RESEARCH ARTICLE Indian Journal of Animal Research, Volume 60 Issue 11: 2019-2026 (November 2026)

Antimycobacterial Activity of Phytochemical Extracts from Herbs against the Non-tuberculous *Mycobacterium fortuitum* Infecting Fish

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ABSTRACT

Background: Non-tuberculous mycobacteriosis (NTM) is widespread in natural and grown edible and ornamental fish and is caused by *Mycobacterium fortuitum*. Mycobacterial infection in fish causes clinical signs like discoloration of the skin and exposed sores affecting the commercial value of fish. Moreover, mycobacteriosis is thought as a zoonotic disease making it much worse for edible fishes.

Methods: The antimycobacterial efficacy of the phytochemical extracts from *Tribulus terrestris, Mimosa pudica, Leucas aspera, Foeniculum vulgare, Cinnamomum verum and Acorus calamus* was studied against *Mycobacterium fortuitum* secluded from a diseased ornamental fish, *Betta splendens*. The antimycobacterial efficiency of extracts of these herbs made with water and methanol was estimated *in vitro* using a 96-well microplate assay technique with a Resazurin dye.

Result: The aqueous and methanolic extracts of *Acorus calamus*, *Mimosa pudica and Cinnamomum verum* (50 µg/ml) exhibited the highest antimycobacterial activity after that *Foeniculum vulgare* and *Tribulus terrestris* (100 µg/ml). The least inhibition activity was exhibited by *Leucas aspera* (200 µg/ml). These results revealed that the extracts of *Acorus calamus, Cinnamomum verum and Mimosa pudica* can be used as an alternative to the present antimycobacterial drugs for the treatment of Mycobacteriosis in edible and ornamental fish. However, further studies on the active compounds involved in the antimycobacterial activities of these herbs are needed.

Key words: MIC, *Mycobacterium fortuitum,* Phytochemical analysis, Resazurin.

INTRODUCTION

Mycobacteriosis in fish is due to the Non-Tuberculous Mycobacterium (NTM) or atypical mycobacterial species *viz*., *M. chelonae, M. fortitum* and *M. marinum* (Gcebe *et al.*, 2018). Mycobacteriosis has been reported to infect edible and ornamental fishes like cobia (Lowry and Smith, 2007), European seabass (Colorni, 1992), *Danio* spp. (Shamsudin, 1990), *Colisa* spp, (Marzouk *et al.,* 2009), *Trichogaster* spp. (Peterson *et al.,* 2013) and *Channa striatus* (Snakehead) (Chinabut *et al.,* 1990). *Mycobacteria* are acid-fast, aerobic, non-motile, gram-positive, non-spore-forming and pleomorphic bacteria having varied phenotypes like colony appearance, growth rate, ecological distribution and fast or slow growing with pathogenic potential (Cook *et al.,* 2009). While discussing fish mycobacteriosis, it is also important to note the broader context of mycobacterial diseases affecting both animals and humans. Tuberculosis is a worldwide contagious and chronic disease of humans as well as domestic animals with zoonotic potential. The *Mycobacterium bovis* and *Mycobacterium tuberculosis* are the main cause of tuberculosis. It has worldwide distribution with significant effect on animals and has public health importance (Basit *et al.,* 2015). Due to the increasing demand for freshwater fish in the local market, food fish are also being imported from mainland India, which may further elevate the risk of introducing and spreading bacterial pathogens (Praveenraj *et al.,* 2024).

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How to cite this article: Gangatharan, S., Uma, A., Tirumurugaan, K.G., Shanmugam, S.A. and Pandian, A.K. (2026). Antimycobacterial Activity of Phytochemical Extracts from Herbs against the Non-tuberculous *Mycobacterium fortuitum* Infecting Fish. I*ndian Journal of Animal Research*. **60(11):** 2019-2026. doi: 10.18805/IJAR.B-5586.

