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Evaluation of Antioxidant activity, Antimicrobial activity, Anticancer activity and DNA Barcoding of *Lepidagathis barberi* Gamble

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KEYWORDS

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ABSTRACT

The present study investigates the antioxidant, antimicrobial, anticancer potential and molecular identification of whole dried plant powder of *Lepidagathis barberi*. The antioxidant activity was evaluated using DPPH, ABTS, NO, SOD assays, The extract exhibited strong free radical scavenging activity with maximum inhibition at 500 µg/ml, showing 84.64% (DPPH), 86.05% (ABTS), 74.36% (NO), and 65.59% (SOD), confirming a dose-dependent effect. The IC_{50} values for DPPH, ABTS, NO, and SOD were 62.35 µg/ml, 60.78 µg/ml, 71.12 µg/ml, and 94.66 µg/ml, respectively, suggesting potent antioxidant potential comparable to ascorbic acid. Antimicrobial activity was assessed against five bacterial strains namely *Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, Proteus vulgaris,* and *Staphylococcus aureus* and three fungal strains *Aspergillus niger, Aspergillus flavus,* and *Penicillium notatum*. The isopropanol extract exhibited the maximum antibacterial activity (19 mm) against *P. aeruginosa* and *S. typhi*, while toluene extracts showed strong antifungal effects against *A. flavus*. Cytotoxic activity of the methanolic extract was confirmed through MTT assay against K562 human myelogenous leukemia cells, demonstrated a concentration-dependent decrease in cell viability, with an IC_{50} value of 61.24 µg/ml. Mitochondrial membrane potential (MMP) analysis and AO/EtBr staining further confirmed apoptotic induction, as evidenced by the appearance of red to greenish colour in mmp and green to orange yellowish in EtBrAo staining in treated cells. Molecular identification through DNA barcoding using the chloroplast rbcL gene confirmed sequence identity (100%) with *Lepidagathis barberi* (accession no. NC-084174.1), and phylogenetic analysis placed it closest *to Chroesthes longifolia*. Overall, the results demonstrate that *Lepidagathis barberi* possesses notable antioxidant, antimicrobial, and anticancer properties, validating its potential as a source of bioactive compounds for pharmacological applications.

1. Introduction

Medicinal plants are the local heritage with global importance and world is endowed with a rich wealth of medicinal plants. Medicinal plants are the richest bio-resource of drugs of traditional systems of medicine, modern medicines nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs (Tambe *et al*.,2021).

Free radicals are closely associated with oxidative damage and antioxidants are reducing agents, which limit oxidative damage to biological structures by donating electrons to free radicals and passivating them. The interaction of oxygen with certain molecules leads to the formation of free radicals and once formed, the chief danger comes from the damage they can inflict when they react with important cellular components including DNA, proteins and the cell membrane. These free radicals interact with the antioxidants, which can eventually neutralize them before damages are initiated. Plants synthesize several compounds as secondary metabolites and many of them act as antioxidants (Lalhminghlui & Jagetia, 2018).

Plants extracts are still the major sources of many therapeutic agents including antimicrobial agents for the treatment of infectious diseases (Mohamed *et al*.,2020). Plants have been used for centuries to treat infectious diseases and are considered as an important source of new antimicrobial agents. Several works have been done to examine the antimicrobial effects of herbal plants extracts, including roots, stem, leaves or flowers (Bereksi1 *et al.,*2018).

Cancer is perceived as one of the top leading causes of death worldwide. It is a heterogenous disease characterized by aberrant cell proliferation and invasiveness of abnormal cells which spread to neighboring tissues (Mandour *et al*.,2023). More than 60% of drugs based on the natural products or herbal raw material are currently used for the treatment of cancer. The recent scientific studies show that various food plants, medicinal plants, and spices contain the primary and secondary metabolites that help in the treatment of cancer (Mykhailenko *et al*.,2020). As a result, the use of natural therapies is an alternative to the adverse effects of synthetic drugs (Benny *et al*.,2022).

DNA barcoding is a method of identifying an organism based on sequence data from one to several gene regions. Barcoding has multiple applications and has been used for ecological surveys, cryptic taxon identification and confirmation of medicinal plant samples. Barcoding works by matching sequence data from a query sample (an unknown specimen) to a reference sequence (from a voucher specimen). DNA barcoding has the potential to uniquely identify medicinal plants and provide quality control and standardization of the plant material supplied to the pharmaceutical industry (Schori and Showalte, 2011).

The experimental plant *Lepidagathis barberi* Gamble. is an endemic medicinal plant. It is a flowering plant commonly known as Holly-leaf Foxglove, Karappan poondu in tamil . It belongs to the family Acanthaceae with more than 100 species and it is native to

Karnataka, Tamil Nadu . It is distributed in tropical and subtropical regions of the world with 148 species. It is represented by 30 species and 7 varities in India, of which 18 species and 1 variety are endemic to the country. It grows on dry sandy gravelly terrains of wastelands and scrub jungles. Its distribution areas receive less rainfall and remain dry for a large part of the year (Red data book). It is distributed in various parts such as Coimbatore, Erode, Theni, Dindigul, Tirunelveli, Virudhunagar. *Lepidagathis* has also some medicinal properties and it is traditionally used to cure some disorders. Some reports are available for the treatment of fever, psoriasis, epilepsy, eczema, skin abscess, mouth ulcer, wounds, skin itching, burns, snake bites and other skin diseases. It also shows some pharmacologically important activities such as antipyretic, antiurolithiatic, anti-inflammatory, analgesic, hypoglycaemic, wound healing, immunosuppressive and boosting fertility, flowering and fruiting is June-January (Kadam *et al*.,2022).

In this study, whole dried plant extract of *Lepidagathis barberi* was used to focused on Free radical scavenging activity by DPPH, ABTS, SOD and NO assay, Antimicrobial activity, Anticancer activity by MTT and MMP assay, EtBrAO staining and DNA Barcoding.

2. Materials And Methods

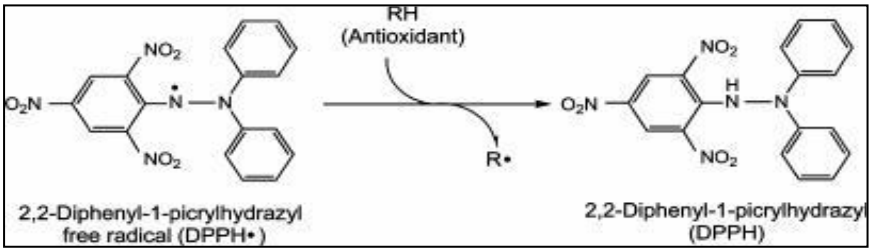
2.1 Plant Material Collection

The experimental plant, *L.barberi* was collected from G.Venkataswamy Naidu, College campus, Kovilpatti, Tamil Nadu, India. Elevation about 130m (Mean Sea Level with 9.1719° N latitude and 77.8726° E longitude). The plant specimens were identified and botanically authenticated by BSI, Kovai,with accession no:651. A voucher specimen was deposited in the herbarium of S.T. Hindu college Nagercoil. Specimens of the experimental plant species were collected and dried at room temperature (30±2ºC) for about two weeks to get a constant weight. The dried plant materials (as the whole plant) were powdered by a mechanical device and stored for further analyses.

DPPH Radical scavenging activity

Principle

The DPPH assay is popular in natural product antioxidant studies. One of the reasons is that this method is simple and sensitive. This assay is based on the theory that a hydrogen donor is an antioxidant. It measures compounds that are radical scavengers. Figure 1, below, shows the mechanism by which DPPH accepts hydrogen from an antioxidant. DPPH is one of the few stable and commercially available organic nitrogen radicals. The antioxidant effect is proportional to the disappearance of DPPH in test samples. Monitoring DPPH with a UV spectrometer has become the most commonly used method because of its simplicity and accuracy. DPPH shows a strong absorption maximum at 517 nm (purple). The color turns from purple to yellow followed by the formation of DPPH upon absorption of hydrogen from an antioxidant. This reaction is stoichiometric with respect to the number of hydrogen atoms absorbed. Therefore, the antioxidant effect can be easily evaluated by following the decrease of UV absorption at 517 nm.



Materials Required

0.1mM DPPH solution, Ascorbic acid, Methanol

0.1 mM DPPH Solution

Dissolve 39 mg of DPPH in 100 ml of methanol and store at -20° C until needed.

Ascorbic acid (Standard)

1mg/ ml of Ascorbic acid

Procedure

1. Briefly, prepare 0.1 mM of DPPH solution in methanol and add 100 µl of this solution to 300 µl of the solution of sample *L.barberi* at different concentration (500, 250, 100, 50 and 10 µg/mL).
2. The mixtures have to be shaken vigorously and allowed to stand at room temperature for 30 minutes.
3. Then the absorbance has to be measured at 517 nm using a UV-VIS spectrophotometer. (Ascorbic acid can be used as the reference).
4. Lower absorbance values of reaction mixture indicate higher free radical scavenging activity.
5. The capability of scavenging the DPPH radical can be calculated by using the following formula.

6. DPPH scavenging effect (% inhibition) = [(absorbance of control-absorbance of reaction mixture)/absorbance of control] X 100.

