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SCREENING OF PHYTOCHEMICAL ANALYSIS AND ANTIMICROBIAL ACTIVITY OF LEAF EXTRACT OF INDIGOFERA LINIFOLIA (L.f) Retz.

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Abstract

Indigofera linifolia (L.f.) Retz (Fabaceae) is an erect, prostrate herb that spread throughout India and was found to be used by people to cure febrile eruptions. The extracts of leaves showed antibacterial and antifungal activities. The current study assessed the phytochemical analysis of the three solvents resembling petroleum ether, ethanol, and aqueous extracts, which were examined for eight phytochemicals. Petroleum Ether contains five phytochemicals among the 8; Ethanol contains five phytochemicals, whereas aqueous includes four. The Antibacterial activities of ethanol extract of *Indigofera linifolia* (L.f.) Retz. Showed a higher zone of inhibition against the selected bacteria Staphylococcus aureus (21mm), E.coli (15mm), Pseudomonas aeruginosa (14 mm), Bacillus subtilis (11mm), and the control microbial antibiotic Amikacin is the less inhibition zone (20mm). Meanwhile, the antifungal activity of ethanol extract from *Indigofera linifolia* (L.f.) Retz. Showed a higher zone of inhibition against the selected fungi *Candida tropicalis* (17mm), *Aspergillus niger* (16mm), and *Candida albicans* (14mm). The results of the current study will create a way to invent herbal medicines for several ailments by using *Indigofera linifolia* (L.f.) Retz, which may lead to the development of novel drugs. The presence of secondary metabolites has been identified from the phytochemical studies. Hence, this plant may be used to treat many diseases.

Keywords: *Indigofera linifolia*, Fabaceae, Antimicrobial activity, Phytochemical analysis.

Introduction

The Fabaceae family is the second-largest family of medicinal plants, with more than 490 species used in traditional medicine (Dzoyem et al., 2014). *Indigofera* is the third-largest genus in this family and consists of approximately 750 species (Schrire, 2013), distributed across all tropical and subtropical regions of the world (Su et al., 2008). Some 75% of these species are restricted to Africa and Madagascar, and many species are also encountered in the Sino-Himalayan region, Australia, and Central and South America (Schrire, 2013). A few species can be found in temperate areas of East Asia (Ponmari et al., 2014). *Indigofera* species comprise mainly herbs, shrubs, or small trees distributed in forests, savannas, and disturbed areas (Kaushik et al., 2016; Marquiafavel et al., 2009). Although many active pharmaceutical ingredients have

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been prepared by the synthetic route, the importance of plants still cannot be ignored, and that is the reason that plants are constantly explored for novel compounds of biological importance (Memariani Z et al., 2021). Medicinal plants are the primary source of pharmaceutical compounds and various valuable medicines derived directly or indirectly (Cordell GA 2000 & Ul Hassan SS et al., 2017). As mentioned, humans have used plants, flowers, and insects to isolate different compounds beneficial for health or curing diseases (Majid M et al., 2022). Natural compounds from plants have been considered the unchallenged foundations of new drug discoveries. However, the newest contest between combinatorial chemistry and computational drug design (Adang A and Hermkens 2001) has ended the supremacy of natural products in drug discovery. The phytochemical study identified the presence of steroids, phenolics, saponins, and flavonoids in chloroform and ethanolic extracts of Indigofera linifolia. In the anti-inflammatory studies, the percentage increase in paw edema was reduced in chloroform and ethanol extract-treated animals compared to standard Indomethacin (Uvarani et al., 2012). The current study's objectives were to evaluate screening of phytochemical analysis and antimicrobial efficacy against selected microorganisms of the leaves extract of *Indigofera linifolia* (L.f) Retz.

Materials and Methods

Materials

The experimental material selected for the present study is *Indigofera linifolia* (L.f) Retz. Belongs to the family Fabaceae. The plant material was collected from the Ayyaneri village of Kovilpatti taluk of the Tuticorin district of Tamil Nadu, India. It is situated 4 km from sub-district Kovilpatti and 60km from district headquarters Tuticorin district. The taxonomic features of collected species have been confirmed with the 'Flora of Presidency of Madras' (Gamble, 1928) and the 'Flora of the Tamilnadu Carnatic' (Mathew, 1981). (Figure -1).

