene, citral bisabolene, farnesene, and $\beta$ -phelladrene
Traditional use	It used as food spice and as a carminative in indigestion and bowel complaints.	It used against diarrhea, dysentery, intestinal parasites and as contraceptive	It used as Antiseptic, antispasmodic, tonic and carminative	It used frequently for dyspepsia, gastroparesis, slow motility symptoms, constipation, and colic and as carminative

(as basal medium) then overloaded with 15 ml of seeded medium previously inoculated with fungal zoospore suspension (2 ml of  $10^5$  zoospores/100 ml of medium) to attain ( $2.0 \times 10^3$ ) zoospores/ml of medium. The plant extracts were prepared by dissolving their intended amounts in 2 ml of methanol, sterilized through disposable Millipore syringe ( $0.22 \mu\text{m}$ ) and loaded over sterilized filter paper discs of (7 mm) in diameter to attain final concentration of 0.5 mg/disc. Filter paper discs loaded with  $1 \mu\text{g}$  of malachite green was used as a positive control and reference fungicide. The plates were refrigerated at  $4^\circ\text{C}$  for two hours then incubated at  $18 \pm 2^\circ\text{C}$  for three days. The presence of inhibition zones were investigated, estimated by Vernier caliper and regarded as indication for antifungal potency.

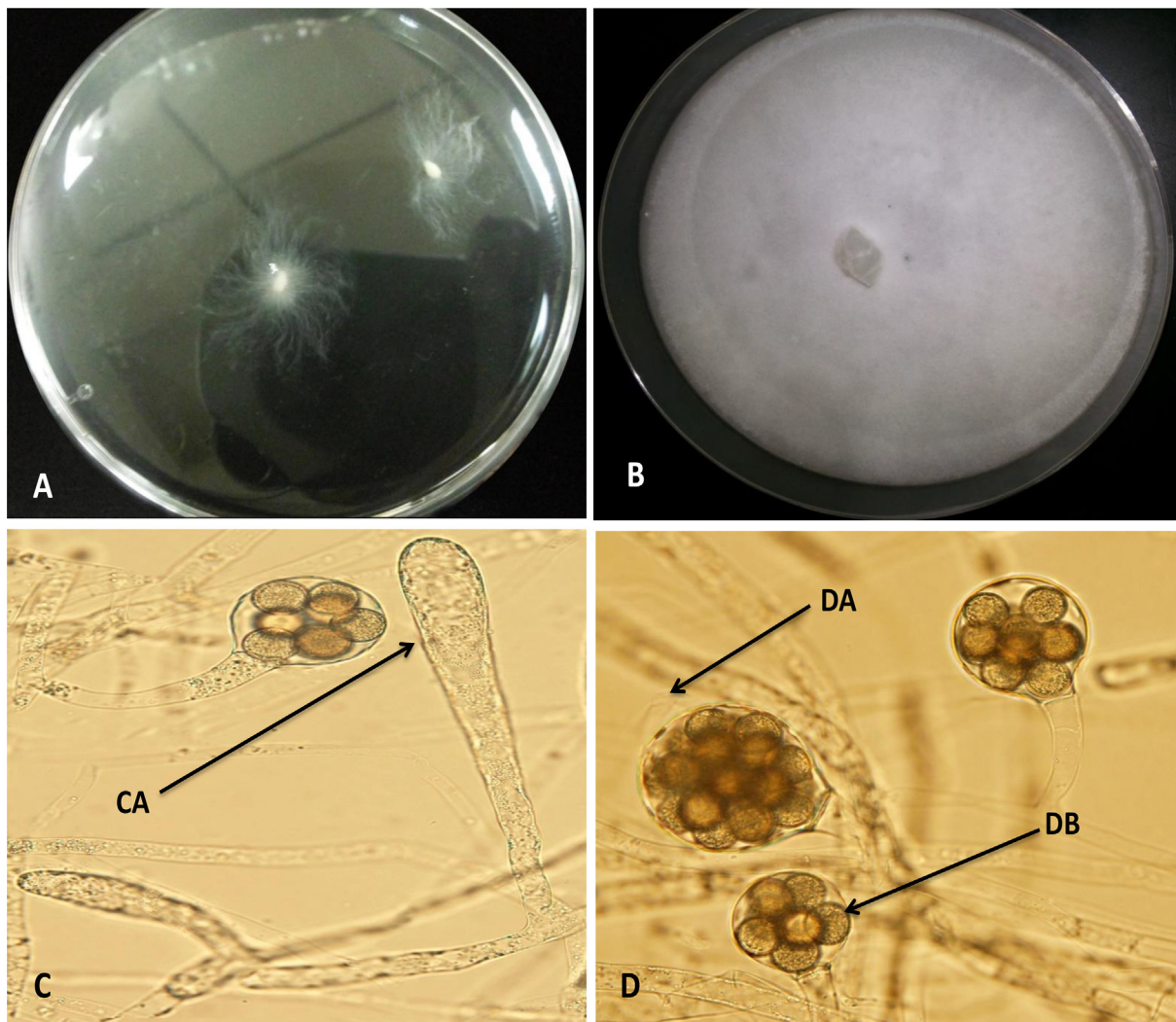
#### 2.4.2. Determination of minimum inhibitory concentration (MIC)

