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Analysis Of Flavonoid Profile In Fruit Extract Of *Terminalia bellirica* ROXB. Using The Hptlc Technique And Evaluation Of Antimicrobial Activity

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Abstract
The Fruit *Terminalia bellirica* was subjected to phytochemical analysis, which identified several bioactive substances with antibacterial potential. Tannins, saponin, steroids, alkaloids, polyphenols, flavonoids, anthraquinone, terpenoids, triterpenoids, coumarins, and glycosides were found in the ethanolic and methanolic extract of Terminalia bellirica, according to several chemical tests and HPTLC analyses. According to research on various harmful bacterial and fungal strains, including *Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Candida albicans,* and *Aspergillus flavus,* the plant component can be utilized to treat illnesses brought on by these bacteria and fungus. The *Terminalia bellirica* fruit exhibits the strongest antibacterial action against bacteria when compared to fungi, according to the ethanolic and methanolic extract. The crude extract's efficacy in treating *Terminalia bellirica*, urinary tract, diarrheal, and gastrointestinal illnesses supported its use in conventional medicine. The aqueous extract exhibited little action and was ineffective against the microbiological strains.

Keywords: *Terminalia bellirica* , HPTLC, antibacterial activity, *Candida albicans*.

1. Introduction

The ailments known as infectious diseases are those brought on by bacteria, fungi, viruses, or parasites [1]. According to the WHO, infectious illnesses account for 50% of all fatalities in tropical countries and are a major source of morbidity and mortality globally. According to various publications over the past ten years, practically all clinically known antibiotic-resistant bacterial strains exist [2-3].

Undoubtedly, the widespread use and abuse of antibiotics have contributed to the proliferation of germs that are resistant to them. Due to the emergence of resistance, newer therapies are proven to be prohibitively expensive while first-line antimicrobials were both efficient and economical. They do not come without negative consequences. As a result, given the yearly increase in deadly opportunistic infections, the quest for novel antimicrobial medicines is becoming a popular subject.

Although many have received traditional pharmaceutical treatments, the public is becoming more interested in using natural products [4]. With their great variety of bioactive chemicals, medicinal plants are thought to be a preferable option [5].

They are regarded as non-toxic, secure, and sometimes the only source of healthcare for the underprivileged [6].

The antibacterial activity of Terminalia bellirica has not yet been reported. a well-known herb in the Ayurvedic System of Medicine and has historically been used to treat hepatitis, bronchitis, asthma, dyspepsia, piles, diarrhoea, coughs, hoarseness of voice, eye diseases, and scorpion stings. Because of this, nearly 80% of people are dependent, either entirely or partially, on plant-based drugs [7].

Methods

Plant material

The Terminalia bellirica Fruit was procured in Tamil Nadu, India's Thiruvarur district, in December 2018. To get rid of any remaining pollutants, distilled water was used to wash the Terminalia bellirica fruit many times. After a thorough inspection, the fruit's older, contaminated, and fungus-damaged sections were taken

out. A grinder combination was used to ground healthy fruit that had been dried at room temperature. Before being transported to the lab, the samples were put into plastic zip-lock containers and labeled appropriately.

Preparation of plant extract

Take one gram of Terminalia bellirica Fruit powder in each extract, which is made in 50 ml of ethanol and methanol solvent. The extract was shaken vigorously for 30 minutes with a free hand before being allowed to sit for 24 hours. The filtrate from the extracts was used for further analysis after the extracts were filtered using Whatman filter paper No. 1 and transferred to and stored in an airtight container.

Phytochemical screening

Preliminary phytochemical screening was carried out by using standard procedures described by Harborne (1984), Brunton (1995), Wagner et al. (1984) [8-9]. were conducted on various Terminalia bellirica extracts. The presence of coumarins, cardiac glycosides, polyphenols, anthraquinones, alkaloids, triterpenoids, terpenoids, steriods, flavonoids, saponins, and tannins was checked in the extracts.

Quantitative analysis of phytochemicals

Determination of phenols

Reagent: Ether,Ammonium,hydroxide,Amylalcohol

The phenolic component was extracted from the fat-free sample by boiling it in 50 cc of ether for 15 minutes. A 50 ml flask was filled with 5 ml of the extract and 10 ml of distilled water. Additionally, 5 ml of concentrated amyl alcohol and 2 ml of ammonium hydroxide solution were added. The samples were prepared as directed and given 30 minutes to react so that the color would develop. The wavelength of this was 505 nm.