7. Percentage inhibition and IC50 value was calculated using Graph Pad Prism 6.0 software (USA).

ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid

Arnao *et al*., 2001)

Principle

A ferryl myoglobin radical is formed from metmyoglobin and hydrogen peroxide. The ferryl myoglobin radical can oxidize ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) to generate a radical cation, ABTS.+, that is green in color and can be measured by absorbance at 405 nm. Antioxidants suppress this reaction by electron donation radical scavenging and inhibit the formation of the colored ABTS radical. The concentration of antioxidant in the test sample is inversely proportional to the ABTS radical formation and 405 nm absorbance.

Metmyoglobin + ~~H2O2~~ → ferryl myoglobin + H2O

ABTS + ferryl ~~myoglobin~~ → ABTS -+ + metmyoglobin

(Antioxidants inhibit the oxidation of ABTS by electron transfer radical scavenging)

Materials Required

ABTS, Potassium persulfate, 1X PBS was from Himedia, (India). 96 well tissue culture plate and wash beaker were from Tarson (India).

Procedure

For ABTS assay, the procedure followed the method of Arnao *et al*., 2001 with some modifications. The stock solutions included 7 mM ABTS solution and 2.4 mM potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 14 h at room temperature in the dark. The solution was then diluted by mixing 1 ml ABTS solution with 60 ml methanol to obtain an absorbance of 0.706 ± 0.01 units at 734 nm using a spectrophotometer. Fresh ABTS solution was prepared for each assay (500, 250, 100, 50 and 10 µg/ ml) were allowed to react with 1 ml of the ABTS solution and the absorbance was taken at 734 nm after 7 min using a spectrophotometer. The ABTS scavenging capacity of the extract was compared with that of ascorbic acid and percentage inhibition calculated as ABTS radical scavenging activity (%)=Abscontrol−Abssample/ Abscontrol where $Abs_{control}$ is the absorbance of ABTS radical in methanol; Abs_{sample} is the absorbance of ABTS radical solution mixed with sample extract/standard. All determinations were performed in triplicate (n = 3).

Nitric Oxide Radical Scavenging Assay :(Marcocci *et al.*,1994 and Ebrahimzadeh *et al.*,2010)

Principle

The procedure is based on the principle that sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions.

Materials

96 well flat bottom plate, Deionized water and Griess Reagent

Procedure

The extracts were prepared from a 50 mg/mL crude extract (Ethanol). These were then serially diluted with DMSO to make concentrations from 500-10 µg/mL of the sample. These were stored at 4°C for later use. The 500, 250, 100, 50 and 10 µg/ml extract was mixed with an equal volume of freshly prepared Griess reagent (150µl). Control samples without the extracts but with an equal volume of buffer were prepared in a similar manner as was done for the test samples. After 30 minutes of incubation period, 100 µL of the reaction mixture was transferred to a 96-well plate. The absorbance was measured at 540 nm using a UV-Vis microplate reader (Molecular Devices, GA, USA).

Formula

Nitic Oxide Scanenge (%) = $A_{control} - A_{test}/A_{control} \times 100$

Percentage nitrite radical scavenging activity: where = absorbance of control sample and = absorbance in the presence of the samples.

Superoxide radicals Assay: (Jeong *et al*., 2010 and Alam *et al*.,2013)

Principle

Briefly, in SOD assay, the reaction system which had xanthine and xanthine oxidase generated superoxide anion free radical ($O^{\cdot-}_2$) as the by-product. The superoxide anion free radical thus formed oxidized hydroxylamine to form nitrite, which appears to be amaranth purple color upon reacting with developing agent and its absorbance can be detected by spectrophotometer by measuring at 550 nm. The presence of SOD in the system specifically inhibits the oxidization caused by superoxide anions and because of it, fewer nitrite anions are generated. This would lower the absorbance of the sample tube compared to the reference tube without SOD.

Materials

Eppendorf tube, 96 well flat bottom plates, EDTA, Pyrogallol, Tris-HCL Were purchased from SRL. Deionized water

Procedure

Total SOD enzymatic activity in the Test sample (*L.barberi*) was measured using pyrogallol as a substrate. In a eppendorf tube, (500, 250, 100 50 and 10 µg/ml) of the Test sample was added and supplemented with 700 µL of a Tris-HCl buffer solution, pH 8.2, and 50 µL of EDTA. Subsequently, 50 µL of the pyrogallol was added, and after 10 s of the reaction, the optical density (OD) changes were determined for one min at 420nm using a microplate reader (Thermo fisher Scientific USA). The absorbance was continuously assessed. The results of enzymatic activity were reported as the U min-1/mg protein using the following equation:

Formula:

SOD activity (%) = [1− (absorbance value of sample/ absorbance value of control)] ×100. The experiment was performed in triplicate.

Antimicrobial activity

Antibacterial activity: (Kirby Bauer disc diffusion method):

Test Organisms:

The test microorganisms used for antibacterial analysis *Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, Proteus Vulgaris* and *Staph aureus* were purchased from Microbial Type Culture Collection and Gene Bank (*MTCC*) Chandigarh. The bacterial strains were maintained on Nutrient Agar (NA).

Materials Required

Mueller- Hinton agar, Antibiotic discs, Cotton swabs, Petri dishes, 0.5 McFarla, Turbidity standard, Inoculum, Forceps, Metric ruler or caliper.

Nutrient Broth Preparation:

Pure culture from the plate were inoculated into Nutrient Agar plate and sub cultured at 37ºC for 24 h. Inoculum was prepared by aseptically adding the fresh culture into 2 ml of sterile 0.145 mol/L saline tube and the cell density was adjusted to 0.5 McFarland turbidity standard to yield a bacterial suspension of 1.5×10^8cfu/ml. Standardized inoculum Used for Anti-bacterial test.

Procedure:

1.The medium was prepared by dissolving 38 g of Mueller-Hinton Agar Medium (Hi Media) in 1000 ml of distilled water. The dissolved

medium was autoclaved at 15 Lbs pressure at 121^{0}C for 15 min (pH 7.3).

2.The autoclaved medium was cooled, mixed well and poured in to Petri plates (25 ml/plate). The plates were swabbed with pathogenic bacterial culture viz. *Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, Proteus Vulgaris* and *Staph aureus* 3.Finally, The Sample or Sample loaded disc was then placed on the surface of Mueller-Hinton Agar medium.

4.Amikacin was used as the control against those five different bacterial strains. Inoculate the Mueller-Hinton Agar plate by streaking the swab three times over the entire agar surface.

5.Allow 3-4 minutes for the surface of the agar to dry before applying the antibiotics disc, by using 100mm diameter petridish, seven disc may be applied, one in centre and six in periphery.

6.The plates were kept for incubation at 37°C for 24 hours. At the end of incubation, inhibition zones were examined around the disc (including disc) and measured with transparent ruler in millimetres.

Antifungal Activity:

Antibiotic susceptibility tests were determined by agar disc diffusion (Kirby-Bauer) method. Fungal strains such as *Aspergillus niger*, *Aspergillus Flaves* and *Pencillium Notatum* were swabbed using sterile cotton swabs on SDR agar plate. Upto 80 µl of each concentration of the extract respectively introduced into to the sterile discs (10 mm) using sterile pipettes. The standard drug Nystatin was used as the control. The disc was then placed on the surface of SDA medium and the compound was allowed to diffuse for 5 minutes and the plates were kept for incubation at 22^{0}C for 48 hours. At the end of incubation, inhibition zones were examined around the disc and measured with transparent ruler in millimeters.

Anticancer activity:

MTT ASSAY (Mosmann, 1983 and Marshall *et al.*, 1995)

Principle

MTT (3-4, 5 dimethylthiazol-2yl-2, 5-diphenyl tetrazolium bromide) assay, is based on the ability of a mitochondrial dehydrogenase enzyme of viable cells to cleave the tertrazolium rings of the pale yellow MTT and form a dark blue colored formazan crystals which is largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. Solubilization of cells by the addition of detergents (DMSO) results in the liberation of crystals which are solubilized. The number of surviving cells is directly proportional to the level of formazan product created. The color can be quantified using a multi-well plate reader.

Materials required

Fetal Bovine Serum (FBS) and antibiotic solution were from Gibco (USA), DMSO (Dimethyl sulfoxide) and MTT (3-4,5 dimethylthiazol-2yl-2,5-diphenyl tetrazolium bromide) (5 mg/ml) were from Sigma, (USA), RPMI medium, 1X PBS, (India). 96 well tissue culture plate and wash beaker were from Tarson (India).

Procedure

Cell culture

K562 (Human myelogenous leukemia cells) were purchased from NCCS, Pune and were cultured in liquid

medium (RPMI) supplemented 10% Fetal Bovine Serum (FBS), 100 µg/ml penicillin and 100 µg/ml streptomycin, and maintained under an atmosphere of 5% CO_2 at 37°C.