Figure. 1. Natural Habit of *Indigofera linifolia* (L.f) Retz.

Phytochemical Analysis

Extraction

Mature and healthy plants were collected and washed thoroughly, and the leaf was cut into small pieces and dried at room temperature. The dried leaves were ground to a fine powder. About 30 gm of plant powder was taken in a digestion flask fitted to the Soxhlet apparatus, and extracts were obtained with different solvents such as petroleum ether and ethanol. The aqueous extract was prepared directly by boiling the powder with double-distilled H_2O. These extracts

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were concentrated and were kept in brown bottles and used for further analysis. The extracts were evaporated to dryness in a water bath. The plant extracts were off with a distillation apparatus, and yielded quantities of extracts in ethanol solvents were obtained and further taken to evaluate the phytochemical studies. (Greenlee, 2007).

1. Phytochemical Qualitative Analysis

The plant leaf extracts of Petroleum Ether, Ethanol, and Aqueous solutions were assessed for the existence of the phytochemical analysis using the following standard methods described by Harbone (1973) and Sofowora (1993) with slight modifications.

1. a. Saponins: 5.0 ml of distilled water was mixed with aqueous crude plant extract in a test tube, and it was mixed vigorously. The frothing was mixed vigorously with a few drops of olive oil, and the foam appearance showed the presence of saponins.

1. b. Tests for Flavonoids

(i) Alkaline Reagent Test: 2 ml of 2.0% NaOH mixture was mixed with aqueous plant crude extract; a concentrated yellow color was produced, which became colorless when we added two drops of diluted acid. This result showed the presence of flavonoids.

1. c. Test for Terpenoids: 2.0 ml of chloroform was added with the 5 ml aqueous plant extract, evaporated on the water path, and then boiled with 3 ml of H_2SO_4 concentrated. A grey color formed, which showed the entity of terpenoids.

1. d. Test for Steroids: 2 ml of chloroform and concentrated H_2SO_4 were added with the 5 ml aqueous plant crude extract. In the lower chloroform layer, red indicated the presence of steroids.

1. e. Tests for Lipids

(i) Emulsification Test: The sample is emulsified with 5ml of water and 5ml of bile salts and detergent. The emulsion is vigorous. Presence of lipids.

1. f. Tests for proteins

(i) Ninhydrin Reagent: Dissolve 1g of ninhydrin in water and make upto 100ml.1ml of test solution.A few drops of ninhydrin reagent heat for 2 minutes. The purple color formed. Presence of Protein.

1. g. Test for Carbohydrates

(i) Molish's Test: Take 2 ml of plant sample extract, and two drops of alcoholic solution of α-naphthol are added. The mixture is shaken well, and a few drops of concentrated sulphuric acid are added slowly along the sides of the test tube. A violet ring indicates the presence of carbohydrates.

1. h. Tests for Amino acids

(i) Millon's Test: Take 2 ml of filtrate and add a few drops of Millon‟ s reagent. A white precipitate indicates the presence of proteins.

2. Antimicrobial Activity

The antibacterial activities of different solvents of leaves of *Indigofera linifolia* (L.f) Retz. Extracts were tested using the agar disc diffusion method (Bauer *et al.*, 1996). The Kirby-Bauer method tested the antibacterial activity of isolated plant extraction pellets. The bacteria

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were collected from Scudder Institute of Laboratory, Nagercoil. The test organisms used for assay were *E. coli*, Pseudomonas *aeruginosa*, *Staphylococcus aureus* and *Bacillus subtilis*. The antibacterial activities of different solvent extracts were evaluated by measuring the zone of inhibition method. The samples for each bacterial strain were sub-cultured in individual agar slants. The name, gram reaction, nature, and distribution of the bacterial strains used for bioassay (Table 2).

2. a. Preparation of plant extract

Extracts were made from air-dried samples. 30 g of the dry powder of *Indigofera linifolia* (L.f) Retz. (powdered test materials) was separately extracted successively with 250 ml of Petroleum Ether (60-80º C), Ethanol, and Double distilled water. This sequence of solvents allowed for the leaching of all compounds based on their polarity. The individual fractions were collected and concentrated to obtain crude extracts. Water extract was prepared separately. Each extracted sample was dissolved in a solvent for experimental use to get a 1/10 solution.

