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An *in-vitro* study of Himalayan plant extracts against oomycetes disease Saprolegniasis in rainbow trout (*Oncorhynchus mykiss*)

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Abstract

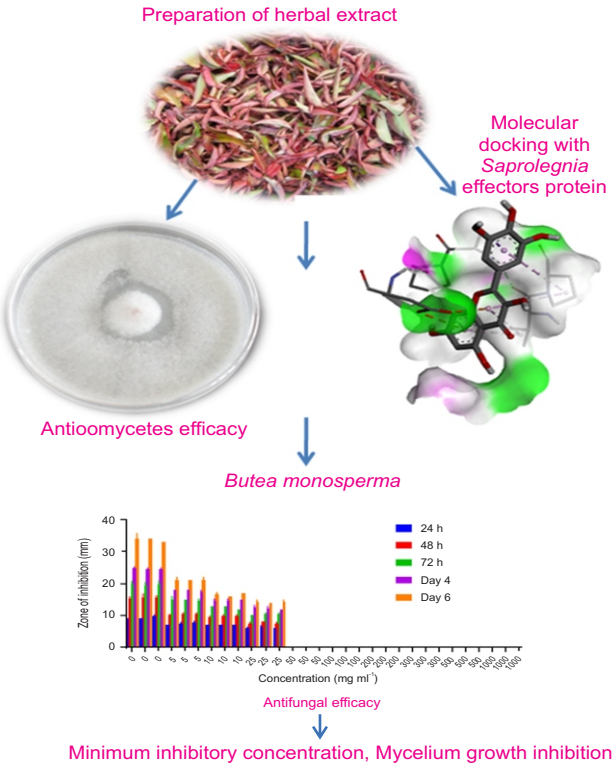
Aim: This study aimed to investigate the effectiveness of ethanolic extract of three Himalayan plants *Myrica esculenta*, *Thymus linearis* and *Butea monosperma* on hyphal germination, colonisation and sporulation of two species of *Saprolegnia* (*Saprolegnia parasitica* and *S. australis*) isolated from rainbow trout, *Oncorhynchus mykiss*. Molecular docking of active ingredients of *M. esculenta,* Myricetin with effector proteins of *S. parasitica* was also performed to investigate the target binding sites for drug development.

Methodology: Minimum inhibitory concentration (MIC), mycelium growth inhibition, spore germination, and inhibition was performed with the most effective concentrations. Molecular docking was carried out with AutoDock Vina software to investigate target binding sites with *S. parasitica*.

Results: Extracts from *Myrica esculenta*, *Thymus linearis* and *Butea monosperma* showed MIC values of the 25, 100, 50 mg ml^{-1} against *S. parasitica* and 25, 50, 25 mg ml^{-1} against *S. australis* hyphal growth, respectively. Nevertheless, malachite green as reference control was effective with a MIC value of 2.5 mg l^{-1}. The concentration required to inhibit *S. parasitica* and *S. australis* spores were (50) *Myrica esculenta*, (25) *Thymus linearis*, (100) *Butea monosperma* in mg ml^{-1} and (50) *Myrica esculenta*, (50) *Thymus linearis*, (100) *Butea monosperma* in mg ml^{-1}, respectively.

Interpretation: The study concludes that *M. esculenta* and *B. monosperma* are effective against Saprolegniasis and could be used as phyto additives.

Key words: Anti-oomycetes, Plant extract, *Saprolegnia australis, Saprolegnia parasitica,* Spore germination



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Introduction

Trout farming has progressed steadily in India, and in the last 15 years, the cold water aquaculture industry has developed tremendously in states and union territory of Jammu & Kashmir, Himachal Pradesh, Sikkim, Uttarakhand and Arunachal Pradesh (Singh, 2020). Intensification of culture practices, climate changes, and genetically fatigue stock are the major bottlenecks for expanding trout farming in India (Barat *et al.,* 2015). In trout farming, major diseases are caused by bacteria, virus, parasites and water moulds (oomycetes). Fungal and fungal-like, oomycetes infections are second only to bacterial disease outbreaks resulting in economic losses in aquaculture (van West, 2006). Saprolegniasis is one of the most severe diseases encountered in rainbow trout, *Oncorhynchus mykiss* farming (Tandel *et al.,* 2020a). Saprolegniales are responsible for the weakening of immune mechanism and death of fishes causing economic losses in salmonids and other aquaculture industries (Masigol *et al.,* 2019; Tandel *et al.,* 2020b). Saprolegniasis is characterised by white or grey cotton-like patches/hyphae on the skin and fins of infected fish (Das *et al.,* 2012). Among several strains of *Saprolegnia*, *Saprolegnia parasitica*, *S. australis* and *S. diclina* are responsible for significant infections in fish and eggs, particularly in aquaculture facilities (van Den Berg *et al.,* 2013).

Previously, *Saprolegnia* infection was effectively controlled by malachite green. However, since 2002, the use of malachite green has been banned worldwide due to its carcinogenic and toxicological effects. Formalin, copper sulphate, hydrogen peroxide, boric acid, ozone, iodophor, sodium chloride and peracetic acid are reported as other antifungal agents to control the saprolegniasis in the eggs of salmonids (Ali *et al.,* 2017; Tedesco *et al.,* 2019). Nevertheless, there are no chemicals presently available that give sufficient protection against the disease after hatching (Srivastava *et al.,* 2018; Good *et al.,* 2020). Hyphal infection is particularly devastating in aquaculture hatcheries and can spread quickly between neighbouring eggs (Smith *et al.,* 1985). At present, extra attention needs to be taken care of the oomycetes infections at early stages of rainbow trout.