Submitted: 21-03-2025 **Accepted:** 31-05-2025 **Online:** 23-06-2025

Non-tuberculosis mycobacterial infections are very common in fish, especially in exhibition aquaria and in concentrated aquaculture systems. These infections are

commonly referred to as simple mycobacteriosis. All fish varieties are vulnerable to these infections while few species of fish are at greater risk. *Mycobacterium* was found in a wider range of fish species irrespective of the water environment. About twenty species of NTMs have been documented to be associated with granulomatous infection in fish and fish handlers in India (Saha *et al.,* 2015; Sengupta *et al.,* 2012).

However, this ornamental fishkeeping sector is increasingly challenged by bacterial diseases that compromise fish health, marketability and export potential. Among these, *M. fortuitum*, a non-tuberculous mycobacterium (NTM), is emerging as a significant pathogen in ornamental fish. A surveillance study conducted from December 2022 to August 2023 in Kolathur revealed that 29.4% of 196 fish samples were infected with bacterial pathogens, with *M. fortuitum* accounting for 5.6% of the infections. Though less prevalent than other bacteria like *Aeromonas veronii* or *Pseudomonas aeruginosa*, *M. fortuitum* poses a unique challenge due to its chronic nature, resistance to conventional treatments and zoonotic potential. Its detection underscores the urgent need for targeted diagnostics, surveillance and the development of alternative therapies to manage mycobacterial infections in ornamental fish (Srinath *et al.*, 2024).

Mycobacteriosis in fish is a continuously spreading infection triggered by different mycobacterial species mostly by *M. chelone* and *M. fortuitum* in both marine and freshwater environments in temperate, cold, tropical and subtropical waterbodies (Hashish *et al.,* 2018).

Herbs have been used for the treatment and control of diseases, since ancient times for their medicinal properties (Reilly *et al.,* 1997). Many herbs possess phytochemicals with different mechanisms of action due to chemical diversity and structural complexity that are active against a wide range of pathogenic microorganisms. Indiscriminate uses of antibiotics to prevent fish diseases are related to antimycobacterial resistance development and the existence of antibiotic remains in edible fishes. The antibacterial activities of the herbs and their use for the control of fish diseases as an alternative have been documented by various researchers.

Herbal extracts are of key importance owing to the presence of phytochemicals and their antimicrobial potential that lack any bacterial resistance to date. Owing to the transformed curiosity in the past decade towards phytochemicals as a source of new therapeutics, herbs were analyzed for many pharmacological effects as well as anti-mycobacterial activity. Phytochemicals have formed the basis for the identification of new drugs with novel mechanisms of action (Okunade *et al.,* 2004; Abreu *et al.,* 2012).

Acorus calamus L. or sweet flag, is a perennial, semi-aquatic herb of the Araceae family with sword-shaped leaves and creeping rhizomes. Found in swampy areas and canal edges, its rhizomes have been traditionally used in Ayurveda as a sedative and for treating asthma, fever, digestive issues, epilepsy, skin problems, mental disorders and rheumatism (Muchtaromah *et al.,* 2019).

Cinnamomum verum is a perennial tree from the family Lauraceae. The extract of its bark was found to have rich sources of compounds possessing antibacterial, antibiofilm, anthelmintic, anticancer and antifungal activity (Singh *et al.,* 2021B).

Foeniculum vulgare, commonly called Fennel is a yearly, flowering herb from the family Apiaceae and is broadly used as a spice and medicine. The seeds and the fennel herb were used in the effective control of several infectious ailment striggered by *Mycobacterium*, fungal and viral infections (Rather *et al.,* 2016).

Leucas aspera belonging to the family Lamiaceae is an aromatic herbaceous plant commonly available in India and used in traditional healthcare systems for treating chronic skin eruptions, wounds, inflammation, pain and asthma (Chetia *et al.,* 2020).