2. b. Microbial strains

The agar disc diffusion method tested the antibacterial activity of isolated plant extraction pellets. The bacterial strains were grown on Muller Hinton (MH) agar plates at 37º C and maintained on nutrient agar slants, while fungi were grown at 30° C and maintained in potato dextrose agar slants. Each organism was maintained in a separate culture medium and was recovered for testing by sub-culturing on a fresh medium. Inoculums of each bacterial strain were transferred in 10 ml of Muller Hinton agar broth and incubated overnight at 37° C.

2. c. Preparation of Muller Hinton Agar Medium

Muller Hinton Agar - 35 gm.

Distilled water - 100 ml.

35 gm of Muller Hinton Agar was dissolved in distilled water and boiled. The content was for the complete dissolution of ingredients. The media, Petri dishes, pipette, and metallic borer were sterilized in an autoclave for 15 minutes at 121º C and 15 psi pressure. The media were poured into Petri dishes under aseptic conditions.

2. d. Muller Hinton Broth

Muller Hinton Agar - 120 gm.

Distilled water - 100 ml

Muller Hinton Agar was dissolved in distilled water. The medium was filtered through a cheesecloth and then autoclaved.

2. e. Preparation of sterile antibiotic discs

Antimicrobial activity was assayed by the filter paper disc diffusion method. Whatman No. 1 filter paper of 5 mm diameter was used. These discs were sterilized before use. The extracts of the medicinal plants were added to the sterile disc. Each sterile disc was incorporated individually with 200-500 µl of an extract of the medicinal plants using a micropipette. Precautions were taken to prevent the solvent extract's flow to the disc's outer surface. The condensed extracts were applied to the disc.

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2. f. Antibacterial assay

An antibacterial assay was conducted using the method described by Lennette (1985) with some modifications. 0.5 ml of the dilute microbial culture was spread on sterile Muller Hinton agar plates. The presoaked and dried discs were placed on the seeded plates and gently pressed down to ensure contact.

Amikacin (10 µg/ml) was used as negative control. The plates were incubated at room temperature for 24 hrs. After incubation, the inhibition zone around the discs was measured and recorded. Three replicates for each concentration were maintained.

2. G.Antifungal study

The standard strains used for the study are Aspergillus flavus, Aspergillus, Candida albicans, and *Candida*. This was grown on Sabourauds dextrose agar (SDA) (Hi Media Laboratories Pvt. Ltd; Mumbai, India) overnight at 37° C for 24 hours and 48 hours. 3-5 colonies of the standard strains of Candida albicans, Candida tropicalis, Aspergillus niger, and *Aspergillus flavus* were suspended in 2 ml of sterile normal saline and vortexed. The turbidity of the homogenous suspension was adjusted to approximately 0.5 McFarland standards.

2. h. Preparation of Sabourauds dextrose agar (SDA) medium

Dextrose - 40 g

Peptone - 10 g

Agar - 15 g

Suspend 65 g of the medium in one liter of distilled water. Heat with frequent agitation and boil for one minute to completely dissolve the medium. Autoclave at 121° C for 15 minutes. Excellent to 45 to 50°C and pour into Petri dishes or tubes for slants. Final pH (at 25° C) 5.6 ± 0.2.

2. i. Preparation of Potato dextrose agar (PDA) medium

Potato - 200 g.

Dextrose agar and distilled water

To prepare potato infusion, boil 200 g sliced, unpeeled potatoes in 1 liter of distilled water for 30 min. Filter through cheesecloth, saving effluent, or use a potato infusion (or commercial dehydrated form). Mix with Dextrose, Agar, and Water and boil to dissolve. Autoclave 15 min at 121° C. Dispense 20-25 ml portions into sterile 15 × 100 mm petri dishes. Final pH, 5.6 ± 0.2.