MTT assay

The Test sample was tested for *in vitro* cytotoxicity, using the K562 cells by MTT assay. Briefly, the cultured K562 cells were harvested and pooled in a 15 ml tube. Then, the cells were plated at a density of 1×10^5 cells/ml cells/well (200 µL) into the 96-well tissue culture plate and treated with various concentrations of the Test sample in a serum-free RPMI medium. Each sample was replicated three times and the cells were incubated at 37°C in a humidified 5% CO2 incubator for 24 h. After incubation, MTT (10 µL of 5 mg/ml) was added to each well and the cells were incubated for another 2-4 h until purple precipitates were clearly visible under an inverted microscope. Finally, the medium together with MTT (220 µL) was aspirated off the wells and washed with 1X PBS (200 µl). Furthermore, to dissolve formazan crystals, DMSO (100 µL) was added and the plate was shaken for 5 min. The absorbance for each well was measured at 570 nm using a microplate reader (Thermo Fisher Scientific, USA) and the percentage cell viability and IC50 value were calculated using Graph Pad Prism 6.0 software (USA).

Formula Cell viability % = Test OD/Control OD X 100

MEASUREMENT OF MITOCHONDRIAL MEMBRANE POTENTIAL

Principle

Mitochondria generate a potential across their membranes due to the activities of enzymes of the electron transport chain. During apoptosis, the collapse of the mitochondrial membrane potential (MMP) coincides with the opening of the mitochondrial permeability transition pores, leading to the release of cytochrome c into the cytosol, which in turn triggers other downstream events in the apoptotic cascade.

JC-10 is used to determine the loss of the MMP in cells. JC-10 is developed to be a superior alternative to JC-1 when a high dye concentration is desired. Compared to JC-1, JC-10 has much better water solubility. JC-10 is capable of selectively entering mitochondria and reversibly changes its color from green to orange as membrane potentials increase. This property is due to the reversible formation of JC-10 aggregates upon membrane polarization that causes shifts in emitted light from 520 nm (i.e. emission of JC-10 monomeric form) to 570 nm (i.e. emission of J-aggregate form). When excited at 490 nm, the color of JC-10 changes reversibly from green to greenish orange as the mitochondrial membrane becomes more polarized.

In normal cells, JC-10 concentrates in the mitochondrial matrix where it forms red fluorescent aggregates. However, in apoptotic and necrotic cells, JC-10 exists in monomeric form and stains cells green. The green emission can be analyzed in fluorescence channel 1 (FL1) and greenish orange emission in channel 2 (FL2).

Materials Required

RPMI medium, Fetal Bovine Serum (FBS) and antibiotic solution were from Gibco (USA), Mitochondrial membrane potential assay kit was from Sigma, (USA), 1X PBS was from Himedia, (India). 96 well tissue culture plate and wash beaker were from Tarson (India).

Procedure

Cell culture

K562 (Human myelogenous leukemia cells) was purchased from NCCS, Pune and were cultured in liquid medium (RPMI) supplemented 10% Fetal Bovine Serum (FBS), 100 µg/ml penicillin, and 100 µg/ml streptomycin, and maintained under an atmosphere of 5% CO_2 at 37ºC.

Measurement of mitochondrial membrane potential

The K562 cells (20,000–50,000 cells/well) were plated to a 24 well plate and treated with 61.24 µg/ml of RRL sample in a serum-free RPMI medium. Again, the plate was incubated at 37°C in a humidified 5% CO2 incubator for 24 hrs. The measurement of mitochondrial membrane potential for the treated and control cells was carried out according to the manufacturer's instructions. Briefly, the cells were incubated with 100 µl/well of JC-10 dye loading solution, and the plate was protected from light. The plate was incubated for 30 – 60 minutes in a 5% CO2 at 37 °C. After incubation, 100 µl/well assay buffer B was added to each sample/well. Finally, the plate was centrifuged at 800 rpm for 2 minutes and the fluorescence was observed using Fluorescent Imaging System (ZOE, Bio-Rad, USA).

EtBr /AO STAINING (Liu *et al.*,2015, Padmapriya *et al.*,2017 *and* Ciniglia *et al.*, 2010)

Principle

Fluorescent dyes with aromatic amino or guanidine groups, such as acridine orange (AO), interact with nucleotides to emit fluorescence. EtBr molecules intercalate inside the DNA double helix. AO can form complexes with either double-stranded DNA or single-stranded DNA and RNA. One molecule of AO can also interact with one phosphate group of single-stranded DNA or RNA to form an aggregated, or stacked, structure that emits red fluorescence with the maximum wavelength at 650 nm. This fluorescent dye is impermeable through the cell membranes of viable cells and can be used as fluorescent indicators of dead cells. Acridine orange is a vital dye and will stain both live and dead cells. Necrotic cells stain orange but have a nuclear morphology resembling that of viable cells, with no condensed chromatin. Ethidium bromide (EtBr) is only taken up by cells when cytoplasmic membrane integrity is lost and stains the nucleus red. EtBr also dominates over AO. Thus, live cells have a normal green nucleus; early apoptotic cells have a bright green nucleus with condensed or fragmented chromatin; late apoptotic cells display condensed and fragmented orange chromatin; cells that have died from direct necrosis have a structurally normal orange nucleus. Ethidium re-emits this energy as yellow/orange light centered at 590 nm. The fluorescence of ethidium bromide in an aqueous solution is significantly lower than that of the intercalated dye.

Materials required

RPMI medium, Penicillin/Streptomycin antibiotic solution was purchased from Gibco (USA), EtBr, and Acridine orange was purchased from Sigma Aldrich (USA).

Procedure

Cell culture

K562 (Human myelogenous leukemia cells) were cultured in liquid medium (RPMI) supplemented 10%

Fetal Bovine Serum (FBS), 100 µg/ml penicillin, and 100 µg/ml streptomycin, and maintained under an atmosphere of 5% CO_2 at 37ºC.

EtBr/AO staining

Briefly, 5 x 10^5 cells/ml of K562 cells were plated into a 96 well tissue culture plate and the cells were treated with 61.24 µg/ml of RRL extract sample in a serum-free RPMI medium. The plate was incubated at 37ºC at a 5% Co2 incubator for 24 hours. After incubation, 10 µl of 1 mg/ml acridine orange and ethidium bromide were added to the wells and mixed gently. Finally, the plate was centrifuged at 800 rpm for 2 minutes and evaluated immediately within an hour, and examined at least 100 cells by a fluorescence cell imaging system using a fluorescent filter.

DNA BARCODING:

Molecular identification of test sample

1. **Materials Required**

- 10 % SDS Lysis Buffer
- Polypropylene tubes (don't use polycarbonate tubes with phenol and chloroform)
- Centrifuge (at least 14,000 x g)
- RNase A Solution (10 mg/ml in water, DNase-free)
- Isopropanol
- 70% Ethanol
- 2 ml polypropylene centrifuge tubes
- Centrifugal Vacuum Concentrator (e.g., SpeedVac)
- TE Buffer (10 mM Tris, pH 8, 1 mM EDTA)
- Phenol/Chloroform/Isoamyl Alcohol (25:24:1 ratio) stored under TE buffer, pH 8

2. **Stock solutions**

0.5 M Tris HCl (pH-8.0)

Tris base
- 3.028 g

Distilled water
- 40 mL

pH was adjusted to 8.0 using HCl and the volume was made to 50 mL, then autoclaved and stored at 4 °C.

0.5 M EDTA (pH-8.0)

EDTA
- 9.31 g

Distilled water
- 40 mL

The pH was adjusted to 8.0, using NaOH was made up the volume to 50 mL, then autoclaved, and stored at 4 °C.

10 mM Tris HCl (pH-7.5)

Tris base
- 0.03 g

Distilled water
- 20 mL

The pH adjusted to 7.5 using HCl was made up the volume to 25 mL, then autoclaved, and stored at 4 °C.

TAE Buffer (50X – 1 Liter)

Trisbase
- 242 g

Glacial acetic acid
- 57.1 mL

EDTA
- 100 mL (0.5 M, pH- 8)

242 g of Trisbase, 57.1 mL of glacial acetic acid mixed. 100

mL of 0.5 M EDTA, (pH 8.0) added and made up with distilled water to 1liter.

Bromophenol blue

0.5% Bromophenol blue - 500 mg

It was made up into a final solution of 100 mL with double distilled water and then autoclaved and stored at 4 °C

3.Working Solutions

Solution 1:

Tris-HCl
- 50 mM (pH 8.0)

EDTA
- 20 mM (pH 8.0)

Solution 2: Saturated NaCl solution - 6 M

SDS
- 10 %

Proteinase K (20 mg/mL)

Proteinase K
- 10 mg

Autoclaved distilled water - 500 µL

Proteinase K is dissolved in distilled water and stored at -20 °C.

TE buffer

Stock 0.5 M Tris HCl (pH-8.0) - 2.0 mL (10 mM)

Stock 0.5 M EDTA (pH-8.0) - 0.2 mL (1 mM)

Made up the solution to 100 mL with distilled water, then autoclaved and stored at 4 °C.

4. DNA isolation Procedure

The test samples were placed in 1.5 mL tubes separately and then added 500 µL of Solution1 and 10% of 10 µL SDS. Homogenize the sample with a sterile homogenizer. And 5 µL of Proteinase K was added (20 mg/mL). The mixture was incubated at 55 °C for 2 hrs in the water bath (with occasional mixing/quick vortex) for easy digestion. After complete digestion, the sample was kept on ice for 10 min. To this, 250 µL of Solution 2 was added (saturated NaCl) and inverted several times to mix.