The effective extracts including *P. granatum* and *T. vulgaris* were achieved to detect their potency in the restriction of pathogenic *S. diclina* strain and to estimate their minimal inhibitory concentration (MIC). Different concentrations of each plant extract (0.2, 0.4, 0.6 and 0.8 mg/ml) were preformed by dissolving their intended amount in 2 ml of methanol, sterilized through Millipore filter ( $0.22 \mu\text{m}$ ) and pipetted over filter paper discs of 7 mm in diameter. Ten ml of PDA basal medium followed by fifteen ml of

seeded medium (previously injected with zoospores suspension of *S. diclina*) were poured into sterile petridishes. Requisite amounts of different concentrations of each plant extract were loaded on sterile filter paper discs and placed over the PDA plates at 3 cm from each other. The plates were refrigerated at  $5^\circ\text{C}$  for 2 h to permit plant extract diffusion then incubated at  $18 \pm 2^\circ\text{C}$  for 3: 5 days. The presence of inhibition zones were recorded and tabulated against plant extracts concentrations.

#### 2.5. Acute fish toxicity of the effective plant extracts

Acute toxicity test of the effective plant extracts on tilapia fish (*Oreochromis niloticus*) was achieved with fish fingerlings of average body weight ( $10 \pm 0.5$  gm). Stock solutions of *P. granatum* and *T. vulgaris* ethanolic extracts were prepared with the concentrations of (0.0, 200, 400, 800, 1200, and 1600 ppm). A total of 60 healthy tilapia fingerlings were held in six glass aquaria containing 60 L of water and supplemented with an air supply and dechlorinated tap water in a rate of 10 fish/aquarium. Through the experiment period, water pH was adjusted to  $7.0 \pm 0.5$  and the temperature was maintained at  $23 \pm 2^\circ\text{C}$ . All fish were acclimated to lab. conditions and were fed twice daily at a rate of 3% of its body weight (6.0 gm/treatment). Six experimental fish groups



**Fig. 1.** A) Isolation of *Saprolegnia diclina* using sesame seeds in sterilized aquarium water; B) Cultural characteristics of *S. diclina* grown on PDA medium; C) Morphological characteristics of *S. diclina* showing clavate zoosporangium (CA); D) Sexual Structures of *S. diclina* showing declinous antheridium (DA) and spherical oogonium with centric oospores (DB).



**Table 2**  
Antifungal screening test of ethanolic plant extracts (0.5 mg/ml) against pathogenic *Saprolegnia diclina* isolate.

Plant species	Inhibition zone diameter (mm)	Plant extract (mg) equivalent to (1 µg) of malachite green potency
<i>Nigella sativa</i>	0.00* ± 0.0	0.00
<i>Punica granatum</i>	17.81* ± 0.6	0.97
<i>Thymus vulgaris</i>	15.75* ± 0.3	1.10
<i>Zingiber officinale</i>	0.00* ± 0.0	0.00
Malachite green	34.53 ± 1.1	–

Asterisks (\*) referred to the significant values ( $P \leq 0.05$ ). Data are mean of triplicates ± standard error.

were exposed to different concentrations of *P. granatum* and *T. vulgaris* extracts for 96hrs without feeding. Dead fish were picked up immediately from the glass aquarium to avoid deterioration of water. The mortality percentage of fish was calculated after 24, 48, 72 and 96 hrs. of exposure for each plant extracts respectively.