Mimosa pudica L. from the family Mimosaceae, commonly called touch-me-not plant or shame plant is a horizontal creeper widely reported to possess wound healing activity, regeneration of sciatic nerve and anti-venom activity (Gandhiraja *et al.,* 2009).

Tribulus terrestris belonging to the family Zygophyllaceae was used in both Chinese and Indian classical medication for treating various diseases. It has various chemical components in various parts of the plant body with analgesic, absorption enhancing, anthelmintic, antibacterial, anticarcinogenic, antidiabetic, anti-inflammatory, antispasmodic, anti-urolithic, aphrodisiac, immunomodulatory, cardiotonic, diuretic, hepatoprotective, hypolipidemic and larvicidal activities (Zhu *et al.,* 2017).

Resazurin indicator is a commonly used compound in microbial viability assays, including antimicrobial susceptibility testing. It is a blue, non-toxic dye that undergoes a color change in the presence of viable cells. The principle behind using Resazurin is based on its ability to undergo a chemical reduction in the presence of metabolically active cells. When added to the wells containing the mycobacterial cells and plant extracts, if the cells are viable and metabolically active, they will reduce the Resazurin dye to its reduced form, resorufin. This reduction process is facilitated by the activity of cellular enzymes and the electron transport chain of actively growing cells.

The reduction of Resazurin leads to a color change in the solution. The dye changes from its initial blue color to a pink or fluorescent color, indicating that the mycobacterial cells are viable and actively metabolizing. This color change occurs only in the presence of live cells that possess the necessary metabolic activity to reduce the dye. On the other hand, if the mycobacterial cells are not viable or their metabolic activity is inhibited by the plant extracts, the Resazurin dye will remain in its oxidized blue form. This lack of color change indicates that the cells are not viable or that their metabolic activity has been significantly impaired.

As effective treatment methods are not available for *Mycobacterium*, there is a need to identify new compounds to control *Mycobacterium* (Roberts *et al.,* 2005). The antimicrobial activity of several herb species has already been tried in comparison with the activity of Rifampicin, a widely used antibiotic for treating mycobacterial infection (Newton *et al.,* 2000).

This present study was aimed to estimate the antimycobacterial activity of *A. calamus, C. verum, F. vulgare, L. aspera, M. pudica* and *T. terrestris* against *M. fortuitum* infecting fish.

MATERIALS AND METHODS

The research work was carried out during the period 2021-2024 at the State Referral Laboratory for Aquatic Animal Health, Tamil Nadu Dr. J. Jayalalithaa Fisheries University, Madhavaram Campus, Chennai, Tamil Nadu, India.

Collection of herbs

Whole herbs of *L. aspera, M. pudica, T. terrestris*, roots of *A. calamus,* the bark of *C. verum and* the seed of *F. vulgare* were collected from a local plant nursery and their identity was confirmed with the help of a botanist.

Synthesis of aqueous herbal extract

The gathered herbs were washed and cut into small pieces, shade-dried and ground to get powdery form. Aqueous extract was prepared with 10 g of the powdered herb in 100 ml of sterile distilled water by keeping it in a shaker incubator for 24 h and filtered through a sterile funnel using Whatman No.1 filter paper (No. 42-125 mm). The filtered solution was kept in sterile petri plates for evaporation in a sterile environment and the final aqueous extract was kept at 4°C for future research.

Preparation of methanol extract

About 40 g of powdered herb was Soxhlet-extracted using methanol at 80°C for 72 h. The herbal powder was taken in a porous bag made of filter paper kept in the thimble. 300 ml of methanol was added to the sterile round bottom flask. Along with the condenser, the apparatus was fitted over the Mantoux heater at a constant temperature of 40 to 50°C. This process continued until there were no traces of residual liquid droplets in the siphon arm. The obtained crude extract was transferred into sterile petri plates and air dried to obtain the extract powder which was preserved at 4°C for future applications (Redfern *et al.*, 2014).