One of the substitutions of these synthetic compounds is the use of natural plant extracts or purified compounds of herbal compounds, which are locally available, abundant, low cost, low residual effects and can be used in large aquaculture ponds (Huang *et al.,* 2015). In many countries, raditional herbal medicines are used to prevent diseases in humans as well as in animals (Sharma *et al.,* 2010). Dichloromethane extract from red seaweed *Ceramium rubrum* elicited antimicrobial activity against *S. parasitica* (Cortes *et al.,* 2014). *Magnolia officianalis* and *Euphorbia fischeriana* demonstrated antifungal activity against *Saprolegnia* sp. isolated from the tail skin of infected grass carp, *Ctenopharyngodon idella* (Huang *et al.,* 2015). Caruana *et al.* (2012) also documented the growth dynamics of *S. australis* by plant extracts of *Rumex obtusifolius, Sophora flavescens*, *Echinacea* and *Zingiber officinalis*.

In the present study, the effectiveness of plant extracts of *M. esculenta, T. linearis* and *B. monosperma* was tested against different life stages of *Saprolegnia* spp. isolated from farms and hatcheries of rainbow trout, *O. mykiss*. Molecular docking of active ingredients of *M. esculenta* with effector proteins of *S. parasitica* was also carried out to investigate the target binding sites for drug development.

Materials and Methods

***Saprolegnia* spp. isolate:** Pure culture of *S. parasitica* and *S. australis*, isolated previously from oomycetes affected rainbow trout, *Oncorhynchus mykiss* were used in the efficacy study. Radial growth edges of pure culture isolates were excised into uniformed sizes (5 x 5 mm) and placed onto the middle of Potato Dextrose Agar (PDA) plates supplemented with antibiotics (250 mg l^{-1} ampicillin, Invitrogen). Hemp seeds and sesame seeds were provided as bait. Seed with hyphal attachments was taken and washed 2-3 times in sterilised distilled water and transferred into PD broth for 48 hrs and then sterilised tap water in 90 mm petri plates (Axygen) to observe zoospore formation.

Collection of plants and extract preparation: The collected plant parts such as leaves of *M. esculenta*, *T. linearis* and flowers of *T. linearis* were adequately washed 2-3 times with tap water and soaked in distilled water for 30 min to remove the dust. They were dried in shade for 5 days and then ground to powder form. The ethanolic extract was prepared by dissolving 2.8 g of dried sample in 35 ml of 70% ethanol and kept in a shaker for 48 hrs at 30°C for proper mixing of the sample. After that, the mixture was filtered through Whatman filter paper (No.1), and the remaining supernatant was centrifuged at 2460 rpm for 10 min. The supernatant was then evaporated at 40°C in a rotary evaporator. The plant extracts were collected and kept at -20°C until further use for efficacy study. Malachite green (Himedia) was used as a reference control for efficacy study.

Anti-oomycetes efficacy tests: Different concentrations of plant extracts were prepared and added to sterilised PDA agar supplemented in 90 mm petri-plates with antibiotics (250 mg l^{-1} ampicillin, Invitrogen) in triplicate and one as the control without herbal extract and malachite green at 2.5 mg l^{-1} as reference control. Following overnight solidification, hyphae from the advancing edge of growing colony of pure culture were cut and placed onto prepared PDA plates and incubated at 20±1ºC and checked after 72 hr. The experiment was done three times, and the results were recorded visually for the presence of mycelium.

Minimum inhibitory concentration tests: Plant extracts showing negative mycelium growth were tested for minimum inhibitory concentration (MIC). The mixture with different concentrations was distributed in 90 mm petri-plates in triplicate and one without extract served as control, while malachite green as a reference control. Following overnight solidification, hyphae from the advancing edge of the growing colony of pure culture were cut and placed onto prepared plates and incubated at 20± 1ºC and checked routinely for

24 hr, 48 hr and 72 hr, 96 hr and 144 hr, respectively. The experiment was replicated three times, and the lowest concentration inhibiting the growth of the mycelium after 6 days of incubation was taken as minimum inhibitory concentrations.

Mycelium growth inhibition assay: *In-vitro* oomycetes activity of the plant extracts were assessed. For the mycelium growth inhibition assay, the radial growth of mycelium was measured for each plate after 24 hr, 48 hr, 72 hr, 96 hr and 144 hr.

Spore germination inhibition and colonisation test: The hyphae of *Saprolegnia* isolates grown on PDA plates were excised and incubated in PD broth at 20ºC for two days to obtain the fungal mat. The newly grown mats were repeatedly washed three to four times in autoclaved tap water and incubated at 20°C for 15 hr. Zoospores number in autoclave tape water (ATW) were counted using a Neubauer hemocytometer and expressed as spores ml^{-1}. 400 microliter of *Saprolegnia* spore suspension (1×10^5 spore ml^{-1}) and 400 µl of each tested drug concentration were incubated with hemp seed in 24 well flat-bottom plates at 20ºC. Sterilised distilled water was used as a non-treated control, and malachite green was used as a positive control. After 24, 48 and 72 hrs, the plates were examined microscopically (Nikon eclipse, Japan).

Molecular docking of myricetin with *S. parasitica* functional and virulent proteins: V-type proton ATPase structures of *S. parasitica* were not available in the PDB bank. So the homology modelling of *S. parasitica* catalytic protein was performed using alignment mode of SWISS-MODEL server (https:// swissmodel. expasy.org). The software aligns the submitted amino acid sequence with the known templates available in the protein data bank. Based on the degree of similarity, the target templates were selected, and the model was built for all the protein sequences. In the Blast P, the desired template was not present for *S. parasitica* host targeting protein. Thus, for developing 3D structure of *S. parasitica* host targeting protein, BLAST-PDB search was initiated to identify potential templates with a default matrix of BLOSUM62. The template with better score listed by pDOMTHREADER was considered for homology modelling using Modeller 9.18 stand alone version.