### 2.6. Statistical analysis

The experiments were performed in triplicates for each treatment. The obtained results were presented as mean ± SE (standard error) and analyzed statistically for significance by one-way ANOVA test at  $P \leq 0.05$ .

## 3. Results

### 3.1. Identification of the saprolegnoid strain

Isolation of pathogenic *S. diclina* was performed from colonized dorsal muscle of diseased tilapia fish (*Oreochromis niloticus*) and the production of reproductive structures was achieved by growing the isolate on sesame seeds in sterilized aquarium water (Fig. 1). The purified strain was identified on the basis of macroscopic and microscopic characteristics with reproductive structures according to methods described by Beakes (1994), Johnson et al. (2002).

The purified strain showed a typical morphology of *Saprolegnia diclina* on sesame seeds and PDA plates (Fig. 1A & B). The isolate cultured in sesame seeds produced sexual structures with a similar morphological characteristic of *S. diclina* like, clavate zoosporangium (Fig. 1C), declinous antheridium (Fig. 1DA) and spherical unpitted oogonium with centric oospores (Fig. 1DB).

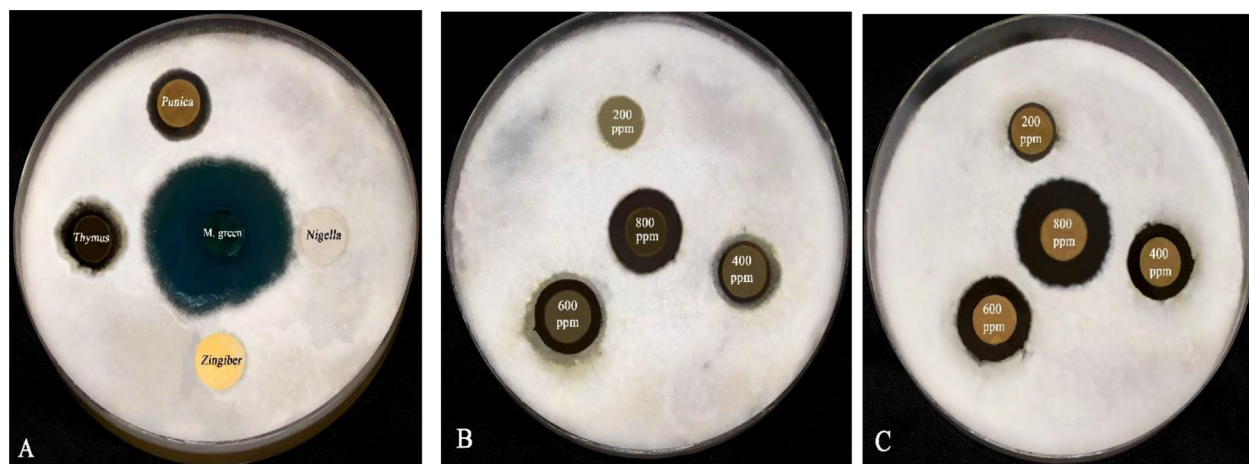
### 3.2. Antifungal screening test

Scientific and classical name of the used plants and their traditional medicine uses are demonstrated in Table 1. Four plants correlated to four botanical families were investigated to detect their antifungal potency against etiological agent of fish saprolegniasis (*Saprolegnia diclina*) using disc diffusion method. Screening of antifungal activities of these plant extracts was represented in Table 2 and demonstrated in Fig. 2. Only, *P. granatum* and *T. vulgaris* extracts were potentially effective in preventing mycelial growth of *S. diclina* at concentration of 0.5 mg/ml while other plant extracts of *Nigella sativa* and *Zingiber officinales* were not effective. *P. granatum* was the most effective extract inhibiting mycelial growth of *S. diclina* and exhibiting inhibition zone of (17.81 mm) while *T. vulgaris* extract was less effective in suppressing mycelial growth of the pathogenic *S. diclina* and recorded inhibition zone of (15.75 mm).