Phytochemical analysis

The aqueous and methanol extracts of the herbal plants were characterized by the existence of phytochemical constituents like saponins, alkaloids, tannins, glycosides, terpenoids and flavonoids following standard procedures (Mujeeb *et al.,* 2014).

Test for tannin

0.5 ml of extract was mixed with 10 ml distilled water, filtered and treated with 5% ferric chloride. A blue-black precipitate indicated tannins.

Test for saponins

0.5 ml of extract was shaken with 5 ml distilled water. Persistent frothing confirmed saponins.

Test for terpenoids

0.5 ml of extract was mixed with 5 ml chloroform, filtered, then treated with acetic anhydride and concentrated sulphuric acid. A pink interphase confirmed terpenoids.

Test for flavonoids

Magnesium and concentrated HCl were added to the extract. An orange or crimson color indicated flavonoids.

Test for glycosides

0.5 ml of extract was treated with 2 ml chloroform and layered with concentrated sulphuric acid. A reddish-brown interphase confirmed glycosides.

Test for alkaloids

0.5 ml of extract was heated with 5 ml 1% HCl, filtered and treated with Dragendorff's reagent. Turbidity or precipitate indicated alkaloids.

Inoculum preparation

M. fortuitum (Gen Bank Accession no. OP754899) isolated from *Betta splendeus* and maintained in the repository of State referral laboratory for aquatic animal health of Tamilnadu Dr.J.Jayalalithaa Fisheries University, Madhavaram campus was used for the study. The isolate was revived by inoculating in Middlebrook 7H9 broth (Becton Dickinson, Sparks, MD) accompanied with 0.2% glycerol (Himedia, India) and 10% oleic acid albumin dextrose catalase (OADC; Becton Dickinson) and maintained at 37°C till exponential growth was attained. The mycobacterial culture was then suspended in an adequate amount of sterilized supplemented Middlebrook 7H9 media to attain a turbidness corresponding to McFarland's No.1 standard. This suspension was further diluted in the ratio of 1:50 using the same culture media to get nearly 6×10^6 CFU/ml instantly prior to use (Akbari *et al.,* 2014) and this was used as the test inoculum.

Resazurin microtitre assay (REMA)

The antimycobacterial activities of crude herbal extracts made from water and methanol were tested against *M. fortuitum* by Resazurin Microtitre Assay (REMA) (Singh *et al.,* 2021A). In order to reduce the evaporation loss from the plates, the outer border of all the wells were filled with 200 µl of sterile water. About 200 µl of supplemented Middlebrook 7H9 broth and LJ media were added to all the wells. The herbal extracts were thawed to room temperature and around 200 µl was added to the first well of each row and two serial dilution series were done from that well over the microplate column. Precisely 100 µl of the *M. fortuitum* (6×10^6 CFU/ml) inoculum was inoculated to all the wells including the test as well as control. Rifampicin was utilized as positive control whereas an extract-free medium wasemployed as negative control. The microplates were

incubated for 5 days at 37°C. After the incubation time, a mixture of 20 µl of resazurin indicator was treated with 12 µl of sterilized 10% Tween 80 and the plates were kept for incubation again at 37°C for 24 h for color development. Clear pink colored wells were marked as positive for growth. The least concentration of the herbal extracts that prohibited the color change was the minimal inhibitory concentration (MIC) (Cantrell *et al*., 2001).

Statistical analysis

All the experimental data were evaluated *via* the IBM SPSS 26 statistical package for Windows. Shapiro-Wilk test was used to determine the normal data distribution and the values were given in Mean ± Standard Deviation. Two-way ANOVA was employed to analyze the interaction between the effects of two independent variables namely the herbal extracts name and their concentrations.