The modelled 3D structures were refined using Mod Refiner (https://zhanglab.ccmb.med.umich.edu/ModRefiner/). To verify these properties RAMPAGE and Procheck servers were used to locate the unique geometry of each residue in all the 3D models. Myricetin ligand 12 were downloaded from Pubchem and converted into PDB coordinate files by using OPENBABEL software. The ligands were virtually screened on the basis of Lipinski's "rule of five" that sets criteria for drug-like properties (Lipinski *et al.,* 2004). The binding sites of each protein were predicted by 3D Ligand stie. The AutoDock Vina software was used to simulate the ligand into active site of protein to calculate the binding energy of ligand-receptor complexes. Computer-based docking predicted nine docking poses for each ligand-protein complex. The preferable binding orientation between protein and compound, with more negative binding affinity score, was considered for further study (Meng *et al.,* 2011). The three-dimensional structures and 2D docking interactions were visualised using discovery studio visualiser.

Statistical analyses: Minimum inhibitory concentration (MIC), mycelium growth inhibition and spore germination inhibition (SGI) were performed in triplicate and results were expressed as mean value±S.E. Statistic at analyses were done with Graph Pad Prism 8.4.2 (Graph Pad Software, San Diego, USA). Graph Pad Prism 8.4.2 were also used to plot the graph of MIS and SGI. Non-linear regression and sigmoid curve were fitted (see Troskie *et al.,* 2012) with 95% confidence intervals and below equation

Y= Bottom + (Top-Bottom)/(1+10^((LogIC50-X)*HillSlope))

Top represent high antioomycetes activity and bottom denotes growth as in control. IC_{50} represents 50% growth inhibition than control. One way ANOVA with multiple comparison test was used for statistical analysis of three biological repeats with triplicate technical repeats for comparison of Ic_{50}.

Results and Discussion

Malachite green, n-methylated diaminodiphenylmethane has been restricted since 2002, as fungicides or ectoparasiticide because of its mutagenicity, teratogenicity and carcinogenicity (Srivastava *et al.,* 2004). Moreover, available chemical treatments and approved formulations like formalin and hydrogen peroxide pose a high risk to humans as well as wildlife and carcinogenic in nature (European Commission, 2014), which are likely to be banned shortly. Therefore, efforts are being made to identify safe and effective alternative antimycotic agents to control oomycetes, *Saprolegnia* pathogens in different life stages of fishes like eggs and adults (Ali *et al.,* 2019).

In this study, *in-vitro* screening of plant extracts available in Indian Himalayan regions having reported antifungal activity were performed against *Saprolegnia* spp. isolated from *O. mykiss*. The antioomycetes activity were performed following the protocols of Huang *et al.* (2015), Madrid *et al*. (2015) and Tedesco *et al.* (2019). Plant extracts of *M. esculenta*, *T. linearis* and *B. monosperma* showed inhibitory effects on the hyphae growth with MIC values of 25,100, 50 mg ml^{-1} against *S. parasitica* and 25, 50, 25 mg ml^{-1} against *S. australis*, respectively (Fig. 1). At the same time, MIC value for positive reference control malachite green were 2.5 mg l^{-1}. Several plant extracts have been reported as anti-oomycetes agent. *Rumex obtusifolius*, *Sophora flavescens*, *Echinacea* and *Zingiber officinale* screened out of twenty-four crude plant extracts were effective against *Saprolegnia australis* isolated from brown trout (Caruana *et al.,* 2012). Petroleum ether extracts of *Aucklandia lappa, Cnidium monnieri* and *Magnolia officinalis* were effective against *Achlya klebsiana*, and *Saprolegnia* sp. infection (Xue-Gang *et al.,* 2013). Similarly, Huang *et al.* (2015), evaluated the effectiveness of thirty naturally occurring plant species against *Saprolegnia* sp. from