On the other hand, malachite green was strongly effective against *S. diclina* suppressing its mycelial growth with inhibition zone of (34.53 mm). However, malachite green still the most effective fungitoxicant suppressing mycelial growth of *S. diclina* than extracts of *P. granatum* and *T. vulgaris* as inhibition zone was (34.53 mm) at concentration of 1 µg/ml while 0.97 and 1.10 mg of the previous plant extracts were required respectively to attain the same effect of malachite green. The MIC of the effective plant extracts (*P. granatum* and *T. vulgaris*) was employed by disc diffusion method to assess their fungicidal properties. The concentration influence of the functional plant extracts on growth parameter of *S. diclina* was represented in Table 3 and demonstrated in Fig. 2(a–b) as their inhibitory effect was increased in proportion to their concentrations. The results in Table 3 revealed that, *P. granatum* extract was strongly effective against *S. diclina* recording MIC of 200 ppm with inhibition zone of (11.34 mm) while *T. vulgaris* extract was less effective and no suppressive potency was detected at the same concentration. MIC of *T. vulgaris* extract against *S. diclina* was 400 ppm with inhibition zone of (15.05 mm). Thus we can conclude from the previous results that, the extract of *P. granatum* and *T. vulgaris* inhibited mycelial growth of the pathogenic *S. diclina* which was highly susceptible to *P. granatum* extract than that of *T. vulgaris*.

### 3.3. Acute fish toxicity assay

The acute fish toxicity of the plant extracts was achieved through exposing of fish to different concentrations of *P. granatum*



**Fig. 2.** A) Screening of antifungal activities of some plant extracts against *S. diclina*; B) MIC of *Thymus vulgaris* extract against *S. diclina* using disk diffusion method; C) MIC of *punica granatum* extract against *S. diclina* using disk diffusion method.

**Table 3**

Mycelial growth inhibition (mm) of *Saprolegnia diclina* after treatment with the effective plant extracts at different concentrations.

Plant extract conc. (ppm)	Plant species	
	<i>Punica granatum</i>	<i>Thymus vulgaris</i>
	Inhibition zone diameter (mm)	Inhibition zone diameter (mm)
200	11.34 ± 0.4	0.00 ± 0.0
400	16.25 ± 1.1	15.05 ± 0.9
600	19.63 ± 0.6	17.82 ± 0.3
800	24.65 ± 0.3	22.41 ± 0.5

Data are mean of triplicates ± standard error.

and *T. vulgaris* for 96 hrs. Results in Table 4 indicated the low toxicity of plant extracts as no fish mortalities were observed in aquaria containing 200, 400 and 800 ppm of plant extracts respectively. Only, 10 and 30% of fish accumulative mortalities were detected at 1200 ppm and 1600 ppm of pomegranate extract exposure for 96 hrs. On the other hand, 10 and 20% of fish mortalities were observed at 1200 and 1600 ppm of Thyme exposure. However, no mortalities were further detected in aquaria and the fish survivors from plant extracts toxicity test didn't show any cytotoxic effect.

#### 4. Discussion

Saprolegniasis of fresh water fish can be controlled by good water quality, air circulation of pond water, good fish nutrition, avoidance of fish crowding to minimize injury and finally application of chemical fungicides as malachite green, formalin and hydrogen peroxide (Sharma et al., 2012). However, excessive application of fungicides in fish disease treatment leads to accumulation of toxic residues in fish flesh, increase risk of environmental pollution and considered to be responsible for many carcinogenic and teratogenic attributes. So, the adverse impression of these fungicides on human health and environment are burning issues and it becomes necessary to develop effective and eco-friendly fungicides. Four ethanolic plant extracts were screened in vitro at 0.5 mg/ml to evaluate their antifungal activity in controlling saprolegniasis. Our results showed that, two plant extracts provided a significant inhibition of mycelial growth of the pathogenic *S. diclina* isolate and its sensitivity to a given plant extract varied greatly. *P. granatum* extract showed a potential suppressive effect on growth parameter of *S. diclina* at concentration of 0.5 mg/ml followed by extract of *T. vulgaris* while the other plant extracts have no suppressive potency at the same concentration. These results