Determination of Flavonoid

Reagents: 80% aqueous methanol

At room temperature, 100 ml of 80% aqueous methanol was used to extract 10 g of the plant material several times. Whatman filter paper No. 42 was used to filter the entire solution (125 mm). The filtrate was then put into a crucible, evaporated over a water bath to dryness, and weighed to a consistent weight.

Histochemical tests

Terminalia bellirica Fruit powder was processed using particular chemicals and reagents. The modified plant powder underwent additional light microscopy analysis. Flavonoids are indicated by the fruit of Terminalia bellirica which was treated with diluted ammonia and H2SO4.

A plant powder that has been treated with 5 drops each of acetic anhydride and H2SO4 to produce a color ranging from violet to blue or green shows the presence of steroids. The presence of polyphenols is shown by the blue-green/red color that plant powder treated with Toluidine blue produced. Terpenoids are present when plant powder that has been treated with a small amount of dinitrophenyl hydrazine becomes orange.

HPTLC studies

To confirm the existence and determine the concentration of phytochemicals, HPTLC (High-Performance Thin Layer Chromatography) examination was carried out. The methanolic extract was subjected to column chromatography on silica gel 60-120 mesh with solvents of increasing polarity beginning with hexane, chloroform, ethyl acetate, ethanol, and acetone in varied ratios to give sub-fractions. TLC examination revealed that the 100% ethyl acetate fraction was efficient. Standard Preparation: Quercetin 10 mg was dissolved in methanol 10 mg. The phase that remains constant: is Silica Gel 60 F254. Mobile phase: Toluene: Ethylacetate: Methanol Acetic acid (2.5:7.0:0.25:0.25) (2.5:7.0:0.25:0.25) Using a Linomat5 sample applicator, 5 l of the standard &5 and 10 l of the test solutions were applied to a silica gel 60 F254 HPTLC plates (E.Merck) that had been precoated. the plate in the solvent system until it was 8 cm away. Using the TLC Scanner3, densitometrically scan the plate. Using the CAMAG REPROSTAR3, the plate was examined under UV light at 254 and 366 nm. 254 nm, wavelength

Microbial strains

The microbial strains employed in the biological assays were *Staphylococcus aureus* (MTCC 3160), *Bacillus subtilis* (MTCC 441), *Escherichia coli*, (MTCC 732) and *Pseudomonas aeruginosa* (MTCC 741). The fungus

Candida albicans (MTCC 183) and *Aspergillus flavus* (MTCC 2813) were obtained from Microbial type culture collection (MTCC) at the institute of Microbial Technology (IMTECH), Chandigarh, India.

Culture media
Bacteria were activated using Nutrient Agar (NA-Himedia) Media, while fungi were activated using Potato Dextrose Agar (PDA-Himedia) Media. NA was also used to determine the minimum inhibitory concentration (MIC).

Preparation of inoculums
By culturing freeze-dried cells in NA for 24 hours at 37 degrees Celsius, bacterial inoculums were made. A loop of each of the microorganisms was suspended in around 10ml of physiological saline in a Roux bottle. Except for the fungal specimen, which was kept at 25°C for 48 hours, each of them was streaked onto the proper culture slants and incubated at 37°C for 24 hours. When the incubation time was through and growth was visible, the tubes were maintained at 2 to 8 C until use.

Preparation of test sample
A 10% aqueous DMSO solution was used to dissolve the aqueous and methanolic extracts to provide a variety of concentrations (30g, 50g, 100l, and 150l). 10% aqueous DMSO was utilized as the negative control (solvent control).

Antimicrobial assays
The disc diffusion technique was used to assess the antibacterial activity, and MIC values were also calculated.

Disc diffusion assay
30 ml of NA/PDA medium for bacteria/fungi was added to Petri plates to prepare them. Using a micropipette, the test organism was inoculated on a hardened agar plate, spread out, and given 10 minutes to dry. We injected bacteria and fungus from a broth culture onto the surfaces of the media. It is done by equally inoculating the Nutrient agar/PDA plate's whole surface with a sterile cotton swab soaked in a standardized bacterial/fungi test solution. In short, inoculums comprising different bacterial species were placed on potato dextrose agar for fungal strains and Nutrient agar plates for bacteria. With the use of sterile forceps, the sterile filter papers (6 mm in diameter) holding the crude extracts (50 l) were placed on the infected agar plate's surface.. The plates were incubated for 24 hours at 37 degrees Celsius for bacteria and for 24–48 hours at 30 degrees Celsius for yeast strains. Each sample was examined three times.