The sterile swab was dipped in suspension and swabbed on dried plates of Sabouraud's dextrose agar to get lawn culture. 6 mm sterile filter paper discs were purchased and sterilized. These were placed and inoculated on dried SDA plates. 10 µl of the extraction was placed on the disc. These plates were incubated at 37° C. Zone of inhibition was noted around the disc at 24 and 48 hrs. These experiments were repeated three times, and the average diameter was recorded. The negative control used in the study was the respective solvents, and the positive control was Clotrimazole.

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Results and Discussion

Proper control of starting material is essential to ensure the reproducible quality of herbal products. Thus, in recent years, there has been an emphasis on the standardization of medicinal plants of therapeutic potential. Despite the modern techniques, pharmacognostic studies' identification and evaluation of plant drugs are still more reliable, accurate, and inexpensive. According to the World Health Organization (WHO, 2000), a medicinal plant's macroscopic and microscopic description is the first step towards establishing its identity and purity. It should be carried out before any tests are undertaken (Anonymous, 2002).

Antibacterial activity Screening

Several synthetic drugs have been used to cure various diseases caused by pathogenic microbes in man. These drugs produced side effects due to overdose. Several medicinal plants have been identified and used to cure bacterial infections in human beings. In the present investigation, the antimicrobial activity of the leaves of *Indigofera linifolia* (L.f) Retz. It was reported with eight pathogens, and their zone of inhibition was tabulated. The results indicated that the plant extracts have good antimicrobial activity against different microorganisms. Recently, the use of leaf extracts against bacteria has increased because of the gradual increase in drug resistance among microorganisms. Several synthetic drugs have been used to cure various diseases caused by pathogenic microbes in human beings. Several medicinal plants have been identified and used to cure bacterial infections in human beings.

The present study shows the antibacterial activities of different solvent extracts of leaves of *Indigofera linifolia* (L.f) Retz. They were investigated and showed promising activity for the zone of inhibition against four human pathogens (Table 2. and Figure 3). The ethanol solvent extract exhibited different zones of inhibition against various microorganisms. Among the different extracts of leaves of *Indigofera linifolia* (L.f) Retz. Showed a maximum level of zone of inhibition. *Staphylococcus aureus* showed maximum level of inhibition against ethanol extract (21 mm), and *E. coli* showed maximum zone of inhibition in petroleum ether extract (19 mm). *Bacillus subtilis* (11 mm) and *Pseudomonas aeruginosa* (12 mm) exhibited a minimum level of inhibition against aqueous extracts (Figure 3).

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Figure No. 3. Antibacterial activity of leaves extracts of *Indigofera linifolia* (L.f) Retz. Using disc diffusion assay