are coincident with that of ALSafah and AL-Faragi (2017) who ascertained that the dietary supplementation of *T. vulgaris* to fish increase survival rate of *Cyprinus carpio* challenged with *Saprolegnia* sp. Moreover, the results were compatible with that of Mostafa et al. (2012) who reported that both of *P. granatum* and *T. vulgaris* extracts were potentially active against *Rhizoctonia solani*, *Fusarium oxysporum* and a species of Oomycetes (*Pythium aphanidermatum*). A variation of antifungal efficacy of the concerned plant extracts may be attributed to considerable variation in their phytochemical constituents and variation in fungal diversity (Al-Rahmah et al., 2013). Thymol and Carvacrol were determined as the most effective antimicrobial component in *T. vulgaris* extract (Omidbeygi et al., 2007) while punicalagin, castagalagin and granatin were determined as the effective antimicrobial compounds in *P. granatum* extract (Dahham et al., 2010; Foss et al., 2014). Some researchers suggested that the effective plant extract contained antimicrobial components which were able to cross fungal cell membranes, blocking their vital enzymes and denaturizing proteins of their cell membrane that destroying selective permeability of fungal cell membrane and suppressing different biochemical process of fungal cell causing cell death (Pane et al., 2011; Madhuri et al., 2012). According to fish toxicity assay of the effective plant extracts, both of *P. granatum* and *T. vulgaris* extracts showed low toxicity to fish as no mortalities were detected at 800 ppm. Considering the low toxicity of these plant extracts, it may be concluded that 200 and 400 ppm of pomegranate and thyme extracts which suppressed the mycelial growth of the *S. diclina* could be safely used for saprolegniasis control through immersion of the diseased fish in aquaria containing the previous concentrations of the potentially active plant extracts for 30 min. Also, these plants extracts may be mixed with the fish feed decreasing their ability to be infested with *S. diclina*.

Hence, the present study indicated that application of pomegranate and thyme extracts can be used as potential and applicable fungicide in fish disease treatment especially saprolegniasis caused by *S. diclina*. Also, the extracts of *P. granatum* and *T. vulgaris* can be considered as a safe alternative to teratogenic malachite green.

#### 5. Conclusion

These effective plant extracts create a new opportunity in development process of safe and eco-friendly fungicides and they can be considered as alternative fungicides to control saprolegniasis avoiding carcinogenic malachite green application. However, further investigations on the effective plant extracts as prophylactic treatment during outbreak of saprolegniasis *in vivo* are required before practical use of these extracts in fish aquaculture.

**Table 4**

Accumulative mortalities of Tilapia sp. (*O. niloticus*) during acute exposure to plant extracts for 96 h.

Plant extracts	Conc. (ppm)	No. of dead fish				Percentage of mortality			
		24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h
<i>P. granatum</i>	0.0	0	0	0	0	0	0	0	0
	200	0	0	0	0	0	0	0	0
	400	0	0	0	0	0	0	0	0
	800	0	0	0	0	0	0	0	0
	1200	0	0	0	1	0	0	0	10
<i>T. vulgaris</i>	160	0	0	1	3	0	0	0	30
	0.0	0	0	0	0	0	0	0	0
	200	0	0	0	0	0	0	0	0
	400	0	0	0	0	0	0	0	0
	800	0	0	0	0	0	0	0	0
	1200	0	0	1	1	0	0	0	10
1600	0	0	1	2	0	0	0	20	

## Declaration of Competing Interest

None.

## Acknowledgment

The authors extend their appreciation to the Deanship of Scientific Research at King Saud University for funding this work through research group No. (RGP-1440-094).

