Biological Forum – An International Journal **15(5): 248-253(2023)**

ISSN No. (Print): 0975-1130
ISSN No. (Online): 2249-3239

In vitro Antibacterial Activity and FTIR Analysis of *Anisomeles malabarica* (L.) Leaves Extract against *Mycobacterium marinum* Affecting Gold Fish (*Carassius auratus*)

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(Received: 20 February 2023; Revised: 14 April 2023; Accepted: 17 April 2023; Published: 20 May 2023)
(Published by Research Trend)

ABSTRACT: In the present study, *Mycobacterium marinum*, a bacteria isolated from infected gold fish (*Carassius auratus*) was tested for its antibacterial potency against *Anisomeles malabarica* leaf – methanol extracts. Disc diffusion plates on agar were used to measure the antibacterial activity. Different concentrations (0.25 µl, 0.5 µl, 0.75 µl and 1.0 µl) of *A. malabarica* leaf-methanol extract was tested against *M. marinum.* The *A. malabarica* leaf - methanol extract was exposed a consequence level of inhibition against *M. marinum*. Maximum zone was measured after 24 hours at 37°C. The result of the present study revealed that the fish pathogen *M. marinum* was exhibited maximum zone of clearance (18 mm) at 1.0 µl of concentration, where as the minimum zone of clearance (8 mm) was recorded at 0.25 µl of concentration. Fourier Transform Infra-Red Spectroscopy (FTIR) of *A. malabarica* leaf – methanol extract revealed the presence of alkenes, nitrogen, aliphatic, oxygen and hydroxyl groups. The Phytochemical analysis of *A. malabarica* leaf – methanol extract showed the presence of tannins, flavonoids, carbohydrates, phenols, proteins, steroids, saponins and coumarins. As per the result of the present study, it can be concluded that qualitative phytochemicals were identified in *A. malabarica* leaf - methanol extract was effective against *M. marinum*, a microbial pathogen that has infected gold fish (*C. auratus*). To verify these results, *in vitro* and *in vivo* experiments are required.

Keywords: *M. marinum, A. malabarica,* phytochemicals, antibacterial activity, FTIR analysis.

INTRODUCTION

Anisomeles malabarica belongs to the family Lamiaceae is found throughout tropical and Subtropical regions of India. It is perpendicular shrub commonly known as Malabar Catmint spread throughout South India (Longman, 1994). The essential oil present in herb is used in uterine affection (Kritikar and Babu 1993). *A. malabarica* is reported to have antibacterial, antipyretic, analgesic, anti – inflammatory, anti - allergic, anthelmintic, antiseptic activities and it also act as genuine herbicide in wheat fields (Dharmasiri *et al*., 2003). The leaves of *A. malabarica* consist of diterpenoids, ovatodiolide, and its derivatives that are used as HTV inhibitors (Alma *et al*., 2003). As a result of indiscriminate use of antimicrobial drugs in the treatment of infectious diseases, pathogenic microbial agents have developed resistance to many old and newly produced antibiotic drugs from plants, this has led to screening of medicinal plants for their potential antimicrobial activity (Sharma *et al*., 2010). Many efforts have been made to discover new antimicrobial compounds from various kinds of sources such as micro –organisms, animals and plants. One such resource is folk medicines. Systematic screening of them may result in the discovery of novel effective compounds (Vinod *et al.*, 2014). There has been growing interest regarding thousands of bioactive compounds that has been produced from plants, compounds are referred to us phytochemicals (Dubey *et al*., 2004). Systematic study of higher plants for detecting antimicrobial activity is of comparatively recent origin (Neeraj Choudhar *et al.*, 2012). The majority of plant extracts exhibited some biological properties that are employed in medical field (Bhavadharaniparkavi and Abirami 2023). However antimicrobial activities which has indeed formed the basis for their applications in some pharmaceuticals, alternative medicine and natural therapies (Reynolds,

1996; Lis- Balchin and Deans 1997). Antimicrobial activity and investigation of phytochemical compounds present in the leaves of *A. malabarica*. This study investigated the in vitro antibacterial activity and qualitative phytochemical screening of *A. malabarica* leaf - methanol extract against the pathogen *M. marinum* - infected gold fish (*Carassius auratus*).

