

ANALYSIS OF THE PHYTOCHEMICALS, ANTIOXIDANTS, AND ANTIMICROBIAL ACTIVITY OF *FICUS TSJAHELA* BURM. F LEAF, BARK, AND FRUIT EXTRACTS

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ABSTRACT

Objective: *Ficus tsjahela* Burm. f is a medicinal tree species, endemic to the Western Ghats having various healing properties. This study focused to check the antioxidant and antimicrobial activities of the leaf, bark, and fruit samples of *F. tsjahela*.

Methods: The plant samples were subjected to Soxhlet extraction for phytochemical analysis and further experimental studies. The test on phytochemical studies indicated the presence of alkaloids, saponins, glycosides, and flavonol glycosides within the plant parts, respectively. The estimation of alkaloids, saponins, *in vitro* antioxidant, and antibacterial activities revealed that the methanol bark extracts have high activity compared to others.

Results: Total alkaloid and saponin content was found to be high in leaf methanol extract was 996.17 mg/g quinine equivalent/g and 957.3 mg/g diosgenin equivalent/g, respectively. *In vitro* antioxidant assays revealed a strong radical scavenging potential of the methanol bark extract against stable (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), phosphomolybdenum, and superoxide radicals. Agar well diffusion method has been used to determine the antimicrobial activities against Gram-positive bacteria (*Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli*, *Acetobacter aceti*, and *Pseudomonas aeruginosa*).

Conclusion: The bark methanol extract of *F. tsjahela* has exhibited remarkable antioxidant activity and significant antibacterial activity ($p < 0.05$) against all tested bacterial strains observed.

Keywords: *Ficus tsjahela*, Western Ghats, Phytochemicals, Antioxidants, Antimicrobial Activity.

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INTRODUCTION

The medicinal value of each plant lies in the bioactive phytochemicals and their supporting defense system against stress conditions and diseases [1]. The utilization of medicinal plants for various ailments has been documented in the history of every civilization. The interest in the application of medicinal plants in the pharmaceutical industry has increased due to its safe and effective principle.

Phytochemistry, along with its chemical structures, metabolism, natural distribution, biological activity and functions, is a wide area of science concerned with the study of chemical constituents in plants. These phytochemicals are mainly responsible for defending themselves from invaders and escaping from a number of environmental stresses [2].

Naturally, the microorganisms are freely available within the surrounding ecosystem and they can easily reach us at any stage of harvesting, slaughtering, processing, and packaging [3]. The World Health Organization reported about 80% of the world's population still depends on traditional plant medicines for the treatment of some chronic diseases [4].

Ficus tsjahela Burm. f, a deciduous tree, grows up to 25 m tall, of family Moraceae distributed to Peninsular India, especially in the Western Ghats and Sri Lanka. It is well known as Kal-aal in Tamil. The tender leaves of the tree were used as a vegetable [5]. Ethnobotanically, the bark has been reported in the treatment of various health issues including antimicrobial activity. It is also used as astringent and slightly bitter and utilized in colic [6]. The study on antioxidant potential and antimicrobial activity validates ethnobotanical claims regarding the plant used in the various disorders.

METHODS**Plant collection and authentication**

F. tsjahela is an evergreen tree commonly seen in the deep forests of Western Ghats. The leaf, bark, and fruits were collected in the month of April-May at Coonoor, Tamil Nadu, Plant identification and authentication were done at Botanical Survey of India Southern Regional Centre, Coimbatore, the plant voucher specimen No: BSI/SRC/5/23/2016/Tech/1062.

Preparation of plant extracts

The collected plant samples were thoroughly washed, shade dried, powdered, and stored in an airtight container. The powder samples of *F. tsjahela* plant parts were subjected to extraction successively.

Soxhlet extraction

The sample extraction was done by the Soxhlet apparatus. About 50 g of powdered plant material was extracted with solvents of various polarity (petroleum ether, chloroform, ethyl acetate, methanol, and aqueous) for 6 h in about (300 mL). The crude extract of *F. tsjahela* leaves, bark, and fruits was filtered and evaporated.