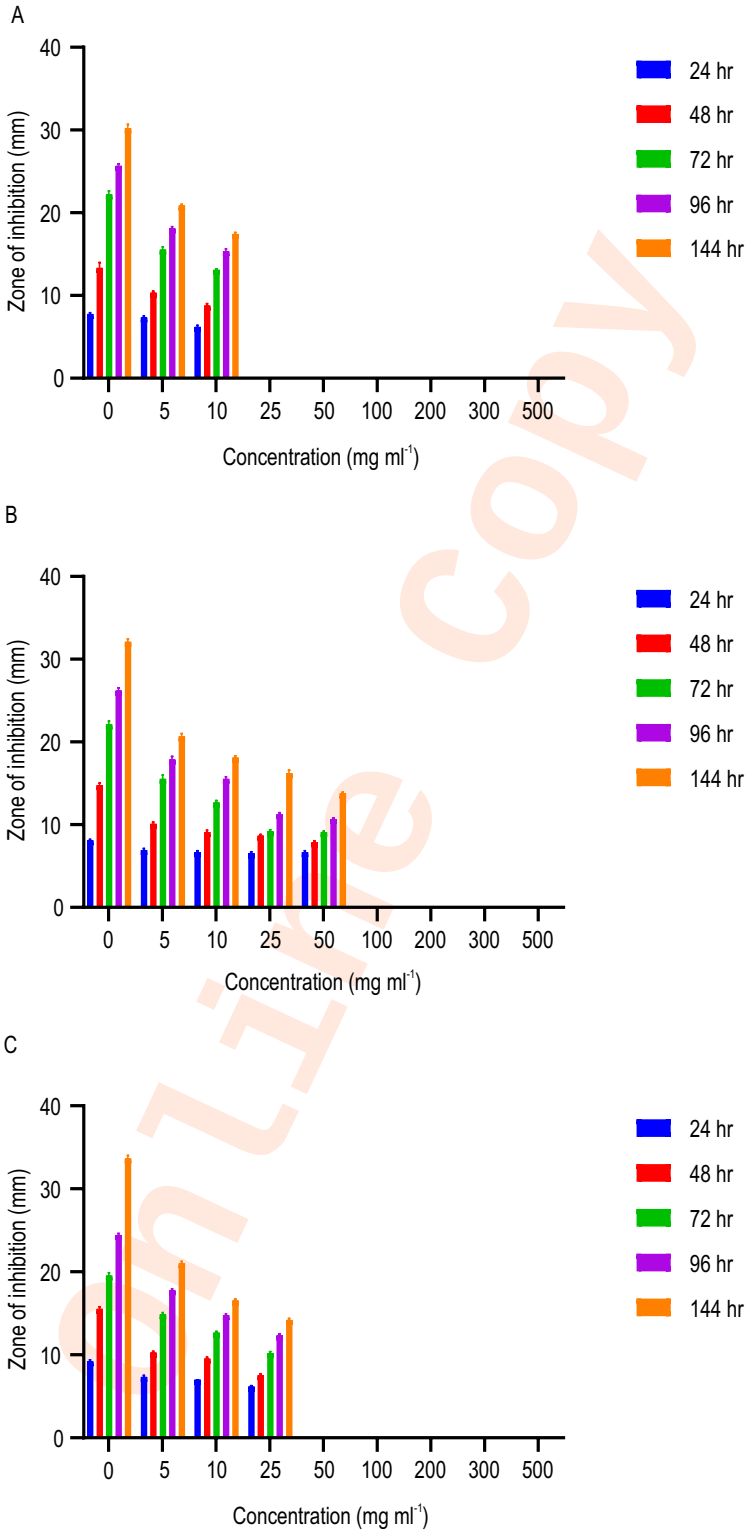

Fig. 1: Minimum inhibitory concentration (zone of inhibition) of three plant extract against *S. parasitica*) *M. esculenta* against *S. parasitica*; B.) *T. Linearis* against *S. parasitica;* C.) *B. monosperma* against *S. parasitica;* The growth of both *Saprolegnia* sp. were evaluated over 24 hr, 48 hr, 72 hr, 96 hr and 144 hr. Each growth measurement is mean of triplicates ± S.E.

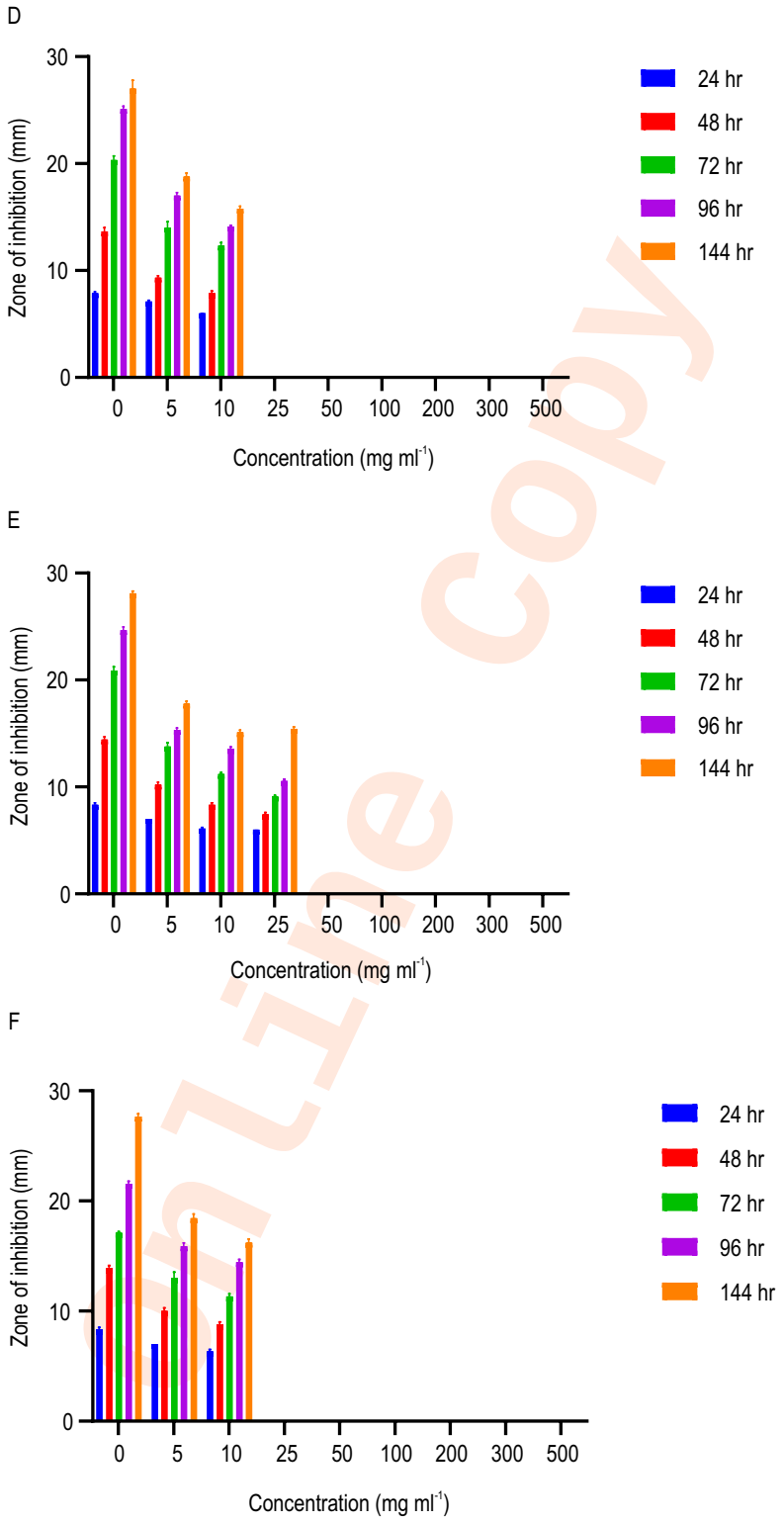

Fig. 1: Minimum inhibitory concentration (zone of inhibition) of three plant extract against *S. australis*. D.) *M. esculenta* against *S. australis;* E.) *T. Linearis* against *S. australis*; F.) *B. monosperma* against *S australis*. The growth of both *Saprolegnia* sp. were evaluated over 24 hr, 48 hr, 72 hr, 96 hr and 144 hr. Each growth measurement is mean of triplicates ± S.E.