Minimum inhibitory concentration (MIC)
According to the twofold serial dilution procedure, the experiment was conducted. A 150 mg/ml stock solution of the test solutions (extracts) was made in nutritional broth and serially diluted up to five times. Each strain's MIC was tested in six assay tubes. A 50 mg/ml concentration of the test chemical solution was applied to the first tube after 1 ml of the sterilized nutrition broth had been infected. Additional dilutions of this solution were created by serially adding 1 ml from the first tube into the second assay tube, followed by 0.1 ml of each test inoculum in each tube. The actions were carried out in sterile circumstances.. The inoculation tubes were held at 37 C1 C for 24 hours for the bacterial test, and at 25 C0.1 C for the incubation time for fungus (A. niger) and fungi (C. Albicans) for three days and seven days, respectively. After the incubation period, tubes were taken out and checked for deposits or turbidity in the solution. They were then shaken to dislodge any bacteria or fungus that may have settled down during the incubation period. We noticed these amounts and considered them to be MIC.

Results and discussion
Preliminary phytochemical screening
In the ethanolic and methanolic extract, the studied plant produced good findings for a variety of phytochemicals, including tannins, saponin, steroids, alkaloids, polyphenols, flavonoids, anthraquinone, terpenoids, triterpenoids, coumarins, and glycoside. (Table-1 and figure1 a and b)

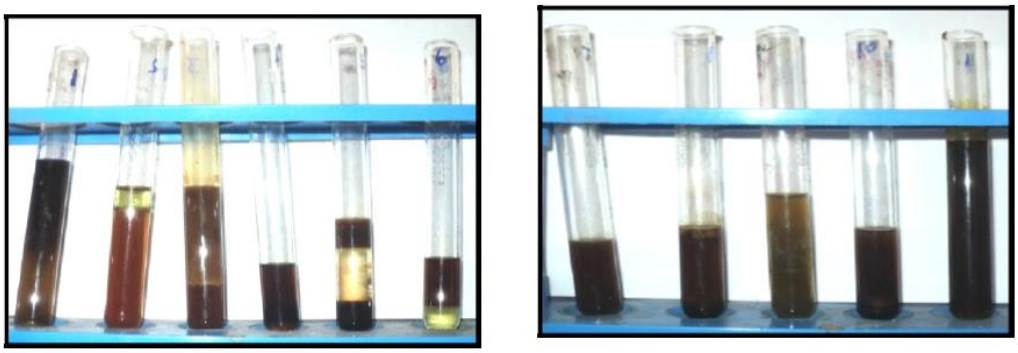

Fig.1 a & b: Qualitative analysis of Phytochemicals in *Terminalia bellirica* Fruit methanolic extract

Table.1: Qualitative analysis of Phytochemicals in *Terminalia bellirica* Fruit extract

S. No	Phytochemicals	Extract	
		Ethanol	Methanol
1	Tannin	++	++
2	Saponin	++	++
3	Flavonoids	++	++
4	Steroids	++	++
5	Terpenoids	++	++
6	Triterpenoids	++	++
7	Alkaloids	+	++
8	Antroquinone	+	++
9	Polyphenol	+	++
10	Glycoside	++	++
11	Coumarins	++	++

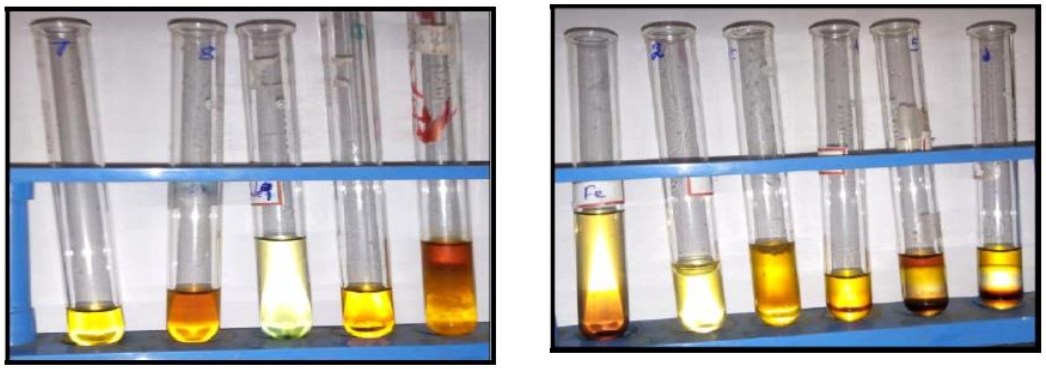

Fig.2 a &b: Qualitative analysis of Phytochemicals in *Terminalia bellirica* Fruit ethanolic extract