RESULTS AND DISCUSSION

Phytochemical analysis of herb extracts

The screening of phytochemicals in the herbal extracts exposed the presence of glycosides, saponins, terpenoids, flavonoids, alkaloids and tannins (Table 1). The presence of these bioactive compounds plays a crucial role in the antimycobacterial activity of the extracts. The antimycobacterial activity against *M. fortuitum* was likely due to one or more of these phytochemicals, as their combined effect contributes to the inhibitory properties of the extracts. The activity of the crude extracts was a result of the cooperative action of complex phytochemicals (Kumar *et al.,* 2014). Metabolites like terpenes, steroids and alkaloids were abundant and served as potential frameworks for developing new anti-mycobacterial drugs (Khameneh *et al.,* 2019).

Resazurin microtitre assay (REMA)

The antimycobacterial activity of both aqueous and methanolic extracts of *L. aspera, M. pudica, T. terrestris, A. calamus, C. verum and F. vulgare* was tested using the Resazurin indicator against *M. fortuitum* by determining the viability of mycobacterial cells in a 96-well plate. The REMA assay is a quick, non-radiometric, high-throughput assay for detecting antibacterial activity based on the redox reaction of Resazurin dye (O'Brien and Pognan, 2001). The dye is reduced in the presence of metabolic activity, resulting in a color change that indicates the extracts' inhibition ability on *M. fortuitum*. A pink color indicated bacterial growth, whereas a blue color signified inhibition (Fig 1).

By observing the color changes in the wells, the antimycobacterial effect of the extracts was determined. The aqueous and methanolic extracts of *A. calamus, M. pudica* and *C. verum* exhibited the highest inhibition activity against *M. fortuitum* at 50 µg/ml. *F. vulgare* and *T. terrestris* required a higher concentration of 100 µg/ml to achieve similar inhibition, while *L. aspera* showed the lowest inhibition activity at 200 µg/ml (Table 2, Fig 2). Importantly, no inhibition was observed at lower concentrations

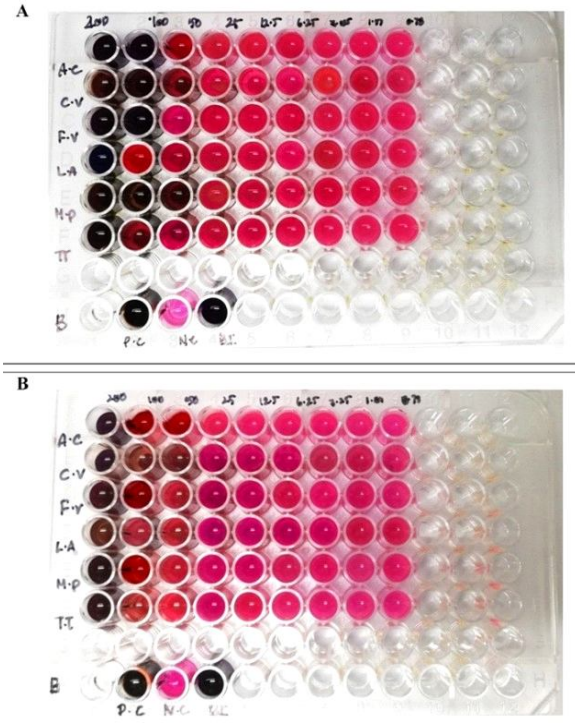
Table 1: Phytochemical constituents in the herbal extracts.

Plant sources	Phytochemical tests																	
	Aqueous extract							Methano extract										
	Glycosides	Saponin	Terpenoids	Flavonoids	Alkaloids	Tannin	Glycosides	Saponin	Terpenoids	Flavonoids	Alkaloids	Tannin	Glycosides	Saponin	Terpenoids	Flavonoids	Alkaloids	Tannin
Leucas aspera	+	+	-	+	+	+	+	+	-	+	+	+	+	+	-	+	+	+
Mimosa pudica	+	+	-	-	+	+	+	-	-	+	+	-	+	-	-	+	+	-
Tribulus terrestris	+	+	-	+	+	-	+	+	-	+	-	+	+	+	-	-	-	+
Acorus calamus	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cinnamomum verum	++	++	+	++	++	++	+	+	+	+	++	+	+	++	+	+	++	+
Foeniculum vulgare	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	++

(+) = Indicates presence; (-) = Indicates absence.

ranging from 0.78 to 25 µg/ml in all tested extracts, indicating their ineffectiveness at lower doses.