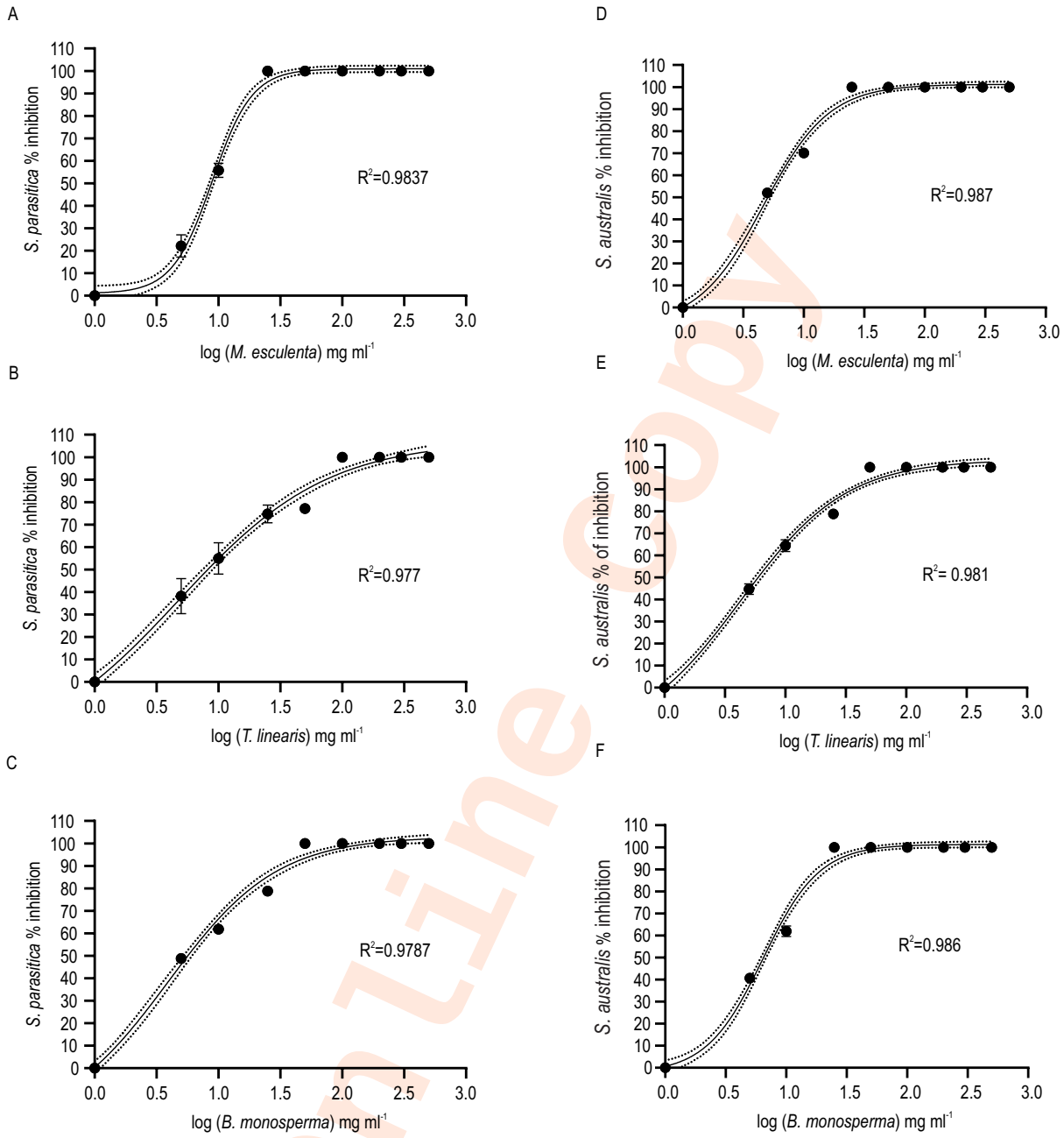

Fig. 2: Mycelium growth inhibition, SGI representative % inhibition response curve of plant extract against *S. parasitica* and *S. australis*; A.) *M. esculenta* against *S. parasitica*; B.) *T. Linearis* against *S. parasitica*; C.) *B. monosperma* against *S. parasitica*; D.) *M. esculenta* against *S. australis*; E.) *T. Linearis* against *S australis;* F.) *B. monosperma* against *S australis*. The dotted line represents the prediction interval value at 95% confidence for the sigmoidal line fit of data.

cultured grass carp (*Ctenopharyngodon idella*) and found that *Magnolia officinalis* and *Euphorbia fischeriana* had antimycotic activity. Cao *et al.* (2014) reported *S. australis* infection from *Carassius gibelio* eggs and suggested *Radix sanguisorbae* extracts as potential anti-*Saprolegnia* agents. Existing synthetic compounds and treatment regimes against *Saprolegnia* infections are limited, uneconomical in large volume ponds, environmental restrictions, having teratogenicity or carcinogenicity and insufficient to constraints losses in aquaculture. Use of phytoadditives as an alternative approach attracts a great deal of attention due to their local availability, effectiveness, low toxicity, environmentally safe and low costs (Lieke *et al.,* 2020). However,