## References

- Agbebi, O., Oyeleke, G., Agbon, A., 2012. Use of *Euphorbia kamerunica* (Spurge) extract in the control of *Saprolegnia* species growth in incubated eggs of *Clarias gariepinus*. *Global J. Sci. Front. Res.* 12, 27–30.
- Al-Rahmah, A., Mostafa, A., Abdel-Megeed, A., Yakout, S., Hussein, S., 2013. Fungicidal activities of certain methanolic plant extracts against tomato phytopathogenic fungi. *African J. Microbiol. Res.* 7 (6), 517–524.
- Alsafah, A., AL-Faragi, J., 2017. Influence of thyme (*Thymus vulgaris*) as feed additives on growth performance and antifungal activity on *Saprolegnia* spp. *Cyprinus carpio*, 1598–1602.
- Barnes, M.E., Saylor, W.A., Cordes, R.J., 2001. Use of formalin treatments during incubation of eyed eggs of brown trout. *North Am. J. Aquaculture* 63 (4), 333–337.
- Battaglin, W., Fairchild, J., 2002. Potential toxicity of pesticides measured in midwestern streams to aquatic organisms. *Water Sci. Technol.* 45 (9), 95–103.
- Beakes, G., 1994. Features which characterize *Saprolegnia* isolates from salmonid fish lesions—a review. *Salmon Saprolegniasis*, 33–66.
- Chukanhom, K., Borisuthpeth, P., Hatai, K., 2005. Antifungal activities of aroma components from *Alpinia galanga* against water molds. *Biocontrol Sci.* 10 (3), 105–109.
- Corcoran, J., Winter, M.J., Tyler, C.R., 2010. Pharmaceuticals in the aquatic environment: a critical review of the evidence for health effects in fish. *Crit. Rev. Toxicol.* 40 (4), 287–304.
- Dahham, S.S., Ali, M.N., Tabassum, H., Khan, M., 2010. Studies on antibacterial and antifungal activity of pomegranate (*Punica granatum* L.). *Am. Eurasian J. Agric. Environ. Sci.* 9 (3), 273–281.
- Earle, G., Hintz, W., 2014. New approaches for controlling *Saprolegnia parasitica*, the causal agent of a devastating fish disease. *Trop. Life Sci. Res.* 25 (2), 101.
- Foss, S.R., Nakamura, C.V., Ueda-Nakamura, T., Cortez, D.A., Endo, E.H., Dias Filho, B. P., 2014. Antifungal activity of pomegranate peel extract and isolated compound punicalagin against dermatophytes. *Ann. Clin. Microbiol. Antimicrob.* 13 (1), 32.
- Ghasemi Pirbalouti, A., Taheri, M., Raiesi, M., Bahrami, H., Abdizadeh, R., 2009. In vitro antifungal activity of plant extracts on *Saprolegnia parasitica* from cutaneous lesions of rainbow trout (*Oncorhynchus mykiss*) eggs. *J. Food Agric. Environ.* 7 (2).
- Hussein, M.M., El-Feki, M.A., Hatai, K., Yamamoto, A., 2002. Inhibitory effects of thymoquinone from *Nigella sativa* on pathogenic *Saprolegnia* in fish. *Biocontrol Sci.* 7 (1), 31–35.
- Ilondu, E.M., Arimoro, F.O., Sodje, A.P., 2009. The use of aqueous extracts of *Vernonia amygdalina* in the control of saprolegniasis in *Clarias gariepinus*, a freshwater fish. *Afr. J. Biotechnol.* 8 (24).
- Johnson, T.W., Seymour, R.L., Padgett, D.E., 2002. *Biology and Systematics of the Saprolegniaceae*. University of North Carolina at Wilmington, Department of Biological Sciences.
- Kumar, A., Shukla, R., Singh, P., Prasad, C.S., Dubey, N.K., 2008. Assessment of *Thymus vulgaris* L. essential oil as a safe botanical preservative against post harvest fungal infestation of food commodities. *Innovative Food Sci. Emerg. Technol.* 9 (4), 575–580.
- Madhuri, S., Mandloi, A., Govind, P., Sahni, Y., 2012. Antimicrobial activity of some medicinal plants against fish pathogens.
- Mitchell, A.J., Radomski, A.A., Straus, D.L., Carter, R., 2009. The effect of hydrogen peroxide on the hatch rate and *Saprolegnia* spp. infestation of channel catfish eggs. *North Am. J. Aquaculture* 71 (3), 276–280.