The REMA assay findings in this study align with previous research demonstrating strong antimycobacterial activity against *M. avium, M. tuberculosis and BCG* (Sudjarwo *et al.,* 2019). Similar results were obtained in *Mycobacterium tuberculosis* isolates, with higher sensitivity and specificity, using the Resazurin assay (Yajko *et al.,* 1995). The antimycobacterial activity of *C. verum* oil against *M. tuberculosis* using the Alamar Blue assay (Sawicki *et al.,* 2018) was comparable to the present findings. Additionally, ethanolic extracts of *M. pudica* exhibited similar inhibitory effects against *M. smegmatis* using the disc diffusion assay. The antimycobacterial activity of *C. indica, C. sophera, M. dioica, U. dioica* and *T. terrestris* was also consistent with these findings (Gupta *et al.,* 2011).



A- Aqueous extract; B- Methanol extract Mycobacterial extract by REMA (A.C: *Acorus calamus*, C.V: *Cinnamomum verum*, F.V: *Foeniculum vulgare*, L.A*: Leucas aspera*, M.P: *Mimosa pudica*, T.T: *Tribulus terrestris,* B: Blank, P.C: Positive control, N.C: Negative Control, I: Blank indicator).

Fig 1: Resazurin microtitre assay.

A related study using the BACTEC MGIT 960 system found that methanolic extracts of *Piper longum*, *Cressa cretica* and *Calotropis gigantea* inhibited *M. tuberculosis* H37Rv, with MICs of 125-250 µg/ml (Tamrakar *et al.,* 2022). Although these concentrations are higher than those observed in our study, the results support the potential of phytochemical-rich plant extracts as effective antimycobacterial agents, reinforcing the role of natural compounds in combating mycobacterial infections.

Significance of the findings

These results provide preliminary evidence that *A. calamus, M. pudica* and *C. verum* extracts hold strong potential for treating non-tuberculous mycobacteriosis caused by *M. fortuitum* in fish. Their highest inhibition activity at a relatively low concentration (50 µg/ml) suggests their effectiveness in combating mycobacterial infections. *F. vulgare* and *T. terrestris* demonstrated inhibition at 100 µg/ml, requiring a higher dosage for comparable effects, while L. *aspera* was the least effective, inhibiting at 200 µg/ml (Fig 3).

These results align with previous studies where a variety of herbal extracts exhibited antimycobacterial activity against *M. tuberculosis* and *M. smegmatis*. The presence of bioactive compounds like alkaloids, flavonoids, phenolic compounds and terpenoids further supports the potential of these plant extracts as natural alternatives to existing

Table 2: Antimycobacterial efficacy of herb extracts and rifampicin.

Herbs	Nature of extract	A_{570} value				
		200 µg/mL	100 µg/mL	50 µg/mL	Positive control (Rifampicin)	Negative control
Acorus calamus	Aqueous	3.292	2.696	1.703		
	Methanol	3.614	3.3	1.183		
Cinnamomum verum	Aqueous	2.969	2.775	1.014		
	Methanol	2.969	2.504	1.114		
Foeniculum vulgare	Aqueous	2.335	1.653	0.52		
	Methanol	2.889	1.739	0.44		
Leucas aspera	Aqueous	1.265	0.51	0.51		
	Methanol	1.521	0.90	0.476	3.932	0.34
Mimosa pudica	Aqueous	3.153	2.892	1.043		
	Methanol	3.319	2.832	1.287		
Tribulus terrestris	Aqueous	2.117	1.394	0.53		
	Methanol	2.646	1.531	0.456		