Instrumentation

From the SHIMADZU UVmini-1240 UV-VIS spectrophotometer, all spectrophotometric records were collected. For absorption recording, glass cuvettes with dimensions (1 cm × 1 cm × 4.5 cm) were used.

Qualitative phytochemical screening

The leaves, bark, and fruits extract of *F. tsjahela* were analyzed for the presence of major phytochemicals such as alkaloids, saponins, glycosides, and flavonol glycosides, according to standard methods [7].

Alkaloids

Hager's test [8]

Solvent-free extract, 50 mg was stirred with 5 mL of dilute hydrochloric acid and filtered. To the filtrate, 2 mL of Hager's reagent (saturated aqueous solution of picric acid) was added. The formation of a bright yellow precipitate indicates that the test is positive.

Saponins

Frothing test [9]

Fifty milligrams of extract were diluted with distilled water and finalized to 20 mL. The content was shaken in a graduated cylinder for 15 min. The occurrence of a 2 cm layer of foam indicates the presence of saponins.

Glycosides

Borntrager's test [10]

Fifty milligrams of extract were hydrolyzed with concentrated hydrochloric acid for 2 h on a water bath and filtered. Two milliliters of filtered hydrolysate were added to 3 mL of chloroform and shaken to remove the chloroform layer. Then, 10% ammonia solution was added to it. The pink color indicates the presence of glycosides.

Flavonol glycosides

Magnesium and hydrochloric acid reduction [11]

Fifty milligrams of extract were dissolved in 5 mL alcohol and a little magnesium ribbon was added. Concentrated hydrochloric acid was dropped slowly into the test tube. The development of pink or crimson color indicated the presence of flavonol glycosides.

Quantification assays

Determination total alkaloid content

The total alkaloid content of the plant sample was measured using 1, 10-phenanthroline method described by Singh *et al.* [12] with little modifications. The extracted supernatant was used for the further estimation of total alkaloids. The reaction mixture contained 1 ml plant extract, 1 ml of 0.025 M FeCl₃ (ferric chloride) in 0.5 M HCl (hydrochloric acid), and 1 ml of 0.05 M of 1, 10-phenanthroline in ethanol. The mixture was incubated for 30 min in a predicament bath with a maintained temperature of 70±2°C. The absorbance of the red-colored complex was measured at 510 nm against reagent blank. Alkaloid contents were estimated and it had been calculated with the assistance of a standard curve of quinine (0.1 mg/ml, 10 mg dissolved in 10 ml ethanol, and diluted to 100 ml with distilled water). The values were expressed as mg/g Quin equivalent per g.

Determination of total saponin content:

Total saponin was determined by the method described by Makkar *et al.* [13]. About 0.5 g of sample was extracted with 25 ml of 80% aqueous methanol by shaking on a mechanical shaker for 2 h, after which contents of the tubes were centrifuged for 10 min at 3000 rpm during a tube an aliquot (0.25 ml) of the supernatant was taken to which 0.25 ml vanillin reagent (8% vanillin in ethanol) and a couple of 5 ml of 72% aqueous H₂SO₄ were added. The reaction mixtures within the tubes were heated during a water bath at 60°C for 10 min. All the test tubes were cooled in ice for 4 min and permitted to acclimatize to room temperature. Subsequently, the absorbance was measured during a UV/Visible spectrophotometer at 544 nm. Diosgenin was used as a reference standard and the results obtained were expressed as mg/g diosgenin equivalent per g.

In vitro antioxidant assays

(2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) (ABTS)+ radical cation scavenging activity

The total antioxidant activity of the samples was measured by ABTS+ cation radical decolorization assay according to the method of Re *et al.* [14]. ABTS⁺ was formed by reacting 7 mM ABTS aqueous solution

with 2.4 mM potassium persulfate in the dark for 12–16 h at room temperature. Before assay, this solution was diluted in ethanol (about 1:89 v/v) and equilibrated at 30°C to give an absorbance of 0.700±0.02 at 734 nm. The stock solution of the sample extracts was diluted such that after the introduction of 10 µL aliquots into the assay, they produced between 20% and 80% inhibition of the blank absorbance. After the addition of 1 mL of diluted ABTS solution to 10 µL of sample or Trolox (final concentration 0–15 µM) in ethanol, absorbance was measured at 30°C exactly 30 min after the initial mixing. The determination of triplicates was made at each dilution of the standard, and the percentage inhibition was calculated against the blank (ethanol) at 734 nm and then was plotted as a function of Trolox concentration. The antioxidant activity was defined as the concentration of Trolox having equivalent antioxidant activity and expressed as µM/g sample extracts.