(+) Presence, (++) High concentrations and (–) Absences

Quantitative analysis

The Terminalia bellirica Fruit Powder has high amounts of flavonoids (29.87mg/gm) and polyphenols (136.73mg/gm), according to a quantitative study (Table2 and figure2 a&b). The aforementioned phytoconstituents underwent routine testing.

Table.2: Quantitative analysis of Phytochemicals in *Terminalia bellirica* Fruit powder

Phytochemicals	Results (mg/ml)
Poly phenol	136.73 ± 9.57
Flavonoids	29.87 ± 2.09

Histochemical analysis of *Terminalia bellirica* Fruit powder

Structure and development, as well as the timing of phytocompound synthesis and dispersion, have all been studied using histochemical approaches (Krishnan et al., 2001). The Terminalia bellirica fruit powder used in this study was treated with diluted ammonia and H2SO4 to give a yellow color that indicates flavonoids, with FeCL3 to give a dark blue to black color that indicates tannin, and with a few drops of toluidine blue to produce a blue color that indicates tannin. Blue, green, or red color denotes Adding a few drops of the Con. H2SO4 reagent to polyphenol produced When saponin is treated with acetic anhydride and con, it becomes yellow. H2SO4 (1:1) reagent produces violet to blue (or) green color, which denotes steroids (Table 3).

The findings further supported the existence of phytochemicals. Plants produce a wide variety of secondary metabolites that are used in defensive mechanisms, and recent years, it has been clear that some of these compounds have positive health impacts, such as antibacterial capabilities. [11] Plants are a source of substances that can fight disease, according to the WHO. In the Indian traditional medical system, Nymphaea nuchal is a well-known medicinal herb. The plant and its components are constantly being studied because of its many biological actions.

Medical studies on flavonoids like quercetin and catechin are getting more popular. [12] Numerous bacterial virulence factors, including toxins, enzymes, and signal receptors, are inhibited by flavonoids, including particular intracellular or surface enzymes. [13]

Table.3: Histochemical analysis of *Terminalia bellirica* Fruit powder intensity of the colour

S. No	Phytochemicals	Results
1	Starch	++
2	Tannin	++
3	Flavonoids	++
4	Glycoside	++
5	Terpenoids	+
6	Steroids	+
7	Saponins	+
8	Polyphenol	++
9	Alkaloids	+

Note: (+) Presence; (++) present with high

HPTLC analysis

With more than 8000 recognized chemicals, polyphenols, also known as phenolic compounds, are a class of secondary metabolites that are abundantly present in medicinal plants. [14] and have undergone testing as antimicrobials in both clinical and experimental research. [15] Reliable and sensitive quantification of an

essential biological active metabolite in the sample helps address quality control, which is a significant difficulty in the current situation. [16] One of the contemporary, advanced methods that may be used to assess the potency, veracity, quality, and purity of unprocessed pharmaceuticals is high-performance thin-layer chromatography. [17] HPTLC provides greater resolution, and an estimate of active ingredients may be carried out quickly and with fair precision.[18] The antimicrobial effects of polyphenols found in medicinal plants have been thoroughly studied against a variety of microorganisms. Tannins and flavonols, in particular, have drawn more attention because of their broad spectrum and the ability of the majority of them to suppress microbial virulence factors [19] The quantification of a few phenolic components including stigmaterol, rutin, and quercetin concentration is thus shown in the current work. (Table 4 and figure3).