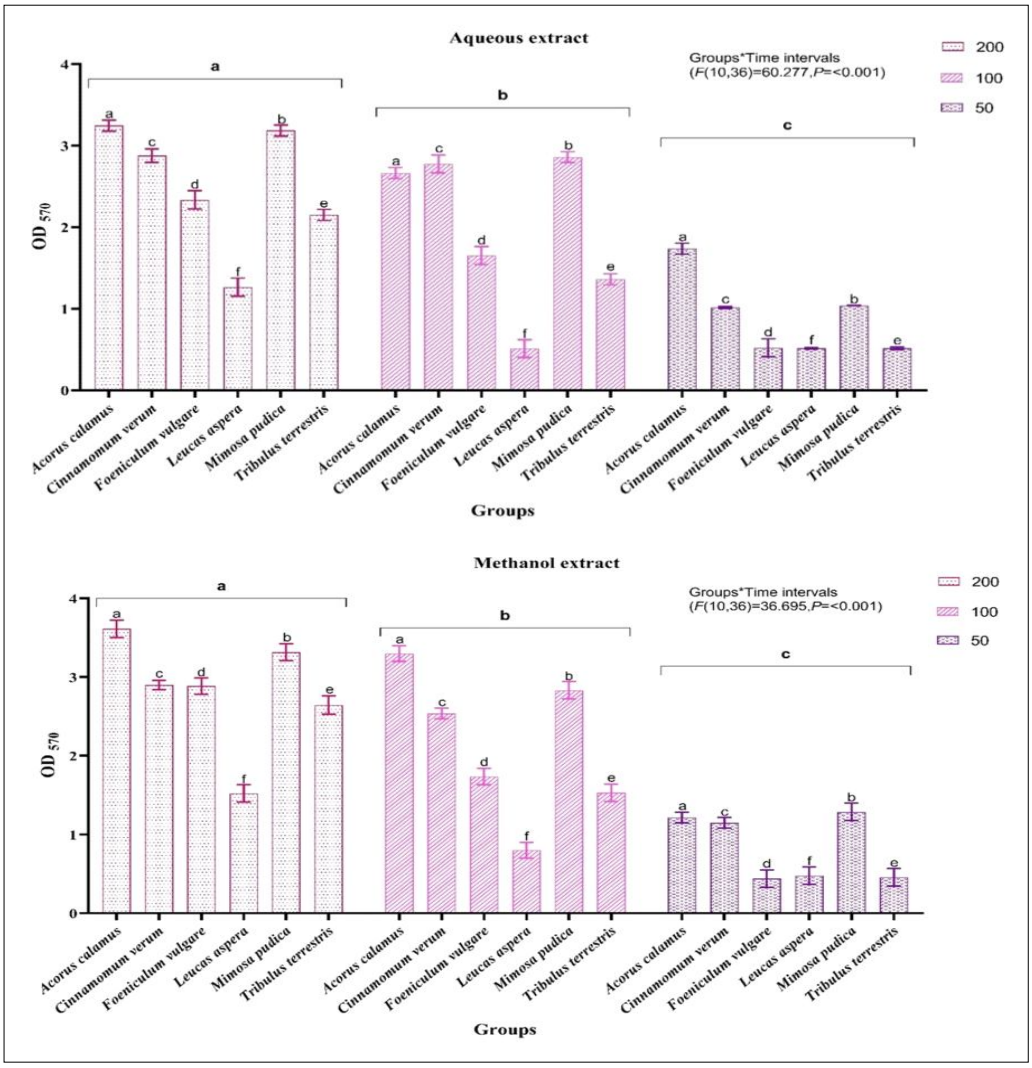


Fig 2: Antimycobacterial efficacy of the herbal extracts.