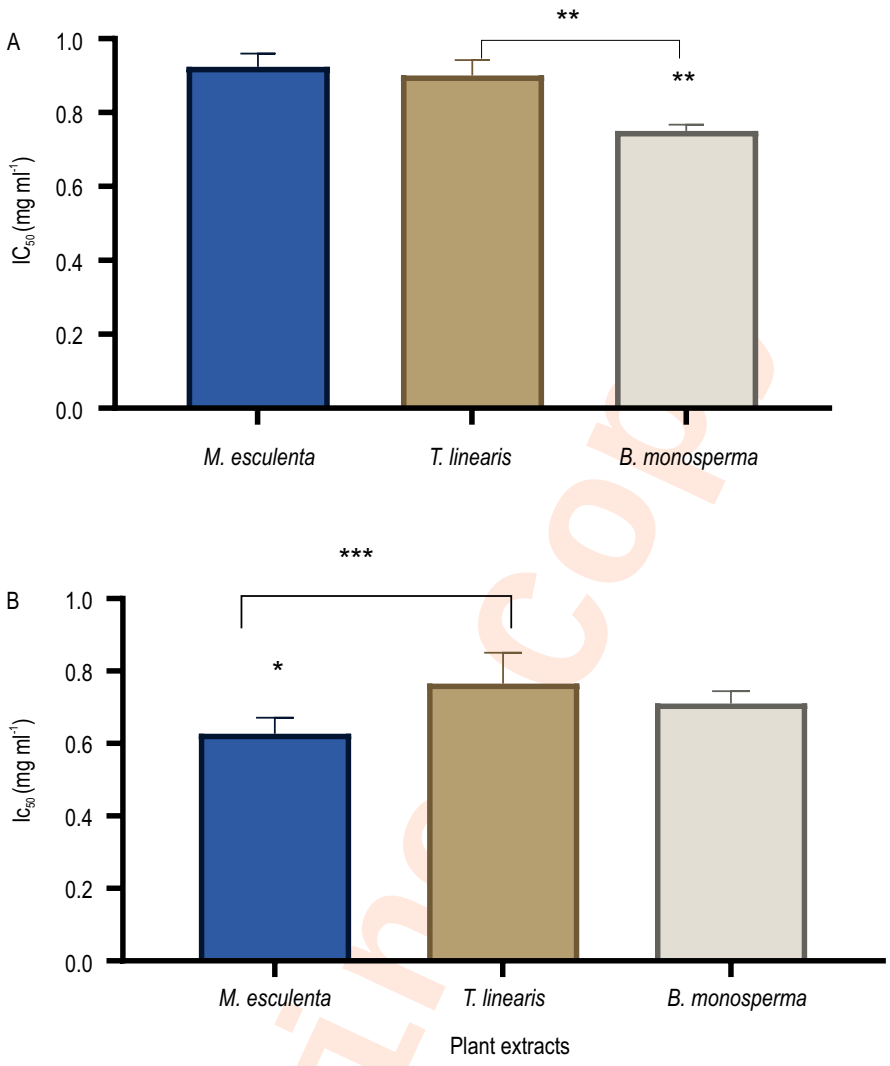

Fig. 3: Comparision IC_{50} value of *M. esculenta*, *T. linearis* and *B. monosperma* against *S. parasitica* (A) and *S. australis* (B).

literature on the effect of sporulation, germination and colonisation of different *Saprolegnia* species isolated from rainbow trout, *O. mykiss* is scarce. Furthermore, anti-oomycetes activity of Himalayan plants has been tested with respect to different life stages of oomycetes, for the first time in this study. The extracts of *T. linearis* and *M. esculenta* exhibited inhibition of spore germination in *S. parasitica* and *S. australis*.

The concentration required to inhibit *S. parasitica* and *S. australis* spores were (50) *Myrica esculenta*, (25) *Thymus linearis*, (100) *Butea monosperma* in mg ml^{-1} and (50) *Myrica esculenta*, (50) *Thymus linearis*, (100) *Butea monosperma* in mg ml^{-1}, respectively. The spore germination inhibition test was performed according to Ali *et al.* (2014). An oomycetes reproduces as swimming zoospores asexually, when reaches a potential host (fish,eggs), it encysts and germinate by producing hyphal growth to develop Saprolegniasis in fish (Paria *et al.,* 2020). IC_{50} value at which 50% growth inhibition of *S. parasitica* and *S. australis* was attained by extracts of *M. esculenta*, *T. linearis* and *B. monospermais* depicted in Fig. 2 and 3. A significant difference was observed in IC_{50} value of *T. linearis* (0.8 mg ml^{-1}) and *B. monosperma* (0.7 mg ml^{-1}) against *S. parasitica* and *M. esculenta* (0.6 mg ml^{-1}) and *T. linearis* (0.7 mg ml^{-1}) against *S. australis* (Fig. 3). *Myrica esculenta*, widely distributed in Kumaun and Garhwal region of Uttarakhand and Himalaya possesses medicinal properties like antimicrobial, anti-inflammatory and antioxidant activities (Shah *et al.,* 2010; Bhandari *et al.,* 2019). Antifungal activity of *M. esculenta* was reported against *Aspergillus niger* and *Candida albicans* due to arylheptanoid myricanone and other bioactive compounds such as saponins, oleanolic acid, p-coumaric acid, and orstigmasterol (Kabra *et al.,* 2019). Similarly, antifungal activity of *T. linearis*