MATERIAL AND METHODS

Collection of *A. malabarica* plant leaves. Fresh leaves of *A. malabarica* were collected from Kanyakumari District of Tamil Nadu, India and transported to laboratory. The leaves were rinsed with distilled water before being dried in the shade under hot air with a maximum temperature of 40°C. The samples were adequately crushed using a mortar and pestle to obtained a fine, homogenous powder, which was then stored in paper bags free from moisture (Saraswathi Krishna *et al*., 2019).

Preparation of plant extracts. *A. malabarica* leaf extracts were obtained utilize a continuous extraction system (Soxhlet extractor) using the method given by (Wang and Weller, 2006) for extracting plant leaves with organic solvent methanol. In addition to 300 mL of methanol, 30 g of plant powder was added to the thimble holder of the Soxhlet device (rate 1:10 w: v). In the thimble-holder of Soxhlet apparatus, a 75% methanol extraction solvent was utilized. Four hours were spent extracting until the solvent that emerged from the thimble turned colorless. Subsequently, in order to concentrate the extracts, they were dried using a rotating vacuum evaporator at temperatures below 40° C until the moisture content reached around 8 % (Dry basis). The crude extracts were filtered to obtain by using Whatman No. 1 filter paper. Samples were transferred in sterile vials and refrigerated at 4 ° C for future studies.

Phytochemical analysis of *A. Malabarica* leaf - methanol extract. The phytochemical analysis of crude *A. malabarica* leaf- methanol extracts were conducted to determine the presence or absence of various bioactive constituents or secondary metabolites such as carbohydrates, coumarins, tannins, saponins, flavonoids, glycosides, phenols, proteins, steroids, and terpenoids using standard protocols (Raman, 2006).

Thin Layer Chromatography of *A. malabarica* leaf extract. Thin-Layer Chromatography (TLC) using petroleum ether: acetone (3:1) solvent systems were used to identify the primary constituents contained in the *A. malabarica* leaf – methanol extract.

The chamber is composed of a petroleum ether: acetone solvent, and the extract is delivered via capillary tubes on a TLC plate that has been pre-coated. To determine the spot of each applied leaves extract on the plate, a thin line and dots are drawn on the plate. After each mobile phase was applied to the TLC plate, it was air-dried and examined under ultraviolet light. The development of the separated bands as reflected by their retention factor (R_f) values was determined (Zeb, 2012).

FTIR (Fourier transform infrared spectroscopy) analysis of *A. malabarica* leaf –methanol extract. Using Fourier transform infrared (Bruker, Alpha T, Germany), the distinctive functional groups in the *A. malabarica* leaf – methanol extract was identified. It is often possible to get clear information about the structure of a molecule from its absorption spectrum. A small amount of extract from *A. malabarica* leaves was combined with dry potassium bromide (KBr). Mixing the sample with potassium bromide in a mortar and compressed it at a pressure of 6 bar produced the disc. The disc was then put in a sample cup of a diffuse reflectance accessory. The IR spectrum was acquired using an infrared spectrometer. The specimen was scanned between 4500 and 500 cm^{-1}. The FTIR peak values were recorded.

Collection of naturally infected goldfish – *C. auratus.* The infected gold fish sample was gathered from an aqua farm at Kanyakumari District of Tamil Nadu, India. Various kinds of clinical signs and behavioral alterations have been discovered and characterized in these infected gold fish. Under a microscope, scrapings from the body surface and fins of diseased gold fish were examined to detect and characterize the presence of molds and other parasites (Lavanya *et al.*, 2010).

Isolation of bacterial strains from infected goldfish (*C. auratus*). Naturally infected gold fish were dissected and the infected epidermis was homogenized with sterile PBS (Phosphate Buffer Saline). The sample was serially diluted to reduce bacterial proliferation (Bullock, 1971). The sample was then inoculated into nutrient agar using the spread plate method (Collins and Lyne 1976). After examining and counting the colonies, the plates were incubated for 24 hours at 37°C. On the basis of physical similarities, colonies were chosen and streaked on nutrient agar media until a pure culture was obtained. Pure colonies were picked up and streaked on nutrient agar slants and stored at 4°C for subsequent identification.