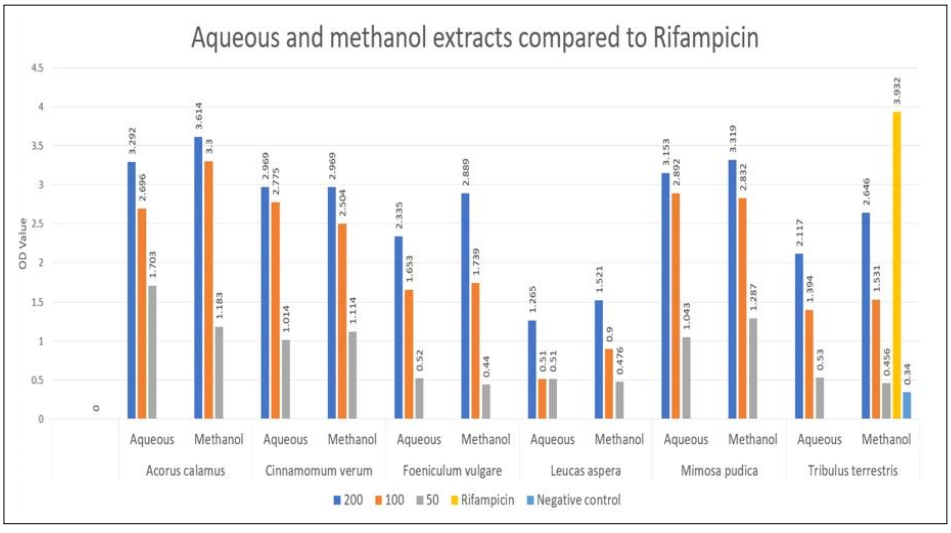


Fig 3: Aqueous and methanol extracts compared to rifampicin.

antimycobacterial drugs. However, further studies are necessary to isolate and characterize the active compounds responsible for antimycobacterial activity. Additional validation studies are required to confirm the efficacy, safety and optimal dosage of these extracts for practical application in treating non-tuberculosis mycobacteriosis in fish populations.

In comparison to a study by Guz *et al.* (2025), which assessed the antibacterial effect of a chloroform extract of *Thymus vulgaris* against both slow- and rapid-growing mycobacterial strains from ornamental fish, several similarities and differences emerge. Both studies focus on non-tuberculous mycobacteria (NTM) in fish, underscoring the need for effective, fish-safe alternatives to conventional antibiotics. While that study investigated a single herb using a chloroform extract, our study examined multiple plant species using aqueous and methanolic solvents, offering a broader comparative approach.

Regarding potency, the *T. vulgaris* extract showed MIC_{50} values of 200 µg/ml for rapid-growing mycobacteria (RGM) and 50 µg/ml for slow-growing mycobacteria (SGM). In comparison, our study found *A. calamus*, *C. verum* and *M. pudica* effective at 50 µg/ml against *M. fortuitum* (a rapid grower), suggesting potentially stronger activity in certain plant extracts, depending on the solvent and compound profiles.

Overall, both studies support the concept that medicinal plants, through diverse phytochemicals, can inhibit NTM strains affecting ornamental fish. Our findings strengthen the case for developing multi-herbal, phytochemical-based strategies for managing mycobacterial infections in aquaculture. However, further studies are necessary to isolate and characterize the active compounds responsible for antimycobacterial activity. Additional validation studies are required to confirm the efficacy, safety and optimal dosage of these extracts for practical application in treating non-tuberculosis mycobacteriosis in fish populations.

CONCLUSION

The primary results presented in this research revealed the inhibitory ability of these 6 herbal extracts against *M. fortuitum*. The extracts of *A. calamus, M. pudica* and *C. verum* showed effective antimycobacterial activity against *M. fortuitum* and it could be used for the control of non-tuberculosis mycobacteriosis caused by *M. fortuitum* in fish. However, further studies on the characteristics of the extracts and *in-vivo* studies using the fish models to understand the active compounds and their role in control of mycobacteriosis.

ACKNOWLEDGEMENT

The authors acknowledge the research facilities extended by Tamil Nadu Fisheries University to carry out this research work.

Conflict of Interest

The authors declare that they have no competing interests.

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