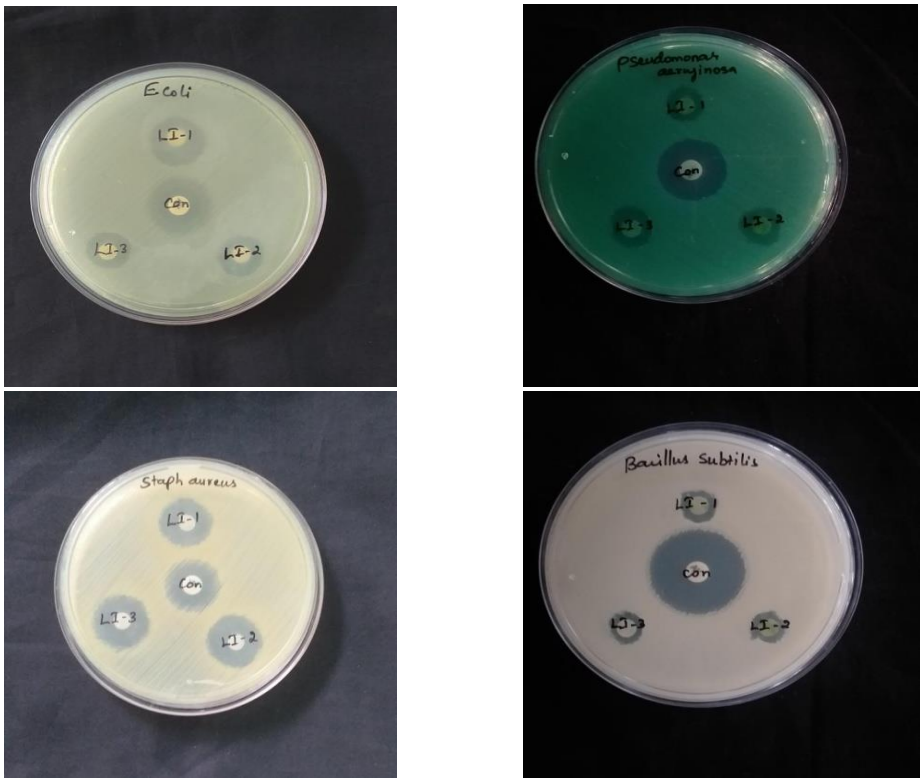
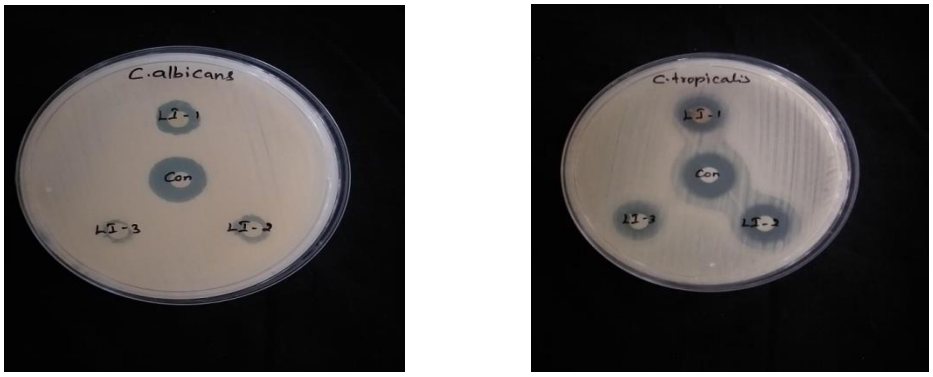


Figure No. 3. Antifungal activity of leaves extracts of *Indigofera linifolia* (L.f) Retz. Using disc diffusion assay



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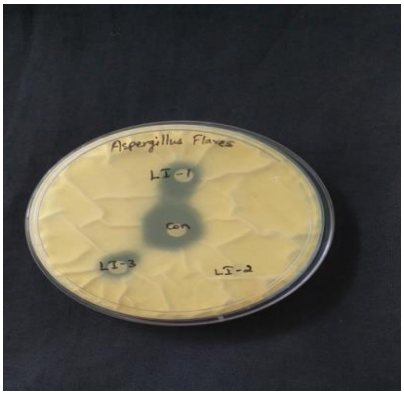

Table No. 2. Antimicrobial activity of leaves extracts of *Indigofera linifolia* (L.f) Retz. Using disc diffusion assay

S. No	Pathogens	Antibacterial activity - Zone of Inhibition (mm)			
		Petroleum Ether	Ethanol	Double Dis. H_2O	Control (Amikacin)
1.	*Escherichia coli*	19 ± 0.5	15 ± 0.3	13 ± 0.6	20 ± 0.4
2.	*Pseudomonas aeruginosa*	12 ± 0.2	14 ± 0.4	12 ± 0.3	21 ± 0.5
3.	*Staphylococcus aureus*	20 ± 0.3	21 ± 0.2	21 ± 0.2	20 ± 0.4
4.	*Bacillus subtilis*	12 ± 0.4	11 ± 0.5	11 ± 0.5	28 ± 0.3