- Mori, T., Hirose, H., Hanjavanit, C., Hatai, K., 2002. Antifungal activities of plant extracts against some aquatic fungi. *Biocontrol Sci.* 7 (3), 187–191.
- Mostafa, A.A., Al-Rahmah, A., Abdel-Megeed, A., Sholkamy, E.N., Al-Arfaj, A.A., El-Shikh, M.S., 2012. Fungitoxic properties of some plant extracts against tomato phytopathogenic fungi. *J. Pure Appl. Microbiol.* 6 (4), 1889–1898.
- Omidbeygi, M., Barzegar, M., Hamidi, Z., Naghdibadi, H., 2007. Antifungal activity of thyme, summer savory and clove essential oils against *Aspergillus flavus* in liquid medium and tomato paste. *Food Control* 18 (12), 1518–1523.
- Pane, C., Spaccini, R., Piccolo, A., Scala, F., Bonanomi, G., 2011. Compost amendments enhance peat suppressiveness to *Pythium ultimum*, *Rhizoctonia solani* and *Sclerotinia minor*. *Biol. Control* 56 (2), 115–124.
- Rai, M., Kaushal, S., Acharya, D., 2002. In vitro effect of five Asteraceae essential oils against *Saprolegnia ferax*, a pathogenic fungus isolated from fish. *The Antiseptic* 99 (4), 136–137.
- Ramanathan, A., Marimuthu, T., Raguchander, T., 2004. Effect of plant extracts on growth in *Pythium aphanideram* spp. *J. Mycol. Plant Pathol.* 34, 315–317.
- Rohani, M., Ebrahimzadeh, M.H., Khosravi, A., Mokhayer, B., Mirzargar, S., Mehrabi, Y.E., 2006. Evaluation of Geranium herbarum essence application in control of fungal contamination in rainbow trout eggs.
- Salehi, M., Soltani, M., Islami, H.R., 2015. In vitro antifungal activity of some essential oils against some filamentous fungi of rainbow trout (*Oncorhynchus mykiss*) eggs. *Aquaculture, Aquarium, Conservat. Legislat.* 8 (3), 367–380.
- Sharma, M., Shrivastav, A., Sahni, Y., Pandey, G., 2012. Overviews of the treatment and control of common fish diseases.
- Shin, S., Kulatunga, D., Dananjaya, S., Nikapitiya, C., Lee, J., De Zoysa, M., 2017. *Saprolegnia parasitica* isolated from Rainbow Trout in Korea: Characterization, anti-*Saprolegnia* activity and host pathogen interaction in Zebrafish Disease Model. *Mycobiology* 45 (4), 297–311.
- Songe, M., Willems, A., Wiik-Nielsen, J., Thoen, E., Evensen, Ø., Van West, P., Skaar, I., 2016. *Saprolegnia diclina* IIIA and *S. parasitica* employ different infection strategies when colonizing eggs of Atlantic salmon, *Salmo salar* L. *J. Fish Dis.* 39 (3), 343–352.
- Straus, D.L., Mitchell, A.J., Carter, R.R., Steeby, J.A., 2009. Optimizing copper sulfate treatments for fungus control on channel catfish eggs. *J. Aquatic Animal Health* 21 (2), 91–97.
- Sudova, E., Machova, J., Svobodova, Z., Vesely, T., 2007. Negative effects of malachite green and possibilities of its replacement in the treatment of fish eggs and fish: a review. *Veterinarni Medicina-Praha- 52* (12), 527.
- Tampieri, M., Galuppi, R., Carelle, M., Macchioni, F., Cioni, P., Morelli, I., 2003. Effect of selected essential oils and pure compounds on *Saprolegnia parasitica*. *Pharm. Biol.* 41 (8), 584–591.
- Thoen, E., Evensen, Ø., Skaar, I., 2011. Pathogenicity of *Saprolegnia* spp. to Atlantic salmon, *Salmo salar* L., eggs. *J. Fish Dis.* 34 (8), 601–608.
- Van Den Berg, A.H., McLaggan, D., Diéguez-Uribeondo, J., Van West, P., 2013. The impact of the water moulds *Saprolegnia diclina* and *Saprolegnia parasitica* on natural ecosystems and the aquaculture industry. *Fungal Biol. Rev.* 27 (2), 33–42.
- Xue-Gang, H., Lei, L., Cheng, C., Kun, H., Xian-Le, Y., Gao-Xue, W., 2013. In vitro screening of Chinese medicinal plants for antifungal activity against *Saprolegnia* sp. and *Achlya klebsiana*. *North Am. J. Aquaculture* 75 (4), 468–473.