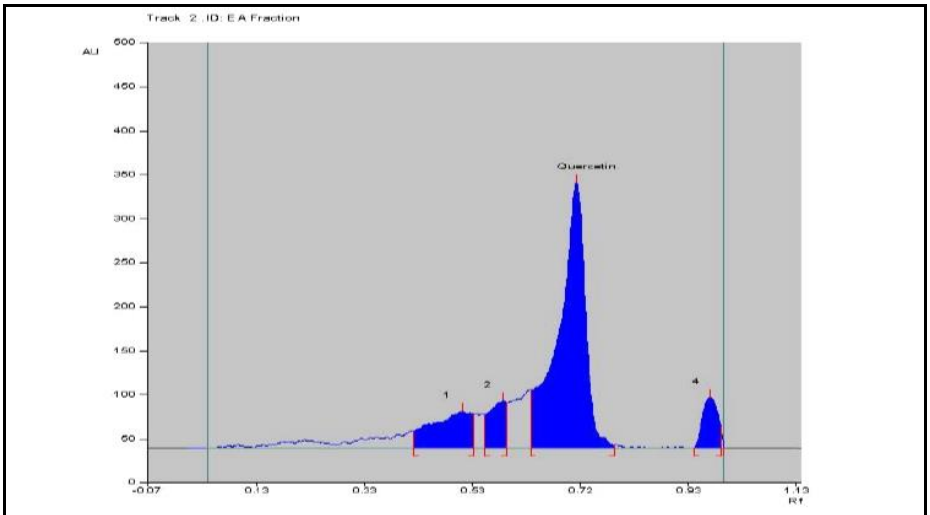

Fig 3: PEAK DISPLAY (5µl of Sample) of HPTLC in *T. bellirica*

Table 4: PEAK DISPLAY (5µl of Sample) of HPTLC in *T. bellirica*

Peak	Start Rf	Start Height	Max Rf	Max Height	Height %	End Rf	End Heigh	Area	Area %	Assigned substance
1	0.42	19.1	0.51	41.2	9.09	0.53	38.7	2291.5	14.31	Stigmaterol
2	0.55	37.2	0.58	53.3	11.78	0.59	51.3	1301.1	8.13	Rutin
3	0.63	65.9	0.72	301.1	66.48	0.79	2.7	11211.8	70.02	Quercetin
4	0.94	0.4	0.97	57.3	12.65	0.99	23.9	1208.9	7.55	Quercetin

Rutin contains Sideritis montana L. subsp. Montana and Nepeta italica L. seeds, both of which contain quercetin. The current study's findings demonstrate they inhibited the development of the microorganisms employed in the experiments at various ratios [20].

Antimicrobial activity

The research of antimicrobial activity can be seen as being of utmost importance, especially at this particular time in human history when bacterial resistance is continually bringing new problems for science. In many impoverished nations, infectious illnesses continue to be the leading causes of death despite significant advancements in pharmacological therapy. Antimicrobial agent resistance has grown to be a serious and urgent worldwide issue. There has been a significant investment in the hunt for novel antimicrobials to combat the growth of resistant microbes.

Bellirica terminalis plant extract Fruit was tested for the presence of the bacteria Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis, and Staphylococcus aureus, as well as the fungus Candida albicans and Aspergillus flavus, using the conventional agar disc diffusion technique. To find out whether a plant extract has antibacterial properties, employ the disc diffusion technique. The test organism was switched out for the hardened Nutrient agar plates, and the samples were then impregnated. The incubation

zone was measured after. The indication of the zone surrounding the disc served as a good indicator of the antibacterial activity of plant extracts.

Table 5: Anti-microbial activity of *Terminalia bellirica* Fruit extract

Microorganisms	Concentrations (µl/ml)				Std. (30µl/ml)
	30	50	100	150	
Bacteria					
Escherichia coli (mm)	3.50	3.50	6.25	7.25	11.75
Staphylococcus aureus (mm)	5.25	7.25	8.50	12.00	12.75
Bacillus subtilis (mm)	6.25	8.25	9.00	9.75	14.25
Pseudomonas aeruginosa (mm)	6.25	8.00	10.50	12.75	15.75
Fungus					
Candida albicans (mm)	6.50	9.50	9.50	11.00	11.50
Aspergillus flavus (mm)	5.50	7.00	6.75	8.25	11.00

The existence of the inhibitory zones shown in photographic (Plate 1) served as a qualitative indicator of the Terminalia bellirica Fruit extract's in vitro antibacterial activity against various bacteria and fungi. Inhibitory activities of the bacteria Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis, and Staphylococcus aureus in culture medium, as well as those of the yeast species Candida albicans and Aspergillus flavus, described in Table 5, were equivalent to those of conventional antibiotics, i.e. fluconazole and chloramphenicol

