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Isolation and Identification of Flavonoid Profile and Evaluation of Antimicrobial Activity of *Belleric myrobalan* Fruit Extracts

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Abstract: The fruits of *Belleric myrobalan* (*Terminalia bellirica*) were the subject of a phytochemical analysis, which made it possible to identify several bioactive substances with potential antibacterial properties. Tannins, anthraquinones, flavonoids, saponins, alkaloids, steroids, triterpenoids, polyphenols, terpenoids, glycosides and coumarins have been found in *Belleric myrobalan* ethanol and methanol extracts according to several chemical tests and HPTLC analyses. According to studies with various harmful bacterial and fungal strains, such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *E. coli* and *Flavus* of *Candida*, this plant component is available to treat diseases caused by these bacteria and fungi. The ethanol and methanol extract of *Belleric myrobalan* fruit has the strongest antibacterial action against bacteria compared to fungi. This crude extract is effective in the treatment of urinary tract diseases, diarrheal diseases and gastrointestinal diseases, and supports its use in conventional medicine.

Keywords: *Belleric myrobalan*, *Terminalia bellirica*, Antibacterial activity, HPTLC, *Candida albicans*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *E. coli*

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Introduction

Infection is a disease caused by bacteria, fungi, viruses or parasites. According to the WHO, infectious diseases account for 50% of all deaths in tropical countries and are one of the leading causes of morbidity and mortality worldwide. According to various publications over the past 10 years, almost all clinically known antibiotic-

resistant bacterial strains exist. There is no doubt that the widespread use and abuse of antibiotics contributes to the growth of antibiotic-resistant bacteria. With the advent of resistance, new treatments proved prohibitively expensive, but first-line antimicrobials were effective and economical. They do not come without negative

consequences. As a result, the search for new antibacterial drugs is becoming a popular topic, given the annual increase in fatal opportunistic infections. Although many undergo traditional pharmaceutical treatment, the general public is more interested in the use of natural products. Due to the wide variety of bioactive chemicals, medicinal plants are considered the preferred choice (Namita and Mukesh, 2012). They are non-toxic, safe and are sometimes considered the only medical source for disadvantaged people. The antimicrobial activity of *Belleric myrobalan* has not yet been reported. An herb famous for its Ayurvedic medical system, it has always been used to treat hepatitis, bronchitis, asthma, dyspepsia, mountains, diarrhea, coughing, hoarseness of the voice, eye diseases and scorpion stings. For this reason, around 80% of people rely on herbal medicines in whole or in part (Gootz, 2010).

Materials and Methods

Plant material:

The *Belleric myrobalan* (*Terminalia bellirica*) fruit was collected from Tamil Nadu in Thiruvallur district, India during December 2018. Before being transported to the laboratory, the sample was placed in a plastic ziplock container and correctly labeled (Abreu *et al.*, 2012). To get rid of the contaminants, the *Belleric myrobalan* fruit was washed several times using distilled water. After a thorough examination, the old parts of the fruit contaminated and damaged by fungi were removed. Using a combination of grinders, healthy crushed fruit were dried at room temperature.

Plant extract preparation:

1 g of *Belleric myrobalan* fruit powder was added to 50 ml of ethanol or methanol solvent. After stirring the extract vigorously for 30 min by free hand, allow to stand for 24 h. The extract was filtered through Watman No. 1 and transferred to an airtight container, stored it, and then used it for further analysis (Venkataswamy *et al.*, 2010).

Phytochemical screening:

Preliminary phytochemical screening by using standard procedures were conducted on various *Belleric myrobalan* extracts (Harborne, 1984).

Quantitative analysis of phytochemicals:

Determination of phenols:

The phenolic component was extracted from a fat-free sample by boiling it for 15 min in ether at 50°C. 50 ml of flask was filled with 5 ml of extract and 10 ml of distilled water. In addition, 5 ml of concentrated amyl alcohol and 2 ml of ammonium hydroxide solution were added. The sample was prepared and given 30 min to react so that the color developed. Its wavelength was 505 nm (Sathyabama *et al.*, 2012).

Determination of Flavonoid:

At room temperature, 10 g of plant material was extracted several times using 100 ml of methanol. Watman no.1 filter paper was used to filter the entire solution. The filtrate was then placed in a crucible, evaporated, dried in a water bath, and weighed to a constant weight. Histochemical tests were followed by standard method (Kuethe *et al.*, 2010).

HPTLC studies:

To confirm the existence and determine the concentration of phytochemicals, HPTLC 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. The phase that remains constant is Silica Gel 60 F254. Mobile phase: Toluene: Ethyl acetate: Methanol Acetic acid (2.5:7.0:0.25:0.25). Using a Linomat5 sample applicator, 5 l of the standard and 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

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

Fig.1: Qualitative analysis of Phytochemicals in *Belleric myrobalan* fruit extract.