Characterization and identification of pathogens. *M. marinum* was identified by its colony form, gram nature, shape and motility. In addition, the isolates were subjected to the following biochemical tests to identify the bacteria based on their reactions: Indole, methyl red, Voges-Proskauer, citrate utilization, nitrate reduction, hydrogen sulphide production, urease, catalase and oxidase. The outcomes of biochemical characterization were compared to those published in earlier studies (Bullock, 1971).

Antibacterial activity of *A. malabarica* leaf – methanol extract. Different concentrations of the extracts 0.25 µl (25 %), 0.5 µl (50 %), 0.75 µl (75 %) and 1.0 µl(100 %) were tested against *M. marinum.* In order to cultivate the bacterial strain, nutrient agar was used. Agar- based diffusion disc plates were utilized to assess the antibacterial activity (Erturk *et al.*, 2003). The antibiotic disc was commercially available. The antibiotic disc was used as the positive control and placed on the center of the agar plates. Added different concentrations of leaf extracts of *A. malabarica* (0.25 µl, 0.5 µl, 0.75 µl, 1.0 µl) on each well. On the agar plate, the zones of inhibition (diameter in mm) were examined after each plate was incubated at 37°C for 24 hours. The antibacterial activity of solvent blanks

produced in the same manner was examined (Hashish et al., 2018).

RESULTS AND DISCUSSION

Identification of pathogens and biochemical analysis. The isolated bacteria from infected goldfish (*C. auratus*) were identified as *M. marinum* based on their biochemical and morphological characterization. The 16S rRNA gene sequencing further confirmed as *M. marinum. M. marinum* is an important bacterial pathogen as it infects gold fish, *C. auratus.* Recent studies have utilized the relatively fast-growing *M. marinum* to examine the host pathogen interfere in natural fish hosts (Leon Grayfer *et al.,* 2011). Antibacterial activity of *A. malabarica* was screened against gram positive and negative bacteria such as *E. coli, S. aureus, P. mirabilis, P. aeruginosa, K. pneumonia* (Kavitha *et al*., 2012).

Qualitative analysis of phytochemical analysis of *A. malabarica* leaf -methanol extract. The phytochemical analysis indicated the presence of bioactive chemical compounds such as coumarins, tannins, saponins, flavonoids, glycosides, phenols, proteins, steroids, terpenoids, as well as carbohydrates. Phytochemicals are defined as bio active chemical compounds produced by plants. In the present study the phytochemical analysis of *A. malabarica* leaf - methanol extract indicated the presence of various compounds including tannins, flavonoids, carbohydrates, phenols, proteins, steroids, saponins and coumarins. Similar findings were reported that the great variety of secondary metabolites present in the *A. malabarica* leaf – acetone extracts are tannins, alkaloids, saponin, glycosides, carotenoids and polyuronides have been a great source of important pharmaceutical compounds (Ulhe and Narkhede 2013). The whole plant of *A. malabarica* contains higher number of flavonoids and phenolic compounds (Packialakshmi and Periyakkal 2015). Herbs having tannins are astringent in nature and are used for treatment of diarrhea and dysentery (Subhuti Dharmanada, 2003).

Table 1 shows the results of a qualitative phytochemical analysis of *A. malabarica* leaf –methanol extract. The phytochemical analysis of A.malabarica leaf –methanol extract showed the presence of tannins, flavonoids, carbohydrates, phenols, proteins, steroids, saponins and coumarins (Plate 1).

Plate 1: Phytochemical analysis of *A. malabarica* leaf – methanol extract

Table 1: Phytochemical constituents of *A. malabarica* leaf – methanol extract.