Phosphomolybdenum assay

The antioxidant activity of samples was evaluated by the green phosphomolybdenum complex formation according to the method of Prieto *et al.* [15]. An aliquot of 40 µL of the sample or ascorbic acid in 1 mM dimethyl sulfoxide (DMSO) (blank) was added with 1 mL of reagent solution containing (0.6 M sulfuric acid H₂SO₄, 28 mM sodium phosphate Na₃PO₄, and 4 mM ammonium molybdate (NH₄)₆Mo₇O₂₄) in a test tube. All the test tubes were enclosed with foil and incubated at 95°C in a water bath for 90 min. Then, the samples were cooled to room temperature and the absorbance of the mixture was measured at 695 nm against the reagent blank. The result values were expressed as mg ascorbic acid equivalents per gram (AAE/g) extract.

Superoxide radical scavenging activity

The superoxide radical scavenging assay was based on the inhibition of formazan formed by scavenging the superoxide radicals generated in riboflavin–light–nitro blue tetrazolium (NBT) system [16]. Each 3 mL reaction mixture contained 50 mM sodium phosphate buffer (pH – 7.6), 20 µg riboflavin, 12 mM EDTA, 0.1 mg NBT, and 40 µL of the aliquot of sample solution or BHA and BHT (standard). The reaction was started by illuminating the reaction mixture with the sample extract for 90 s. After illumination, the absorbance was measured at 590 nm against the reagent blank (reaction mixture without plant sample). Identical tubes with reaction mixture kept in the dark served as the negative control. The superoxide anion scavenging activity was calculated as follows:

$$\text{Scavenging activity (\%)} = \left(\frac{[A_0 - A_1]}{A_0} \right) \times 100$$

Where, A₀ is taken as the absorbance of the control and

A₁ is taken as the absorbance of the sample extract/standard.

Evaluation of antimicrobial activity

The methanol extract of the leaf, bark, and fruit of *F. tsihela* was evaluated for its antimicrobial activity.

Microbial strains

Antimicrobial effect of *F. tsihela* leaf, bark, and fruit methanol extract against the selected enteric pathogens was carried out by the agar well diffusion method. Four standard human enteric pathogenic bacteria, namely, Gram-positive bacteria (*Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli*, *Acetobacter aceti*, and *Pseudomonas aeruginosa*), (procured from IMTECH, Chandigarh, India) were utilized in this study. Each bacterial suspension was swabbed on Muller-Hinton Agar (MHA) medium and wells were made on plates by employing a cork borer (6 mm). Overnight grown cultures of isolates were individually centrifuged at 5000 rpm for 10 min the supernatant was poured into the wells made on plates using a micropipette and was incubated at 37°C. The zones of inhibition were observed against pathogens after 24 h of incubation.

Agar well diffusion method:

The agar well diffusion assay described by Perez [17] was adopted. Different extraction with the various solvent systems of both tea plants was to gauge antimicrobial activity against various pathogens. The microbial cultures (100 µl) of different bacteria were separately

swabbed on MHA plate. Wells (5 mm in diameter) were made with the assistance of a sterile borer each extract (80 µl) obtained as above and added to each well. All plates were incubated at 37 ±1°C in an incubator for 24 h and the zone of inhibition was measured after 24 h. The zone of inhibition of test organism growth around each well was measured in mm. Each test was administered in triplicate [18].