Subsequently, the samples were chilled on ice for 5 min. Then the samples were centrifuged at 8000 rpm for 15 min. After that, about 500 µL of clear supernatant were collected into new-labeled 1.5 mL tubes. Then twice the volume of 100% molecular biology grade ethanol was added to precipitate the DNA. Then the samples were centrifuged at 11,000 rpm for 15 min. After that, the supernatant was removed and 500 µL of ice-cold 70% ethanol was added to the precipitate for washing. The sample was spun at 11000 rpm, for 5 min. Carefully, remove the supernatant, then pipetted out excess liquid and allowed to partially dry with lid-off at room temperature. Partially dried DNA was re-suspended in 100 µL of 1x TE buffer.

5. Quantification of DNA

The quantity of the extracted DNA was checked in a UV spectrophotometer (Labman, India) by taking the optical density (OD) at 260

nm and 280 nm. The quality was checked by measuring the ratio of absorbance at 260 nm and 280 nm (260/280). The value between 1.7 - 1.8 indicates good quality DNA without protein/RNA contamination. DNA quantification was done according to the following calculation: a sample showing 1 OD at 260 nm is equivalent to 50 µg of DNA/mL. The OD of each DNA sample at 260 nm was measured and quantified accordingly.

6. DNA quality determination by agarose gel electrophoresis

The quality of DNA was checked on 0.8% agarose gels.

The casting of 0.8% agarose gel

The unit is set according to the manufacturer's instructions.

0.8% agarose gel

Agarose - 0.24 g

1X TAE - 30 mL

The (0.24 g) agarose powder was soaked in 30 mL of 1X TAE buffer and boiled until it formed into a clear solution. Then it was allowed to cool, down to approximately 50 °C. Then added 1.5 µL of ethidium bromide and mixed well. It was poured into a gel casting plate with an already adjusted gel comb and kept at room temperature for 1/2 hrs for solidification. The gel was soaked in 1X TAE buffer in the electrophoresis tank. 3 µL of DNA with 3 µL of gel loading dye was loaded in the wells using micropipettes. It was run at 70 V for 15 to 20 min. The orange color (DNA) bands were observed in the UV gel documentation system.

7. PCR amplification

Primers used

Gene	Direction	Sequence (5′ – 3′)
matK	Forward	CCTATCCATCTGGAAATCTTAG
	Reverse	GTTCTAGCACAAGAAAGTC

The PCR amplification was carried out in a PCR thermal cycler (PCR System, Himedia).

PCR amplification profile

3.9.1 matK gene PCR condition

95 ºC - 1 min

95 ºC	-	30 sec	}
50 ºC	-	30 sec	35 cycles
72 ºC	-	30 sec	
72 ºC	-	10 min	
4 ºC	-	∞	

8. ExoSAP-IT Treatment

ExoSAP-IT (GE Healthcare) consists of two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase (SAP), in a specially formulated buffer for the removal of unwanted primers and dNTPs from a PCR product mixture with no interference in downstream applications.

Five microliters of PCR product is mixed with 2 µl of ExoSAP-IT and incubated at 37ºC for 30 minutes followed by enzyme inactivation at 80ºC for 15 minutes.

9. Sequencing using BigDye Terminator v3.1

The sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) following manufacturer protocol.

The PCR mix consisted of the following components:

PCR Product (ExoSAP treated) - 10-20 ng

Primer - 3.2 pM (either Forward or Reverse)

Sequencing Mix - 0.28 µl

DMSO - 0.30 µl

5x Reaction buffer - 1.86 µl

Sterile distilled water - make up to 10µl

The sequencing PCR temperature profile consisted of a 1st cycle at 96ºC for 2 minutes followed by 30 cycles at 96ºC for 30 sec, 50ºC for 40 sec and 60ºC for 4 minutes.

10. Post Sequencing PCR Clean up

1. Master mix I of 10µl milli Q and 2 µl 125mM EDTA per reaction and master mix II of 2 µl of 3M sodium acetate pH 4.6 and 50 µl of ethanol was prepared.
2. 12µl of master mix I was added to each reaction containing 10µl of reaction contents and was properly mixed.
3. 52 µl of master mix II was added to each reaction.
4. Contents were mixed by inverting and incubated at room temperature for 30 minutes
5. Spun at 14,000 rpm for 30

minutes

6. Decanted the supernatant and added 100 µl of 70% ethanol
7. Spun at 14,000 rpm for 20 minutes.
8. Decanted the supernatant and repeated 70% ethanol wash
9. Decanted the supernatant and air-dried the pellet.

The cleaned-upair-dried product was sequenced in ABI 3500 DNA Analyzer (Applied Biosystems).

11. BLAST Analysis

With the database of NCBI Gene bank, the matK region sequence was used to carry out BLAST. The first ten sequences were selected and aligned using the multiple alignment software program Clustal Based on the extreme identity score program Clustal W. Distance matrix was generated and the phylogenetic tree was constructed using MEGA 11.

Results and Discussion:

Free radical scavenging activity:

The free radical scavenging activity of the whole plant sample of *L.barberi* is carried by DPPH, ABTS, NO and SOD assay

DPPH:

DPPH is an important free radical, which damages the cell membrane. The free radical scavenging activity is directly proportional to the amount of phenolic compound present in the extract (Gupta *et al*.,2022). The methanol extract of the plant *L.barberi* showed highest percentage of inhibition which was observed at 500 µg/ml (84.64%), indicating strong free radical scavenging ability. This activity gradually decreased with lower concentrations with 80.65% at 250 µg/ml, 72.88% at 100 µg/ml, 49.68% at 50 µg/ml, and 30.25% at 10 µg/ml demonstrating a dose-dependent response (Table 1.1, Plate 3.1 and graph 2.1). According to the findings, the maximum absorption was found at 517 nm. The methanol extracts of the plant samples showed better antioxidant potential when compared to the standard ascorbic acid. The IC50 value of the *L.barberi* was calculated as 62.35 µg/ml (Table 1.2). In DPPH assays, a lower IC_{50} indicates stronger antioxidant activity, as it reflects the smaller concentration required to neutralize 50% of free radicals. While the extract's IC_{50} is higher than that of the standard ascorbic acid, it still demonstrates moderate to strong antioxidant potential. This suggests that the plant contains bioactive compounds, such as phenolics and flavonoids, capable of scavenging free radicals efficiently. The dose-dependent increase in percentage inhibition further supports the extract's effective radical-scavenging ability.These findings are consistent with previous studies on medicinal plants such as *Ocimum sanctum* and *Asparagus racemosus* which have demonstrated similar antioxidant profiles in methanolic extracts Priyadarshini *et al*., 2019 and Behera, 2018. Overall, the results suggest that *Lepidagathis barberi* could serve as a promising natural source of antioxidants for pharmacological and therapeutic applications.

Table 1.1: DPPH assay of the methanolic extract of *L. barberi*

S. No	Tested sample concentration (µg/ml)	Percentage of inhibition (in triplicates)			Mean value (%)
1.	Ascorbic acid	97.5	96.56	96.25	96.77
2.	500 µg/ml	86.25	84.09	83.59	84.64
3.	250 µg/ml	81.40	80.46	80.09	80.65
4.	100 µg/ml	79.53	70.03	69.09	72.88
5.	50 µg/ml	60.18	44.87	44.00	49.68
6.	10 µg/ml	30.90	29.93	29.90	30.25

Graph 2.1: Percentage of Inhibiton of the methanolic extract of *L. barberi*

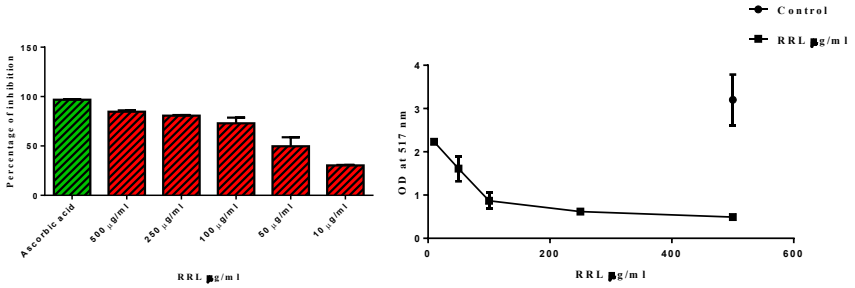
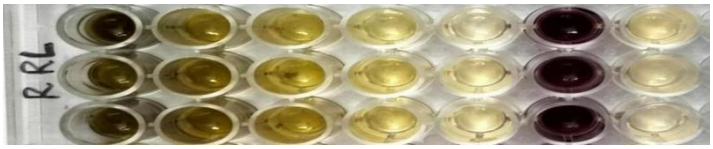


Table 1.2: IC50 Value of tested sample *L.barberi*

log(inhibitor) vs. normalized response -- Variable slope		
Best-fit values		
LogIC50		1.795
HillSlope		-2.548
IC50		62.35
Std. Error		
LogIC50		0.02780
HillSlope		0.4460
95% Confidence Intervals		
LogIC50		1.735 to 1.855
HillSlope		-3.512 to -1.585
IC50		54.30 to 71.60
Goodness of Fit		
Degrees of Freedom		13
R square		0.9608
Absolute Sum of Squares		876.1
Sy.x		8.209
Number of points		
Analyzed	3	15

Plate 3.1: DPPH activity of methanolic extract of the plant sample *L. barberi*



500 µg/ml 250 µg/ml 100 µg/ml 50 µg/ml 10 µg/ml Control std

ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) :

ABTS assay is based on the scavenging of light by [2,2'- Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)] radicals. An antioxidant shows a marked

ability to donate a hydrogen atom which would consequently quench the stable free radical (Rao *et al.,*2013). The ABTS radical scavenging assay revealed that the methanolic extract of *L.barberi* exhibits significant antioxidant activity in a dose-dependent manner. The percentage of inhibition increased with higher concentrations of the extract, ranging from 67.44% at 10 µg/ml to 86.05% at 500 µg/ml. The standard ascorbic acid showed slightly higher activity, with a mean inhibition of 89.87% (Graph 2.2, Table 1.3, and Plate 3.2). However, the value was dose-dependent reaching the maximum value among the treated samples at 500 µg/ml. IC50 value of tested sample was 60.78 µg/ml (Table 1.4). This represents moderate to strong antioxidant activity, confirming that the extract can scavenge ABTS radicals efficiently. It was similar to the previous findings Priyanga *et al*., 2015 and Deepika *et al*.,2018.