± Standard Error, + Present, – Absent

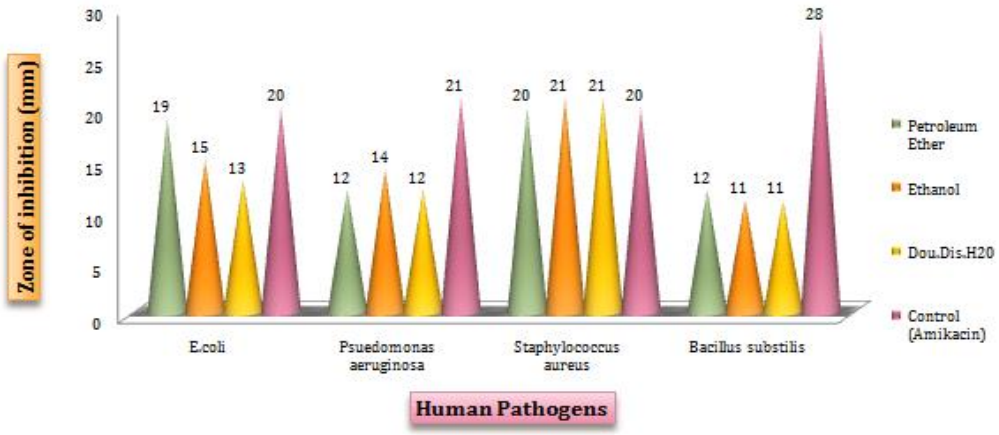

Table No. 3. Antifungal activity of leaves extracts of *Indigofera linifolia* (L.f) Retz. Using disc diffusion assay

S. No	Pathogens	Antifungal activity - Zone of Inhibition (mm)			
		Petroleum Ether	Ethanol	Double Dis. H_2O	Control (Nystatin)
1.	*Candida albicans*	12 ± 0.5	14 ± 0.3	09 ± 0.6	17 ± 0.4
2.	*Candida tropicalis*	15 ± 0.2	17 ± 0.4	16 ± 0.3	20± 0.5

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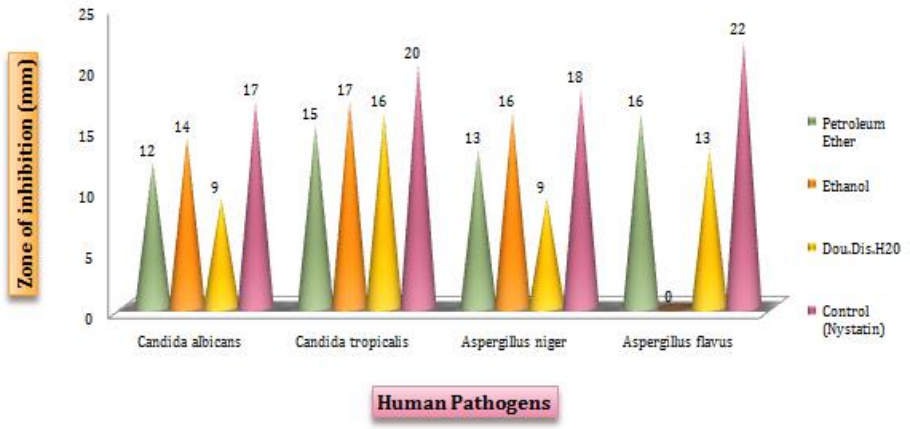
3.	*Aspergillus niger*	13 ± 0.3	16 ± 0.2	09 ± 0.2	18 ± 0.4
4.	*Aspergillus flavus*	16 ± 0.4	-	13 ± 0.5	22 ± 0.3

± Standard Error, + Present, – Absent



Antifungal activity

The antifungal activity of various solvent extracts of leaves of *Indigofera linifolia* (L.f) Retz.were carried out against four strains *viz. Candida albican, Candida tropicalis, Aspergillus niger* and *Aspergillus favus*. The results of the antifungal activity of different extracts of *Indigofera linifolia* (L.f) Retz.were presented in Table 2. *Candida tropicalis* growth is controlled by a maximum zone of inhibition (17 mm) in the ethanol leaves extract and a minimum zone of inhibition (12 mm) in the petroleum ether extract. In *Aspergillus flavus*, petroleum ether extract has a maximum level of zone of inhibition (16 mm) and minimum zone of inhibition (9 mm) in aqueous extract of *Indigofera linifolia* (L.f) Retz (Figure 4).

Analysis of Qualitative Phytochemical Screening

The phytochemical analysis of the four solvents, Ethyl acetate, Acetone, Ethanol, and Aqueous extracts, were tested for 13 phytochemicals (Table 4)**.** The procedure for the phytochemical analysis is carried out using standardized protocols.