Sr. No.	Phytochemical screening	Methanol
1.	Tannins	Positive
2.	Flavonoids	Positive
3.	Carbohydrates	Positive
4.	Terpenoids	Negative
5.	Phenols	Positive
6.	Proteins	Positive
7.	Steroids	Positive
8.	Saponins	Positive
9.	Coumarins	Positive
10.	Glycosides	Negative

Thin Layer Chromatography of *A. malabarica* leaf –methanol extract. Thin Layer Chromatography (TLC) was utilized using petroleum ether: acetone (3:1) solvent systems in order to identify the primary components that were found in the most effective extracts of *A. malabarica* leaves. Thin Layer Chromatography (TLC) is the method mainly used investigate the presence of chemical constituent qualitative and quantitatively in the plant extract. In the present study explore the Neoxanthine, Pheophytin, Chlorophyll - a and chlorophyll - b are the pigments identified in TLC plate. Similar findings reported that TLC analysis of *A. malabarica* leaf – methanol extract three different separations of compounds have been identified (Ushir *et al.*, 2010). Retention factor (R_f) values were obtained to express the movement of the separated bands (Plate 2).

$$R_f = \frac{\text{Distance traveled by solute}}{\text{Distance traveled by solvent}}$$

Table 2: **Identifying the pigment and R_f value of leaf extract**

Sr. No.	Color of spot	R_f value of sample	Pigment
1.	Yellow	0.16	Neoxanthine
2.	Grey	0.93	Pheophytin
3.	Light green	0.42	Chlorophyll b
4.	Green	0.49	Chlorophyll a

Plate 2: Thin Layer Chromatography analysis of *A. malabarica* leaf – methanol extract.

Thin-layer chromatography can be used to separate and distinguish the pigments in leaf extract (Table 2).

FTIR Spectrum of *A. malabarica* leaf – methanol extract. The FTIR spectrum was performed to determine the functional groups of the bioactive constituents in *A. malabarica* leaf – methanol extract based on the maximum value in the region of infrared radiation.

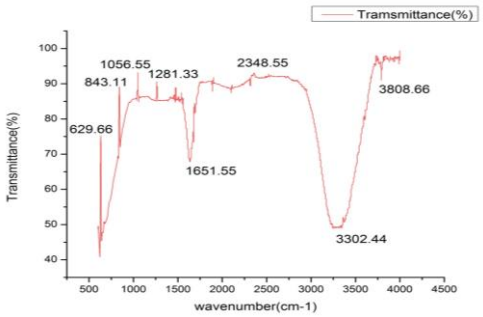

Plate 3: FTIR Spectrum of *A. malabarica* leaf methanol extract.

The results of the FTIR analysis of *A. malabarica* leaf – methanol extract showed in Plate 3.

The graph shows the distribution of different functional groups within the sample. Plate 3 indicates that an FTIR analysis A.malabarica leaf in methanol extract were performed in the wave number range of 500-4500 cm^{-1}. The graphs indicate that the presence of various chemical groups and FTIR signals are obtained from corresponding to (629.66 cm^{-1}) C=C stretching of alkenes, (843.11 cm^{-1}) N-H stretching of nitrogen groups, (1056.55 cm^{-1}) N-H stretching of aliphatic groups, C=O vibrations of oxygen(1281.33cm^{-1}), C=C stretching of alkenes (1651.55cm^{-1}), (2348.55 cm^{-1}) C=C stretching of alkenes, (3302.44 cm^{-1}) N-H stretching of nitrogen groups, (3808.66 cm^{-1}) OH stretching of hydroxyl groups. Fourier Transform Infra-Red (FTIR) was used to identify the functional groups in the extract. In the present study the results of *A. malabarica* leaf extracts of FTIR analysis confirmed the presence of C=C stretching of alkenes, N-H stretching of nitrogen compound N-H stretching of aliphatic compound, C=O vibrations of oxygen, C=C stretching of alkenes and O-H stretching of hydroxyl compounds. Similar findings reported that FTIR analysis of *A.malabarica* leaf extract contain –O-H stretching of carboxyl group, C-H stretching alkanes, C=O stretching carbonyl compounds and C-O starching (primary alcohol and ester) (Antil *et al*., 2019).