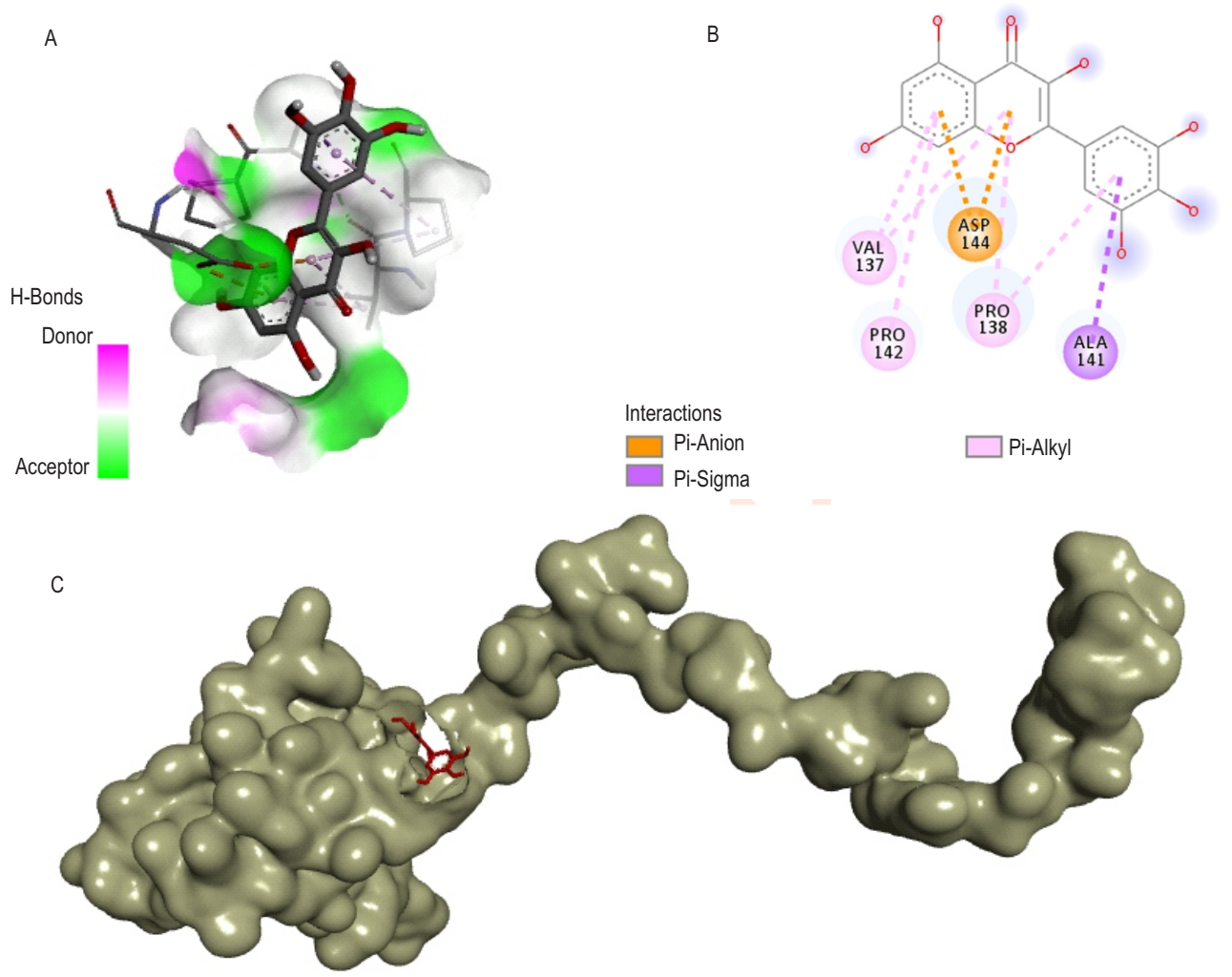

Fig. 4: 2D (A-B) and 3D (C) presentation of docked H bonds on the active sites of host targeting protein of *S. parasitica* with myricetin.

(Shah *et al.,* 2019) and *T. vulgaris* were reported against *C. albicans* (Gedikoglu *et al.,* 2019). Therapeutic importance of *B. monosperma* mainly includes the treatment of inflammation, diabetes, worm and skin infection (Das and Smita, 2018). Although, the antifungal activity of *B. monosperma* has been reported against *A. niger* (Singh , 2011).

The detailed 2D and 3D interactions between the active sites of *S. parasitica* proteins and myricetin (Bhat *et al*., 2020a), an phytochemical compounds present in *M. esculenta* are given in Fig. 4 and 5. The molecular docking result showed better binding affinities with *S. parasitica* htp^{-1} and V type proton kinase with the binding energy of -5.1 and -6.8 Kcal mol^{-1}, respectively. Besides several hydrophobic interactions, two conventional hydrogen bonds were reported between the V type proton kinase and myricetin, thus followed the Lipinski's "rule of five (Lipinski *et al.,* 2004; Bhat *et al.,* 2020b). There are around 969 proteins responsible for virulence of *Saprolegnia* (Jiang *et al.,* 2013), among these V-Type Proton ATPase and host targeting protein has crucial role for the virulence of oomycetes species through vacuolar, and cytoplasmic pH homeostasis (Hayek *et al.,* 2014; Srivastava *et al.,* 2018).