Graph 2.2: ABTS activity of methanolic extract of *L.barberi*

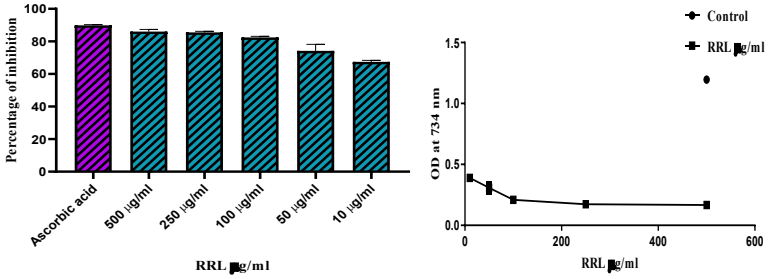


Table 1.3 : ABTS activity of *L. barberi*

S. No	Tested sample concentration (mg/ml)	Percentage of inhibition (in triplicates)			Mean value (%)
1.	Ascorbic acid	90.29	89.87	89.45	89.87
2.	500 µg/ml	84.60	86.27	87.28	86.05
3.	250 µg/ml	85.69	86.10	84.76	85.52
4.	100 µg/ml	82.51	83.09	81.75	82.45
5.	50 µg/ml	78.41	73.72	70.54	74.22
6.	10 µg/ml	68.53	66.94	66.86	67.44

Table1.4: IC_{50} Value of tested sample *L. barberi*

log(inhibitor) vs. normalized response -- Variable slope	
Best-fit values	
LogIC50	1.784
HillSlope	-2.841
IC50	60.78
95% CI (profile likelihood)	
LogIC50	1.716 to 1.847
HillSlope	-4.444 to -1.831
IC50	51.95 to 70.30
Goodness of Fit	
Degrees of Freedom	13
R squared	0.9527
Sum of Squares	1120
Sy.x	9.281
Number of points	

# of X values	15
# Y values analyzed	15

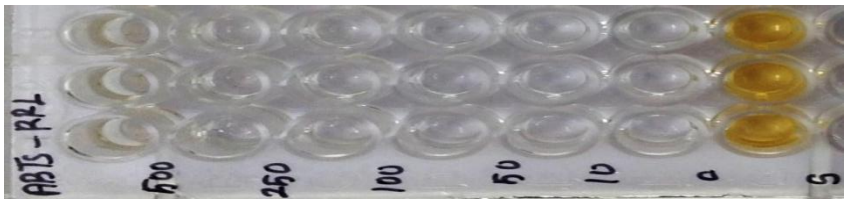


Plate 3.2: ABTS activity of *L.barberi*

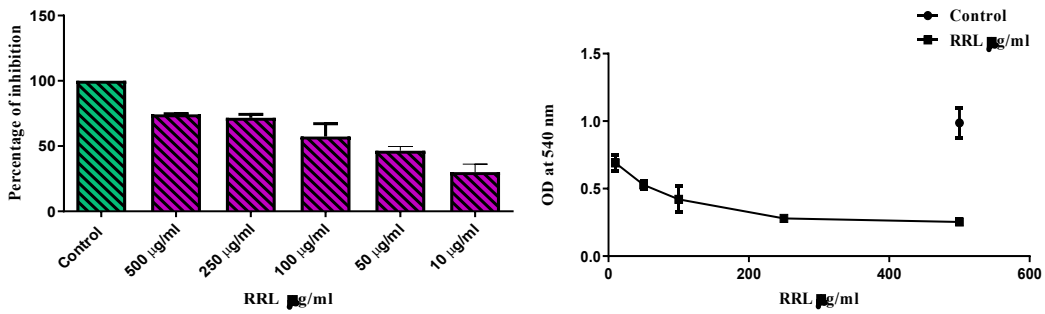
Nitric Oxide Radical Scavenging Assay

Nitric oxide is regarded as an important mediator of acute and chronic inflammation, which can easily react with superoxide anion to form peroxynitrite (ONOO-), a potent oxidizing molecule capable of eliciting lipid peroxidation and cellular damage (Rao *et al*.,2013). The nitric oxide (NO) scavenging assay demonstrated that the methanolic extract of *L. barberi* exhibits dose-dependent radical scavenging activity. (Table 1.5, graph 2.3 and plate 3.3).The percentage inhibition increased with extract concentration, ranging from 30.05% at 10 µg/ml to 74.36% at 500 µg/ml, while the control represents 100% NO levels. IC50 Value of tested sample is 71.12 µg/ml (Table1.6), indicating moderate to strong nitric oxide scavenging activity. The previous study Lalhminghlui & Jagetia 2018 and Lalrinzuali *et al*.,2015 revealed a concentration-dependent rise in its scavenging. Overall, the antioxidant activity of these extracts increased in a concentration-dependent manner.

Table 1.5: NO assay activity of *L. barberi*

S. No	Tested sample concentration (µg/ml)	Percentage of inhibition (in triplicates)			Mean value (%)
1.	Control	100	100	100	100
2.	500 µg/ml	74.87	74.16	74.06	74.36
3.	250 µg/ml	73.15	73.35	68.69	71.73
4.	100 µg/ml	68.59	52.98	50.45	57.34
5.	50 µg/ml	48.53	48.12	42.65	46.43
6.	10 µg/ml	35.96	30.49	23.70	30.05

Graph2.3: Percentage of inhibition of the methanolic extract of *L. barberi*



log(inhibitor) vs. normalized response -- Variable slope	
Best-fit values	
LogIC50	1.852
HillSlope	-1.868
IC50	71.12
95% CI (profile likelihood)	
LogIC50	1.742 to 1.952
HillSlope	-2.887 to -1.256
IC50	55.20 to 89.49
Goodness of Fit	
Degrees of Freedom	13
R squared	0.9244
Sum of Squares	1679
Sy.x	11.36
Number of points	
# of X values	15
# Y values analyzed	15

Table 1.6: IC_{50} Value of tested sample *L. barber*

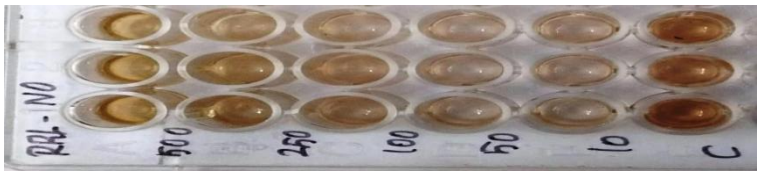


Plate 3.3: NO assay of ethanolic extract of *L. barberi*

Superoxide scavenging Assay:

The superoxide anion produces H2O2, which in turn generates hydroxyl free radicals in the presence of metals. Thus, neutralization of superoxide radical will inhibit the chain of ROS generation and protect the cells from the oxidative stress (Lalrinzuali *et al*.,2015). The Superoxide scavenging (SOD) activity assay demonstrated that the methanolic extract of *L.barberi* inhibits superoxide radicals in a dose-dependent manner. The percentage of SOD activity increased with higher concentrations of the extract, ranging from 19.53% at 10 µg/ml to 65.59% at 500 µg/ml (Table 1.7, Graph 2.4, Plate 3.4). The control, representing 100% activity, confirms the baseline superoxide levels.The calculated IC_{50} value of the extract was 94.66 µg/ml (Table 1.8), reflecting moderate antioxidant potential in superoxide radical scavenging, slightly less potent than DPPH and ABTS scavenging activity. The previous studies Lalhminghlui & Jagetia 2018 and Lalrinzuali *et al*.,2015 showed a concentration-dependent increase in the inhibition of superoxide generation.