Table 3: **Antibacterial activity of different concentrations of *A. malabarica* leaf – methanol extract.**

Sample	Solvent Used	Zone of inhibition					
		Concentrations (µl)				Positive control Ampicillin (mm)	Negative control DMSO (mm)
		0.25µl	0.5 µl	0.75 µl	1.0 µl		
A. malabarica	Methanol	8	12	14	18	19	No Zone

Antibacterial Activity of *A. malabarica* leaf – methanol extract against *M. marinum*. The antibacterial activity of samples was evaluated against the identified strains of bacteria. The inhibition zone of sample extract was varying depending on the microorganism and the solvent used for the extraction. The zone of inhibition indicated that the action of these samples against the bacteria. The antimicrobial activity of the *A. malabarica* leaf – methanol extract samples were initially evaluated by agar plate diffusion method using *M. marinum* isolated from infected goldfish (*C. auratus*).

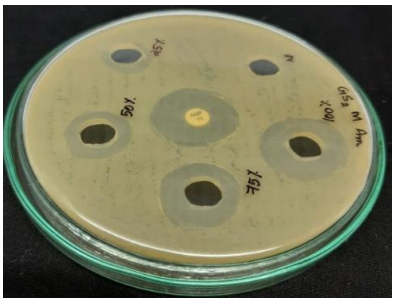

Plate 4: Antibacterial activity of different concentrations of *A. malabarica* leaf – methanol extract.

The efficacy of methanol extract to inhibit the in vitro growth of *M. marinum* was showed in Table 3. The methanol extracts of *A. malabarica* leaves were tested with four different concentrations of 0.25 µl (25 %), 0.5 µl (50 %), 0.75 µl (75 %) and 1.0 µl (100%) respectively, against the fish pathogens of *M. marinum* (Plate 4). In the present study, *A. malabarica* leaf - methanol extract showed minimum zone of inhibition at the concentration of 0.25 µl (8 mm) and maximum zone of inhibition (18 mm) at 1.0 µl concentration, whereas the 0.5 µl and 0.75 µl concentrations the moderate zone of clearance as 12 mm and 14 mm respectively. Similar findings reported the methanolic extracts of *A. malabarica* leaf showed considerably high activity against Pseudomonas auregenosa (Kavimani *et al*., 2015).

The ethanolic extract of *A. malabarica* exhibited best clearance activity against S. aureus, B. subltillis and K.pneumoniae comparison with standard drug tetracycline (Ushir *et al*., 2010).The ethanol leaves extract of *A. malabarica* revealed the remarkable antimicrobial activity comparison with the s tandard antibiotic drug (Vinod *et al*., 2014). Jeba Preethi and Jeni Chandar, 2022 reported that *A. malabarica* showed the highest zone of inhibition against *P. aeruginosa*. The methanol extract from *A. malabarica* leaves can be used as a substitute treatment for bacterial strains that cause fish disease. This study revealed that the *A. malabarica* leaf – methanol extract in effective in vitro against *M. marinum*. Treatment with an extract *A. malabarica* leaves seems most promising effect for the treatment of *M. marinum* infection in *C. auratus* gold fish.

CONCLUSIONS

The medicinal plants continue to be important therapeutic agents in both conventional and modern healthcare systems. The plant derived extracts are utilized to treat a variety of fish ailments in order to promote natural behavior and immunity as well as to prevent the transmission of infection. *A. malabarica* is a remarkable medicinal herb with a wide range of therapeutic characteristics, according to phytochemical, TLC and FTIR analysis of its leaves. In this study, *M. marinum*, a bacterium isolated from infected goldfish (*C. auratus*) was resistant to *A. malabarica* leaf – methanol extract, which shown promising antibacterial activity.

FUTURE SCOPE

A. malabarica's antibacterial and anti-microbial properties are helpful in treating a variety of ailments in fisheries. There are numerous undiscovered bioactive characteristics of this plant which opens doors for future researchers.

Acknowlegement. I extend my sincere thanks to my guide, Dr. R. Raja Jeya Sekar, for giving me proper guidance for carrying out this research work.
Conflict of Interest. None.

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How to cite this article: S. Jeba Sheeba, R. Raja Jeya Sekar, A. Harish and S.M. Vijila (2023). In Vitro Antibacterial Activity and FTIR Analysis of *Anisomeles malabarica* (L.) Leaves Extract against *Mycobacterium marinum* Affecting Gold Fish (*Carassius auratus*). *Biological Forum – An International Journal, 15*(5): 251-253.