The docking results revealed that, the strong molecular docking interaction of myricetin with V-type ATPase which indicates the inhibitory virulence of *S. parasitica* may act as a potent anti-oomycetes agent. In another study, the myricetin forms 11 hydrogen bonds and showed inhibitory activity against *Aeromonas hydrophila* (Bhat *et al.,* 2020a). With regards to anti-oomycetes activity of myricetin, no previous reports are available to collaborate our study. The results were consistent with *in vitro* studies, possibly due to its inhibitory activity and decrease

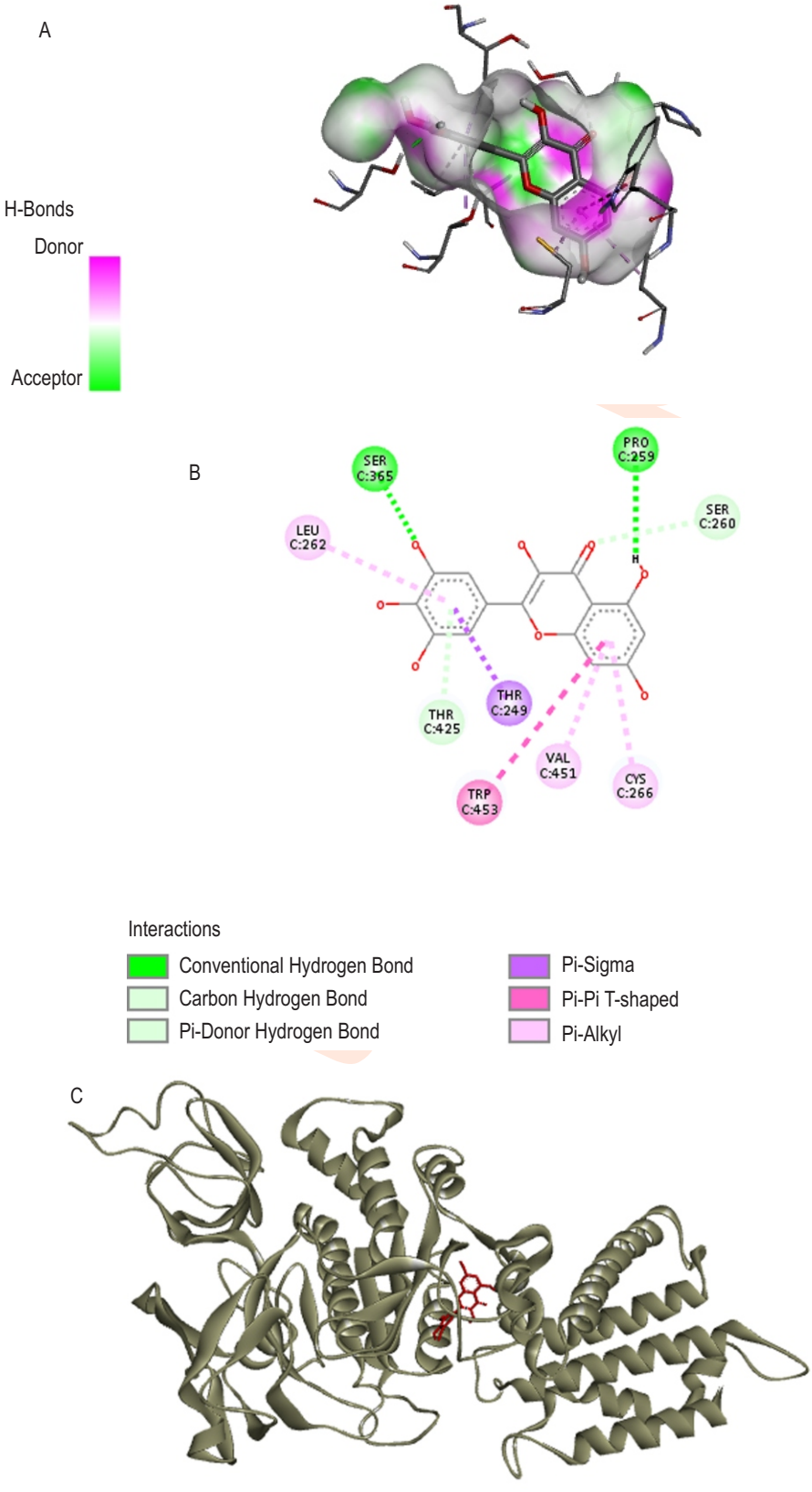

Fig. 5: 2D (A-B) and 3D (C) presentation of docked H bonds on the active sites of V type protein kinase of *S. parasitica* with myricetin.

virulence of *S. parasitica*. The present investigation concludes that the ethanolic extract of *M. esculenta*, *T. linearis* and *B. monosperma* showed anti-oomycetes activity against *S. parasitica* and *S. australis* pathogen under *in-vitro* conditions. The anti-oomycetes action was due to inhibition of hyphal growth and spore germination of *Saprolegnia* pathogen. The binding prediction of *M. esculenta* with target proteins demonstrated that it has more specificity towards the Htp site and TKL protein kinase of *S. parasitica*. This study will enable us to develop a safe anti-oomycetes agent for effective control of different life stages of Saprolegnia spp. in rainbow trout farming. In final note, further research is needed on safety and efficacy of use of *M. esculenta*, *T. linearis*, *B. monosperma* extract and its compound with respect to different life stages of fish, and these plant extract can be used as a potent anti-oomycetes agent in aquaculture.

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Add-on Information

Authors' contribution: R.S. Tandel: Conceptualization, Methodology; N. K. Chadha: Project Administration; **P. Dash, R.A.H. Bhat:** Data curation, Editing & reviewing; **P B Sawant, N.N. Pandey, S. Chandra, D. Thakuaria:** Supervision.

Research content: The research content of manuscript is original and has not been published elsewhere.

Ethical approval: All applicable international, national, and/or institutional guidelines were followed (Institutional Animal Ethics Committee of ICAR-DCFR/Ref. No. IAEC/44(127)/2008/DC/3842-48) for sampling, maintenance, handling during experiments.

Conflict of interest: No conflict of interest was reported by the author(s).

Data from other sources: The supplement data generated during and analysed during the current study are available from the corresponding author on reasonable request.

Consent to publish: All authors agree to publish the paper in *Journal of Environmental Biology*.

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