In Petroleum ether, leaves extracts of *Indigofera linifolia* (L.f) Retz. Contains saponins, terpenoids, lipids, proteins, and amino acids. Flavonoids, Steroids, and Carbohydrates are absent in the leaves extract of Indigofera linifolia (L.f) Retz..in the Petroleum ether extract of the *Dodonaea viscosa* (L.) Jacq. Ethanol leaves extracts of *Indigofera linifolia* (L.f) Retz. It contains Saponins, Flavonoids, Terpenoids, Lipids, and Carbohydrates. Steroids, Proteins, and Amino acids are absent in the Ethanol extract of *Indigofera linifolia* (L.f) Retz. Where the Aqueous extract of the *Indigofera linifolia* (L.f) Retz. Flavonoids, Steroids, Carbohydrates, and Amino acids are present, and Saponins, Terpenoids, Lipids, and Protein steroids are absent in the Aqueous extract of *Indigofera linifolia* (L.f) Retz.

Table No. 4. Qualitative analysis of phytochemical constituents of leaves extracts of *Indigofera linifolia* (L.f) Retz.

S.No.	Phytochemical Tests	Petroleum Ether	Ethanol	Double dis.H_2O

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1.	Saponins	++	++	--
2.	Flavonoids	--	++	++
3.	Terpenoids	++	++	--
4.	Steroids	--	--	++
5.	Lipids	++	++	--
6.	Proteins	++	--	--
7.	Carbohydrates	--	++	++
8.	Amino acids	++	--	++

The phytochemical study evaluated the remarkable occurrence of steroids and flavonoids in all extracts. Other metabolites and bioactive compounds were identified, such as saponins, present in Petroleum ether and Ethanol extracts, while absent in the other extracts. Maximum amount of all the compounds such as Flavonoids, Saponins, Terpenoids, Lipids and Carbohydrates present in Ethonolic extract and Saponins, Terpenoids, Lipids, Proteins and Amino acids are indicated in the Petroleum ether extract.

The present phytochemicals study from the solvents like Petroleum ether, Ethanol, and aqueous extracts were tested for eight phytochemical investigations carried out using standardized protocols. It showed in Petroleum ether and Ethanol extracts of *Indigofera linifolia* (L.f) Retz. Contains five phytochemicals are present. In comparison, aqueous extract contains four phytochemicals that are present and remain absent.

Summary and Conclusion

Biodiversity, the variety of ecosystems, species, and genes, is recognized as one of the critical elements for human existence, as it provides vital goods and services such as food, carbon sequestration, or waste water purification. The biodiversity loss rate is considered to have passed its safe boundaries already. Notwithstanding significant uncertainties linked with the complexity of ecological systems and lack of consensus on distinct cause-and-effect relationships and the proper position of thresholds, it can be said with some confidence that Earth cannot sustain the current loss of biodiversity without reducing its capacity to provide valuable services.

The present investigation of screening phytochemical analysis and antimicrobial activity studies on selected plants was carried out. The selected plant is one of the essential ethno-medicinal plants used in different parts of the world. The plant is collected from the Ayyaneri village of Kovilpatti taluk of the Tuticorin district of Tamil Nadu.

* The antimicrobial activities of the plant extracts obtained in petroleum ether, ethanol, and double dis.H2O are carried out against four human pathogenic bacteria and four fungi. The outcomes are accounted for based on the diameter of the zone of inhibition around each disc (in mm). In the present study, all selected plant extracts generally show moderate to good antimicrobial activity. Positive control Amikacin and Nystatin were used and showed remarkable. Ethanol extracts showed a maximum zone of inhibition against selected

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pathogenic bacteria and fungi compared to other solvent extracts. Each solvent extract of the plants had its own choice of inhibiting microbial growth.

* Antibacterial activity studies were carried out with four human pathogenic bacteria and four fungi. The maximum zone of inhibition is observed in Ethanol extract against Bacillus subtilis, and the minimum zone of inhibition is observed in Staphylococcus aureus. Meanwhile, Fungi Candida tropicalis reveals a higher inhibition zone than *Candida albicans*.
* The phytochemical qualitative analysis of solvents like Petroleum ether, Ethanol, and Aqueous extract of Indigofera linifolia was tested for eight phytochemicals such as Saponins, Flavonoids, Steroids, Terpenoids, Carbohydrates, Lipids, Proteins, and Amino acids.

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