Graph 2.4: SOD assay activity of *L.barberi*

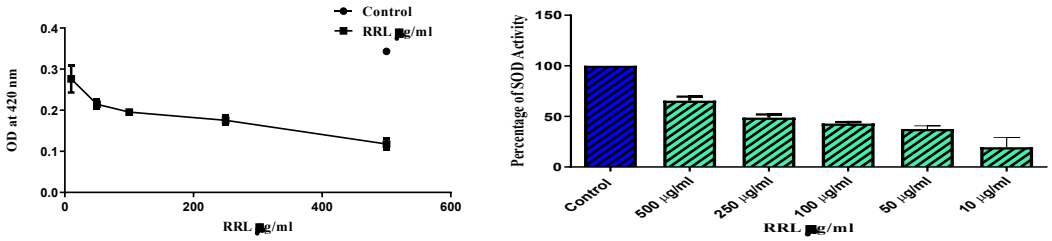


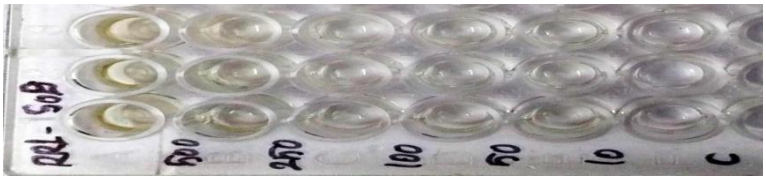
Table 1.5: SOD assay activity of *L.barberi*

S. No	Tested sample concentration (ml)	Percentage of SOD Activity (in triplicates)			Mean value (%)
1.	Control	100	100	100	100
2.	500 µg/ml	65.88	69.38	61.51	65.59
3.	250 µg/ml	50.72	45.18	50.43	48.78
4.	100 µg/ml	44.60	42.56	41.69	42.95
5.	50 µg/ml	40.23	38.19	34.11	37.51
6.	10 µg/ml	28.57	9.32	20.69	19.53

Table 1.6 : IC50 Value of tested sample *L.barberi*

log(inhibitor) vs. normalized response -- Variable slope	
Best-fit values	
LogIC50	1.976
HillSlope	-1.175
IC50	94.66
95% CI (profile likelihood)	
LogIC50	1.808 to 2.135
HillSlope	-1.758 to -0.7847
IC50	64.20 to 136.3
Goodness of Fit	
Degrees of Freedom	13
R squared	0.8584
Sum of Squares	2426
Sy.x	13.66
Number of points	
# of X values	15
# Y values analyzed	15

Plate 3.4: SOD activity of the plant sample *L.barberi*



VI. Anti-microbial activity of *Lepidagathis barberi*

1.Antibacterial activity:

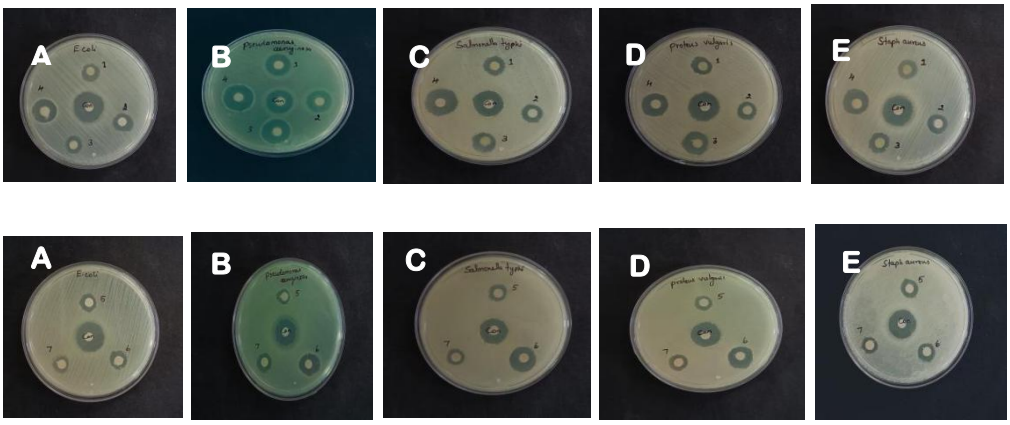
Studies on the anti-microbial activity of the whole plant powdered sample of *L.barberi* were carried out. This study showed that anti-microbial activity of *L.barberi* by using five bacterial strains such as *Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, Proteus vulgaris* and *Staph aureus* with petroleum ether, hexane, toluene, isoproponal, ethanol, aqeuous and acetone solvent extracts was recorded which is clearly visible in Table 1.9 and Plate 3.5. Among the tested solvents, the isopropanol extract exhibited the highest antibacterial activity, showing inhibition zones of 16.2 mm for *E. coli*, 19 mm for *P. aeruginosa*, 19 mm for *S. typhi*, 15 mm for *P. vulgaris*, and 16 mm for *S. aureus*. These inhibition zones were comparable to the positive control (Amikacin), which exhibited zones ranging from 17–21 mm, confirming the assay validity. The hexane and toluene extracts also showed moderate activity against *P. aeruginosa* (16–18 mm) and *P. vulgaris* (15 mm), while the aqueous and acetone extracts displayed the least activity (11–13 mm). The negative control showed no inhibition, indicating that solvent residues alone did not contribute to antimicrobial effects. Previous study in *Euphorbia hirta* linn showed higher inhibition in isopropanol extract Ali *et al*.,2023 and Murarkar *et* al.,2019 studied inhibition in isopropanol extract in fresh spinach leaves and pumpkin juice. The overall results demonstrate that the isopropanol extract of the plant possesses the most potent antibacterial activity among all tested solvents, followed by hexane and toluene, indicating the presence of active secondary metabolites with broad-spectrum antibacterial potential.

Table 1.9:showing the measurement of zone of inhibition against bacteria with different solvents

Bacteria	Petroleum ether	Hexane	Toluene	Isoproponal	Ethanol	Aqeuous	Acetone	Control (Amikacin) +ve	Control -ve
E.coli	13mm	14mm	11.4mm	16.2mm	13mm	13mm	11mm	21mm	NZ
P.aeruginosa	16mm	16mm	18mm	19mm	12mm	17mm	13mm	17mm	NZ
S. typhi	13.2mm	13mm	14mm	19mm	11mm	16.2mm	12mm	18mm	NZ
P.vulgaris	13mm	12mm	15mm	15mm	11mm	16.2mm	12mm	19mm	NZ
S. aureus	12.5mm	13mm	12.4mm	16mm	14mm	12.5mm	11mm	19mm	NZ

NZ = No Zone

Plate 3.5 : Antibacterial activity of *L.barberi* against A. *E.coli,* B. *Pseudomonas aeruginosa,* C. *Salmonella typhi,* D. *Proteus vulgaris,* E. *Staph aureus*



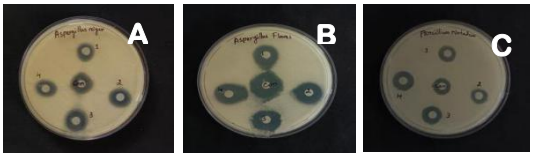
2.Antifungal acivity:

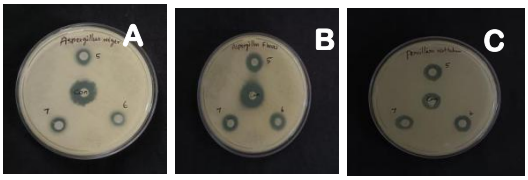
In this study three fungal strains (*Aspergillus niger*, *Aspergillus Flaves* and *Pencillium notatum*) were used against the whole plant powder of *L.barberi.* Nystatin is used as the control. Among the tested solvents, the toluene extract exhibited the highest zone of inhibition (21 mm) against *A. flavus*, followed by the hexane extract (18 mm) and the isopropanol extract (18 mm) for the same organism. For *A. niger*, the hexane and toluene extracts showed a maximum inhibition of 15 mm, while the isopropanol extract recorded the highest inhibition (15 mm) against *P. notatum*. In contrast, the aqueous and acetone extracts displayed comparatively lower inhibition zones ranging from 11 mm to 13 mm. The positive control (Nystatin) exhibited inhibition zones between 14 mm and 18 mm, confirming the validity of the assay, whereas the negative control showed no activity.(Table 1.10 and plate 3.6). This study was similar to Gupta *et al*.,2013. and Thippeswamy *et al.,*2012. Toluene has the highest overall inhibition zone, followed by Hexane and Isopropanol as effective solvents for antifungal compounds.

Table 1.10 showing the measurement of zone of inhibition against fungi with different solvents

Fungi	Petroleum ether	Hexane	Toluene	Isoproponal	Ethanol	Aqueous	Acetone	Control-Nystatin +ve	-ve Control
A.niger	14mm	15mm	15mm	14mm	12mm	11mm	11mm	16mm	NZ
A. flaves	17mm	18mm	21mm	18mm	14mm	13mm	12.4mm	18mm	NZ
P.notatum	14mm	12mm	14mm	15mm	13mm	12mm	11mm	14mm	NZ

Plate 3.6: Antifungal activity of *L.barberi* against fungi : A. *Aspergillus niger*, B.*Aspergillus flaves*, C. *Pencillium notatum*





ANTI-CANCER ACTIVITY STUDIES

The anticancer activity of the plant extract against leukemic (K562) cells was evaluated using the MTT and MMP assays, followed by dual fluorescent staining with Ethidium Bromide/Acridine Orange (EtBr/AO) to distinguish live, apoptotic, and necrotic cells after treatment.