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 (Brunton, 1995).

Antimicrobial activity:

The microbial strains employed in the biological assays were *Escherichia coli*, (MTCC 732), *Bacillus subtilis* (MTCC 441), *Staphylococcus aureus* (MTCC 3160), and *Pseudomonas aeruginosa* (MTCC 741). The fungus *Aspergillus flavus* (MTCC 2813) and *Candida albicans* (MTCC 183) were obtained from Microbial type culture collection (MTCC) at the Institute of Microbial Technology (IMTECH), Chandigarh, India. The antimicrobial activity was carried out by disc diffusion method.

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 conditions. The inoculation tubes were held at 37°C for 24 h for the bacterial test, and at 25°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 (Borges *et al.*, 2013).

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 (Fig. 1).

Quantitative analysis:

The *Belleric myrobalan* fruit Powder has high amounts of polyphenols (136.73 mg/g), and flavonoids (29.87 mg/g) according to a quantitative study (Table 1). The afore mentioned phytoconstituents underwent routine testing.

Table 1: Quantitative analysis of Phytochemicals in Belleric myrobalan Fruit powder

Phytochemicals	Results (mg/g)
Poly phenol	136.73 $\pm$ 9.57
Flavonoids	29.87 $\pm$ 2.09

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

Fig. 2: Phytochemical analysis of *Belleric myrobalan* fruit powder intensity of the colour.

Phytochemical analysis of Belleric myrobalan fruit powder:

The structure and timing of the appearance of plant compound synthesis and dispersion have all been studied by a histochemical approach (Gupta and Gupta, 2012). The *Belleric myrobaran* fruit powder used in this study was treated with diluted ammonia and H₂SO₄ to give a yellow color indicating flavonoids, FeCl₃ gave a dark blue to black color indicating tannins and added a few drops of toluidine blue to produce a blue color showing tannin. Production of polyphenol reagent H₂SO₄ Yellowing occurs when saponins are treated with acetic anhydride. H₂SO₄ (1:1) reagents produce a purple to blue (or) green color that represents the steroid (Fig. 2).

The results further confirmed the presence of phytochemicals. Plants produce a wide variety of secondary metabolites used in defense mechanisms, and it has been revealed that some of these compounds have a positive effect on health, such as antimicrobial ability (Ebrahimi

and Schluesener, 2012). According to WHO, the plant is a source of substances capable of fighting disease. In the traditional Indian medical system, *Nymphaeanuchal* is a famous medicinal plant. Plants and their components are constantly studied for their biological actions. Medical studies on flavonoids like quercetin and catechin are getting more popular. Numerous bacterial virulence factors, including toxins, enzymes, and signal receptors, are inhibited by flavonoids, including particular intracellular or surface enzymes (Cushnie and Lamb, 2011).

HPTLC analysis:

With more than 8,000 recognized chemicals, polyphenols, also called phenolic compounds, are a type of secondary metabolite abundant in medicinal plants and have been tested as antimicrobial in clinical and experimental studies (Shirolkar *et al.*, 2013).

One of the modern and advanced methods that can be used to evaluate the potency, veracity,

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

Fig. 3: Peak Display of HPTLC in *T. bellirica*.

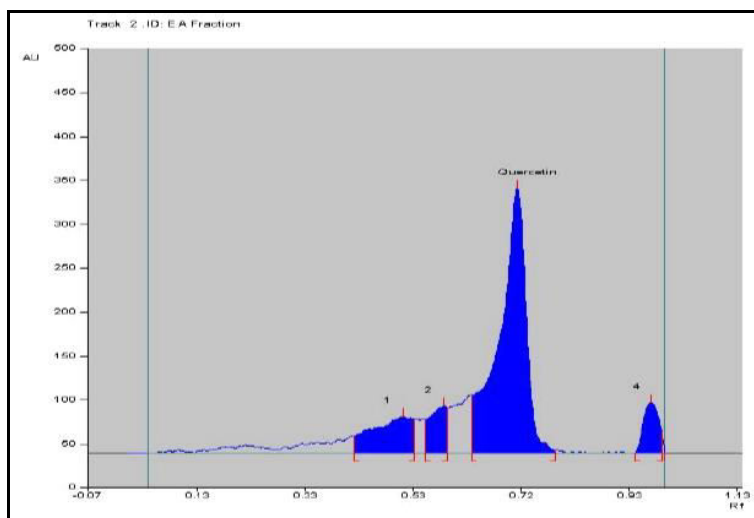


Fig. 4: HPTLC analysis of *T. bellirica*.

quality and purity of crude drugs is high performance thin layer chromatography (Syed *et al.*, 2013). HPTLC offers higher resolution and the estimation of the active ingredient can be carried out quickly and with fair precision. The antibacterial effect of polyphenols contained in medicinal plants has been studied in depth against various microorganisms (Daglia, 2012). In particular, tannins and flavonols attract more attention due to their broad spectrum and ability to suppress pathogenic factors of microorganisms for the most part. Thus, current research shows the quantification of some phenolic components, including stigmaterol, rutin and quercetin concentrations (Figs. 3, 4).