ROS quantification assay

To estimate the reactive oxidative species produced within the microbial cell was determined by the method of [19, 20]. Methanol extract of *F. tsjahela* plant parts were prepared (1 mg/ml, 2% NBT solution was added to the plant extracts). The mixtures were incubated for 1 h at room temperature in the dark. This step is followed by centrifugation and supernatant was discarded. Pellet was washed with phosphate-buffered saline and centrifuged at 8000 g for 2 min. After centrifugation, pellets were washed again with methanol and centrifuged at 8000 g for 2 min. The pellets were collected after centrifugation was suspended in 2 M KOH for the disruption of the cell wall. In addition to the present, 50% DMSO solution was mixed and incubated for 10 min at room temperature to dissolve formazan crystals and centrifuged at 8000 g for 2 min. After centrifugation, 100 mL supernatant was transferred to 96-well plates, and absorbance was recorded at 620 nm using a microplate reader. LB media was taken as control and water used blank.

Statistical analysis

Values are expressed as mean±SE. One-way analysis of variance (ANOVA) was used to determine statistical significance and values with $p < 0.05$ were considered significant.

RESULTS

The results of preliminary phytochemical screening were given in Table 1. The screening results of *F. tsjahela* leaf, bark and fruit has expressed with significant report. The bark showed the richest presence of alkaloid and saponin compared to other parts. Flavonol glycosides were present in leaf extract but absent in fruit parts.

Determination of total alkaloid content

The quantitative phytochemical estimation indicates that the methanol extract of *F. tsjahela* bark, leaf, and fruit contains a significant amount of alkaloid content followed by aqueous extract (Fig. 1). The alkaloids content was quantitatively estimated and was found within the range of 970.83 mg/g Quin equivalent per g. Phenolic compounds such as phenols and phenolic acids, quinones, flavones, flavonoids and flavonols, tannins, alkaloids, terpenoids, and essential oils contribute to major groups of antimicrobial compounds [21].

Determination of total saponin content

The results of the present study show that saponin is present in *F. tsjahela* leaf aqueous extract in some significant amounts (957.3 mg/g diosgenin equivalent per g) compared to other solvent extracts (Figs. 2 and 3). Saponins possess a wide range of pharmacological activities including antimicrobial, anti-inflammatory, hypocholesterolemic, and hypoglycemic property [22].

In vitro antioxidant assays

ABTS radical cation scavenging activity

The Trolox equivalent antioxidant capacity was measured using the improved ABTS⁺ radical decolorization assay, one of the most commonly employed methods for antioxidant capacity, which measures the ability

of a compound to scavenge ABTS radical cation. The assay result was expressed as µM Trolox equivalent/g of extract. The results of ABTS⁺ cation radical scavenging activities of different parts of leaf, bark, and fruit extracts of *F. tsjahela* are shown in Table 3. The methanol and ethyl acetate extracts of bark showed higher radical scavenging activities (17,509.4 and 10,860.69 µM TE/g extract, respectively) as compared to that of other solvent extracts. The higher scavenging activity of fruit was observed in its ethyl acetate extract (4562.97 µM TE/g extract).

Phosphomolybdenum assay

The total antioxidant capacity was determined based on the reduction of Mo (VI) and to Mo (V) and the formation of green phosphate/Mo (V) complex with the maximal absorption at 695 nm. The total antioxidant capacity of different solvent extracts of leaf, bark, and fruit of *F. tsjahela* was analyzed and shown in Table 2. Among different parts used, bark showed higher activity in most of its solvents compared to the extracts of leaf and fruit. The better antioxidant capacity was shown by ethyl acetate extract of bark (1194.76 mg AAE/g extract). The lower antioxidant capacities of the fruit extracts were found to be ranging from 165.54 to 230.34 mg AAE/g extract. Among the different solvents used, the ethyl acetate extracts of all the parts showed better antioxidant capacity as compared to other solvent extracts. Thus, the antioxidant capacity observed from the extracts of *F. tsjahela* can be correlated with its free radical scavenging activity equivalent to that of natural antioxidant ascorbic acid.

Superoxide radical scavenging activity

Superoxide radical is known to be a very harmful species to cellular components as a precursor of more reactive oxygen species. The superoxide radical can result in the formation of H₂O₂ through a dismutation reaction.