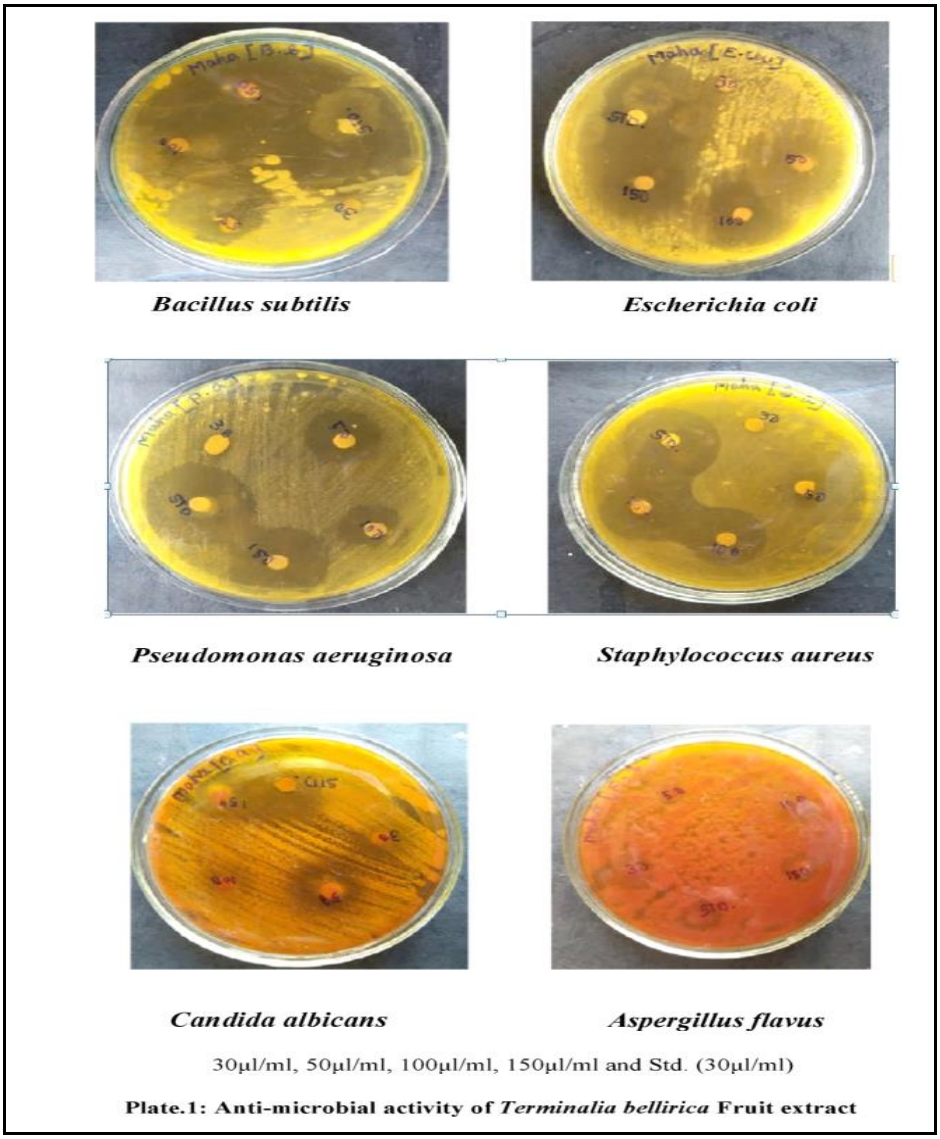

Plate.1: Anti-microbial activity of *Terminalia bellirica* Fruit extract

Conclusion

The evidence indicates *Terminalia bellirica* Fruit can be a source of bioactive compounds, including action against gram-positive and gram-negative bacteria, fungi, and yeast. Stigmaterol, rutin, and quercetin from fruit extract of *Terminalia bellirica* are also reported to have undergone HPTLC examination in the study. Its antibacterial action must have been influenced by the extract that included the phytocompound. Thus, the whole study highlights *Terminalia bellirica*'s potential as a new, sustainable source of a wide range of antimicrobial products. Due to this plant's intriguing biological activity, specialists are becoming increasingly interested in it. The molecule that has contributed to the extract's antibacterial activity and its precise method of action must be identified via more study, though.

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