Antimicrobial activity:

The study of antimicrobial activity can be considered of paramount importance, especially

at this particular time in human history, when bacterial resistance continues to pose new problems for science. In many poor countries, infectious diseases remain the leading cause of death, despite significant advances in pharmacological therapy. Antimicrobial resistance is a serious and pressing problem around the world. Significant investments have been made in the search for new antimicrobials to combat the growth of resistant microorganisms (Emre *et al.*, 2011).

T. bellirica fruits extracted from plants were tested for the presence of antimicrobial activity for *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Staphylococcus aureus*, *Candida albicans* fungi and *Aspergillus flavus* using conventional agar diffusion techniques. To find out if plant extracts have antibacterial properties,

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

Fig. 5: Anti-microbial activity of *T. bellirica* fruit extract.

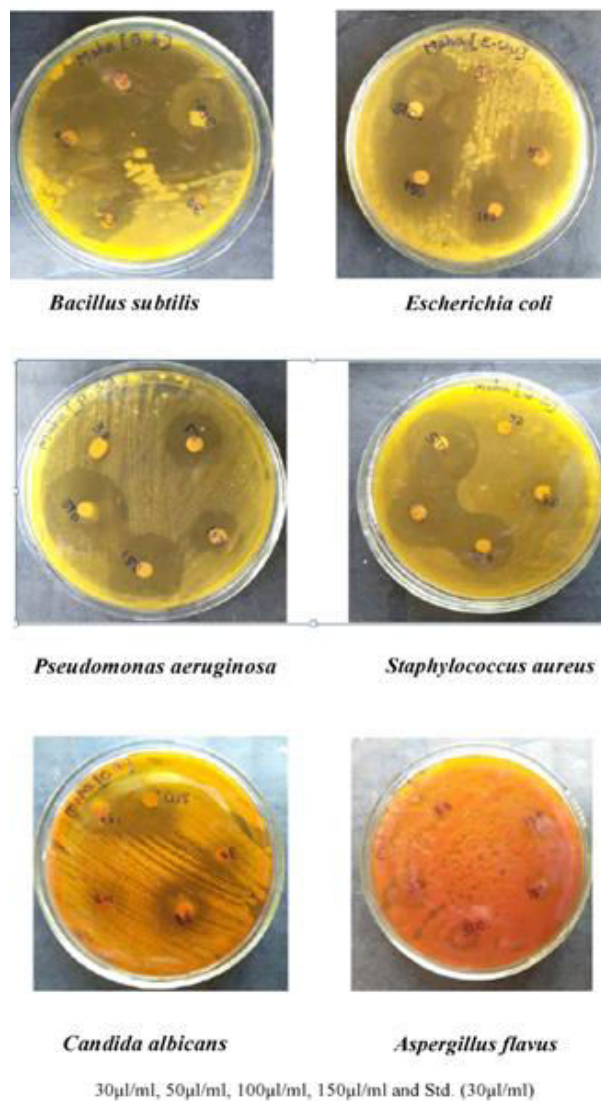


Fig. 6: Anti-microbial activity of *T. bellirica* fruit extract.

a disk diffusion technique is used. The test organism was replaced with sclerotrophic agar plates and then impregnated into the sample. The incubation zone was then measured (Figs. 5, 6). The display of the area surrounding the disc served as a good indicator of the antibacterial activity of plant extracts.

The presence of the inhibitory zone shown in Figure 6 served as a qualitative indicator of the *in vitro* antibacterial activity of *T. bellirica* fruit extract against various bacteria and fungi. The inhibitory action against *E. coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Staphylococcus aureus* and *Staphylococcus aureus* in the culture media, as well as the inhibitory action against the *Candida albicans* and *Aspergillus flavus* species illustrated in Figure 5, were comparable to conventional antibiotics.

Conclusion

Evidence shows that *T. bellirica* fruit may be a source of physiologically active compounds, including Gram-positive and Gram-negative bacteria and fungi. Stigmaterol, rutin and quercetin from *T. bellirica* extract were also undergone HPTLC testing in this study. Its antibacterial action must have been influenced by extracts containing plant compounds, therefore, this whole study highlights the potential of *T. bellirica* as a new sustainable source of a wide range of antibacterial products. Due to the interesting biological activity of this plant, experts are increasingly interested in it. However, the molecules that contributed to the antimicrobial activity of the extract and its precise way of acting should be identified through further research.

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