MTT assay

MTT (3-4, 5 dimethylthiazol-2yl-2, 5-diphenyl tetrazolium bromide) assay, is based on the ability of a mitochondrial dehydrogenase enzyme of viable cells to cleave the tertrazolium rings of the pale yellow MTT and form a dark blue colored formazan crystals which is largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. K562 cell lines showed significant cytotoxicity with the IC_{50} value of 61.24 µg/ml (Table :1.12). The results showed an increase in the absorbance value with a decrease in the concentration of the extracts. Cell viability was found to decrease with the increasing concentration of the extract used in *Lepidagathis barberi.* The value ranges between 50.56 to 74.18. The absorbance value was found as the highest in the low sample concentration (10µg/ml) with a value of 84.23% which was next to the control with 100 and the least was observed in the sample with the highest concentration of 500µg/ml with a value of 45.97%. The most potent antitumor activity has been shown at concentrations 500 µg/ml. The obtained microscopic images revealed the morphological changes in the K562 cell lines after the treatment with the plant extract at all the higher concentrations. The cells reacted by shrinking and the sizes of cells were reduced as compared to the control cells. The cell viability percentage was calculated using the standard formula along with their mean value percentage. The cell viability percentage for *L.barberi* was analyzed for both the control and samples treated with various concentrations of plant extracts was measured at 570 nm using a microplate reader. As for the *L. barberi* was concerned, the mean value of cell viability was found to be 100% for the control, next to the control it was more in 10µg/ml concentration with a viability percentage of 84 % and the minimum mean value of viability was found in 500 µg/ml concentration which was about 45%. Results of the MTT assay were presented in Plate 3.7, graph 2.5 & Table 1.11, which showed the presence of viability values against K562 cells which were incubated with the samples for 24 hrs. The result was quite similar to Pour *et al.,*2009, Benny *et al*., 2022 and Krishna *et al*.,2015 shows dose dependent decrease in the viability of cells exposed to different concentrations of extract with the viability.

Graph 2.5: MTT assay for *Lepidagathis barberi*

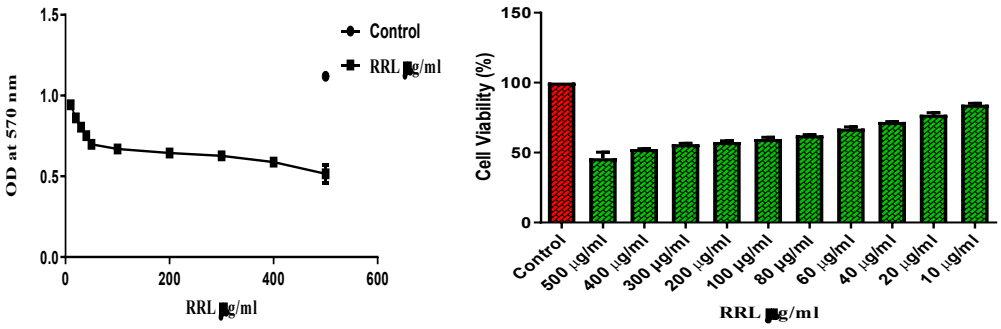


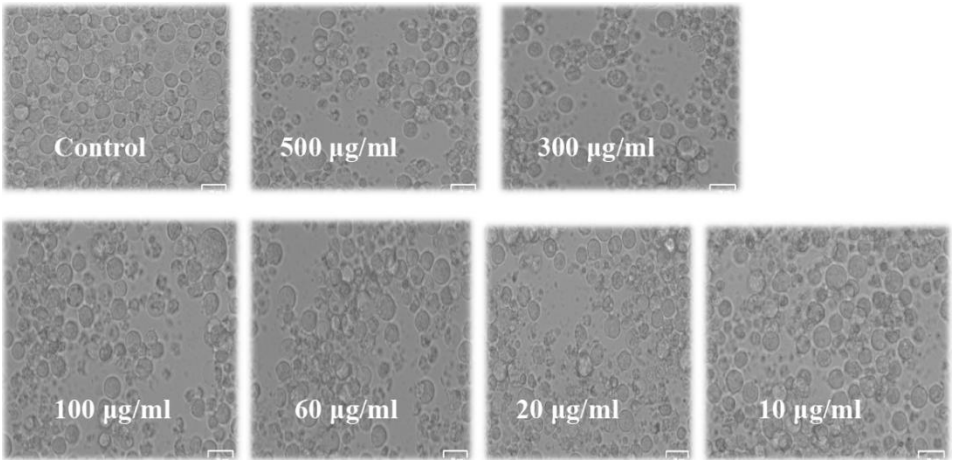
Table 1.11: MTT assay for ethanolic extract of *Lepidagathis barberi*

S. No.	Tested sample concentration (µg/ml)	Cell viability (%) (in triplicates)			Mean Value (%)
1	Control	100	100	100	100
2	500 µg/ml	41.3043	46.6968	49.9129	45.971357
3	400 µg/ml	52.7174	52.6697	52.3519	52.579664
4	300 µg/ml	56.25	56.4706	55.4878	56.069464
5	200 µg/ml	57.971	58.19	56.7073	57.622792
6	100 µg/ml	60.4167	60.4525	58.5366	59.801914
7	80 µg/ml	62.4094	62.8959	61.8467	62.384013
8	60 µg/ml	65.942	67.4208	68.2056	67.189473
9	40 µg/ml	72.1014	72.0362	71.6028	71.913479
10	20 µg/ml	76.2681	78.733	76.2195	77.073553
11	10 µg/ml	83.0616	85.0679	84.5819	84.237116

Table 1.12 : IC50 Value of tested sample *L.barberi*

log(inhibitor) vs. normalized response -- Variable slope	
Best-fit values	
LogIC50	1.787
HillSlope	-1.038
IC50	61.24
Std. Error	
LogIC50	0.04064
HillSlope	0.1023
95% CI (asymptotic)	
LogIC50	1.704 to 1.870
HillSlope	-1.247 to -0.8284
IC50	50.56 to 74.18
Goodness of Fit	
Degrees of Freedom	28
R squared	0.8971
Sum of Squares	2662
Sy.x	9.751
Number of points	
# of X values	30
# Y values analyzed	30

Plate 3.7 :Images of Control and Treated cells



MMP ASSAY:

The K562 cells (20,000–50,000 cells/well) cells were used to measure the impact of the ethanolic extract of the experimental plants on cytotoxicity. In normal cells, JC-10 concentrates in the mitochondrial matrix where it forms red fluorescent aggregates. However, in apoptotic and necrotic cells, JC-10 exists in monomeric form and stains cells green. The green emission can be analyzed in fluorescence channel 1 (FL1) and greenish orange emission in channel 2 (FL2).

However, in the cancer cells treated with the plant extracts, greenish to orange yellow colour was observed which showing that the cells were undergoing early or late apoptosis. Anti-cancer drugs induce multiple mechanisms of response from the cancer cells and most of them showed apoptosis as the strategy of preventing cell proliferation. In our experimental study, also there is a clear evidence that the membrane integrity is broken which leads to the failure of building the membrane potential across the membrane. Results of Mitochondrial Membrane Potential are shown in Plate 3.8 and 3.9. The previous study was similar to Benny *et al*., 2022 and John *et al* 2020.

Plate 3.8: Control cells

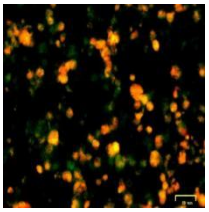
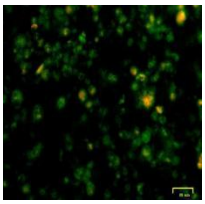


Plate 3.9: Cells treated with 61.24 µg/ml



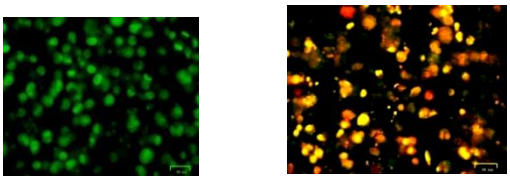
EtBr /AO STAINING

Apoptosis is an important preventive mechanism against carcinogenesis as it eliminates genetically defective cells. Induction of apoptosis is therefore a highly desired mechanism for cancer management. The major drawback of MTT assay is its inability to distinguish between apoptosis and necrosis as the cause of cell growth inhibition. The AO/EB staining was done to assess morphological and apoptotic mechanism of cell, which gives a clear contrast between live, early and late apoptotic cells (Benny *et al*., 2022). Thus, live cells have a normal green nucleus; early apoptotic cells have a bright green nucleus with condensed or fragmented chromatin;

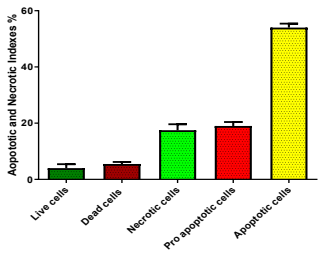
late apoptotic cells display condensed and fragmented orange chromatin; cells that have died from direct necrosis have a structurally normal orange nucleus.

The results revealed that AO stained both living and dead cells, but EtBr exclusively stained those that had their membrane integrity disrupted. Green stained cells indicate live cells, yellow stained cells indicate early apoptotic cells, and orange stained cells indicate late apoptotic cells (Plate 3.8, 3.9). Control cells in this study were uniformly green, but *L.barberi* extract-treated cells displayed yellow, orange, and red signals. In control the percentage of dead cells was found to be 6, Necrotic cells 16, Pro-Apoptotic cells 20, Apoptotic cells 53 and live cells 5 were observed. In cells treated with *L.barberi,* 5 of the cells found to be dead cells, 19 Necrotic cells, 18 Pro-Apoptotic cells, 55 Apoptotic cells and 3 live cells were found (Table 1.13, Graph 2.5). It was shown in table, graph and figure. The previous study was similar to Benny *et al*., 2022 and Gopalakrishnan *et al*., 2025.

Plate 3.8 : Control cells **Plate 3.9 : Cells treated with 61.24 µg/ml**



Graph2.6: Result of EtBr/Ao staining for *L.barberi*



S.No	Dead cells	Necrotic cells	Pro-Apoptotic cells	Apoptotic cells	Live cells
1.	6	16	20	53	5
2.	5	19	18	55	3

Table 1.13: Result for EtBr/Ao staining for *L.barberi*

DNA BARCODING

DNA from the leaves of the plant was extracted and the chloroplast gene rbcL was amplified by PCR and sequenced. The sequence was subjected to a BLAST analysis to compare it with that of other species and a phylogenetic tree was constructed. A significant similarity was noticed between the rbcL sequences obtained by PCR amplification and the rbcL sequenced already available in the database. For *Lepidagathis barberi*, the rbcL gene amplified in our work showed a

100 % match with the same species in the database with an accession number NC 084174.1. The phylogenetic tree constructed using the sequences of maximum similarity showed the closest species to *Lepidagathis barberi* was *Chroesthes longifolia* followed closely by several *Barleria* species such as *B. prionitis, B. lupulina*, and *B. cuspidata*, all showing identities above 95% and similarly high coverage.In this study, the matK barcode provided clear matches within Acanthaceae, reflecting prior findings that matK generally has high species identification success within this group, though with potential overlaps for very closely related taxa. It showed their closeness and common ancestry as they belong to the same family confirmed by taxonomic observation too. The previous study Meenakshi *et al*.,2016 used matK and rbcL to identify and study genetic relationships between several *Barleria* species, finding that matK successfully distinguished between subgenera with very close affinities. The sequence, chromatogram and phylogenetic tree of both samples was shown in Figure 4.1 and 4.2 and 4.3.

Sample Code		*Lepidagathis barberi* Sequence results
L.barberi	matK	CTTTTTTGTAGAAGATCCGCTATGATAATGAAAAAGATTTCTGCATATACGCCCAAATCGTTCAATAATATTCGAATCTGATAAA TCAGCCCAAACCGGCTTACTAATAGGATGCCCTATTACGTTACAAAATTTCGCTTTAGCCAACGACGCAATCAGAGGAATAAT TGGAATAAGGGTATCAAACTTCTTAATAGCATTATTGATTGGAAATGCATTTTCGAGAATTTGGCTCCGTACCACTGAAGGTTT GATCTTCACGCTTGAAAGATAGCCAAAAAATTCAAGGAAATGATTGGATAATTGGGTTCTATAAATCCTTCTTGGATGAAACC ACAGCGAAAAACGCCATTGCCAAAAAGTGAGAAGGTAACATTTCCATTTATTCATGAAAAGTGACGTCCCTTTTGAAGCCA AGATGAATCTTCTTTGATACCTAATATAATGCATGCAAGGTTCCTTGACCACCCATAGGTTCACCTGAAAACCCTTAACCTTAA CTAAGACGTTCACAAGACGTTCTATTTTTCCATAGAAATAGATTCGTTCAAGAAAAACTCCAGAAGATGTTGATCGGAAATG AGAAGATTGGTTACGTAAAAATATGAAAATGGATTCATATTCCCATACATGAGAATTATATAAGAATAAGAATAATCTTTGATTT CTTTTTGAAAAAGAGGAAGCGGCTTTTTTTGGCCTAATACTAATAAGAGTATTCCAATTACAATACTCGTTGAGAAAGAATCG TAATAAATGCAAAGAAGAGGCATCTTTTAACCAATAGTGAAGAGTTTGAACCAAGATTTCCA

Figure 4.1: rbcL sequence of *L.barberi*

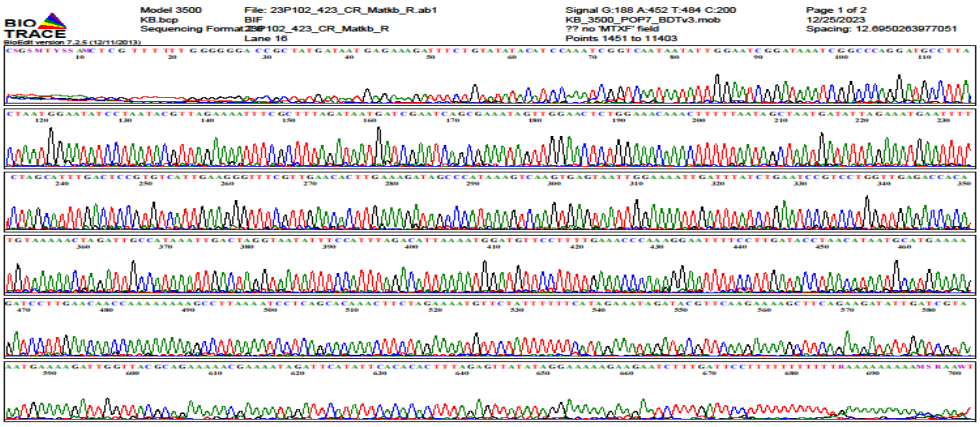


Figure 4.2: Chromatogram of *L.barberi*

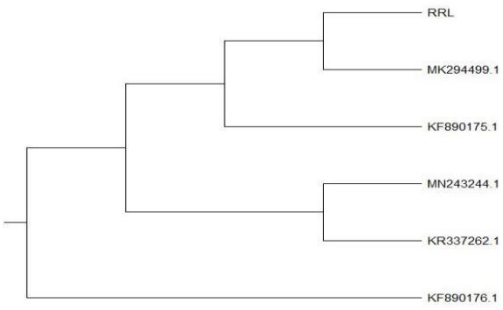


Figure 4.3: Phylogenetic tree of *L.barberi*

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Chroesthes longifolia chloroplast, complete genome	Chroesthes lon...	1500	1500	100%	0.0	100.00%	152594	NC_084174.1
Barleria prionitis maturase K (matK) gene, partial cds; plastid	Barleria prionitis	1312	1312	100%	0.0	95.94%	904	KF890176.1
Barleria prionitis chloroplast, complete genome	Barleria prionitis	1306	1306	100%	0.0	95.81%	152217	NC_048478.1
Barleria lupulina voucher A. Chaveerach 1096 chloroplast, complete genome	Barleria lupulina	1306	1306	100%	0.0	95.81%	152273	NC_070082.1
Barleria sp. CP226 maturase K (matK) gene, partial cds; chloroplast	Barleria sp. CP...	1299	1299	98%	0.0	96.01%	857	MN243244.1
Barleria cuspidata maturase K (matK) gene, partial cds; plastid	Barleria cuspidata	1293	1293	98%	0.0	95.89%	834	KF890175.1
Barleria prionitis voucher CMPR8789 maturase K (matk) gene, partial cds; chloroplast	Barleria prionitis	1284	1284	97%	0.0	95.97%	818	OL580741.1
Cystacanthus paniculatus voucher CPG25312 maturase K (matK) gene, partial cds; chloroplast	Cystacanthus p...	1279	1279	100%	0.0	95.07%	1407	KX526467.1
Barleria acuminata isolate SR301-BA maturase K (matK) gene, partial cds; chloroplast	Barleria acumin...	1267	1267	100%	0.0	94.95%	898	KR337262.1
Barleria cristata voucher A. Chaveerach 1095 chloroplast, complete genome	Barleria cristata	1267	1267	100%	0.0	94.95%	151977	NC_070081.1
Justicia adhatoda voucher AUS-MP24 maturase K (matK) gene, partial cds; chloroplast	Justicia adhatoda	1260	1260	99%	0.0	94.70%	836	JN228938.1
Whitfieldia lateritia voucher IDRCG679-13_FHO_DB7119 maturase K (matK) gene, partial cds; chloroplast	Whitfieldia lateritia	1251	1251	96%	0.0	95.54%	798	MN370405.1

Table 1.14 : sequence alignments of *L.barberi*

Conclusion

The investigation into the bioactive properties of *Lepidagathis barberi* methanolic extracts revealed significant antioxidant, antimicrobial, antifungal, and anticancer activities. The plant demonstrated potent free radical scavenging capabilities in DPPH, ABTS, nitric oxide, and superoxide dismutase assays, with activity increasing in a dose-dependent manner and IC50 values comparable to common antioxidant standards. The extract exhibited strong antibacterial effects, especially in isopropanol solvent extracts, effectively inhibiting pathogens like *Pseudomonas aeruginosa* and *Salmonella typhi*. Similarly toluene extracts showed remarkable antifungal activity against *A. flaves*.

Cytotoxicity evaluation via MTT assay highlighted considerable anticancer potential, with a dose-dependent decrease in K562 leukemia cell viability, supporting the extract's role in inducing apoptosis or growth inhibition. DNA barcoding confirmed the species identity and provided a phylogenetic context for *L. barberi*.

These multifaceted pharmacological activities validate the traditional medicinal use of *Lepidagathis barberi* and underscore its potential as a valuable source of natural therapeutic agents. Further in-depth studies on isolation, characterization of active compounds, and mechanistic insights are warranted to harness its full pharmacological potential for drug development.

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