The results of superoxide anion scavenging activities of bark, fruit, and leaf of *F. tsjahela* are shown in Fig. 4. The plant extracts were found to be a capable scavenger of superoxide radicals generated in riboflavin-NBT-light system *in vitro*. The scavenging activity on ethyl acetate extract of bark, fruit, and leaf was found to be 47.90%, 50.81%, and 48.49%, respectively, whereas, that of methanol extracts of bark, fruit, and leaf was found to be 48.81%, 49.35%, and 49.57%. The methanol and ethyl acetate extracts showed moderate free radical scavenging activity to that of BHT and BHA. All the parts of *F. tsjahela* were found to have good scavenging activity. From this assay, using different extracts of *F. tsjahela*, it is noted that the inhibition of the formation of blue formazan and also the percentage inhibition is directly proportional to the concentration of the plant extract.

Antimicrobial activity

The antimicrobial potential of the experimental plants was evaluated according to their zone of inhibition against various pathogens and also the results were compared with the activity of the standards, streptomycin (1.0 mg/disc).

The result has shown that plant samples have potent antimicrobials action toward all the microorganisms studied (Fig. 5). The antimicrobial properties of the methanol extract of *F. tsjahela* leaf, bark, and fruit have been assessed during this study. For the entire tested microorganisms, bark methanol showed maximum antibacterial activity. The maximum inhibition zone diameter was

Table 1: Phytochemical screening of *Ficus tsjahela*

Test	Name of the test	Observation	Results		
			Leaf	Bark	Fruit
Alkaloids	Hager's test	Prominent yellow precipitate	++	+++	+
Saponins	Frothing test	The layer of foam indication	++	++	+
Glycosides	Borntrager's test	Pink color	+	++	-
Flavonol glycosides	Magnesium and hydrochloric acid reduction	The crimson color indicated	+++	++	-

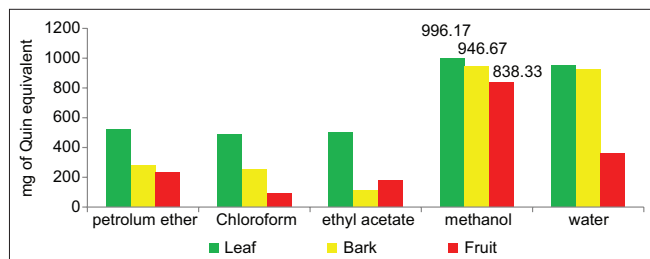
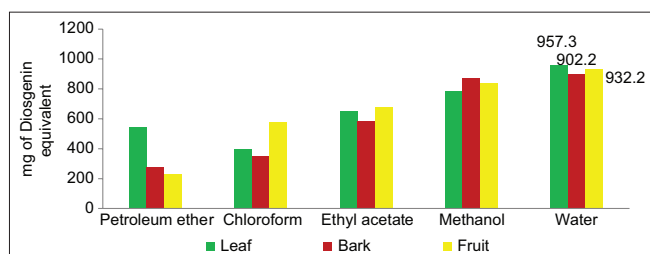
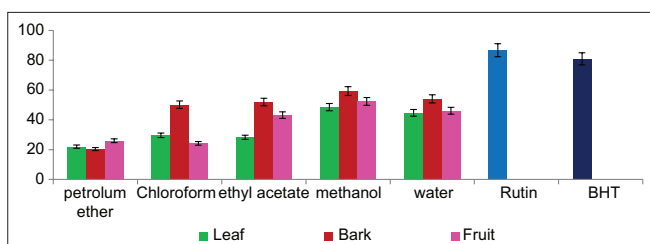
Table 2: Determination of phosphomolybdenum reduction by *Ficus tsjahela*

Sample extracts	Bark (mg AAE/g extract)	Fruit (mg AAE/g extract)	Leaf (mg AAE/g extract)
Petroleum ether	717.34±23.18 ^c	494.0±35.15 ^c	356.66±2.3 ^d
Chloroform	539.34±44.66 ^d	700.66±34.07 ^b	450.6±20.03 ^d
Ethyl acetate	916.0±12.49 ^a	750.0±6.92 ^b	558.60±20.03 ^c
Methanol	946.0±8.71 ^a	894.0±69.54 ^a	871.0±2.01 ^a
Water	812.66±74.00 ^b	754.0±4.0 ^b	749.33±11.7 ^b

Values are mean of triplicate determination (n=3) ±standard deviation.

Statistically significant at p<0.05 where ^a > ^b > ^c > ^d in each column

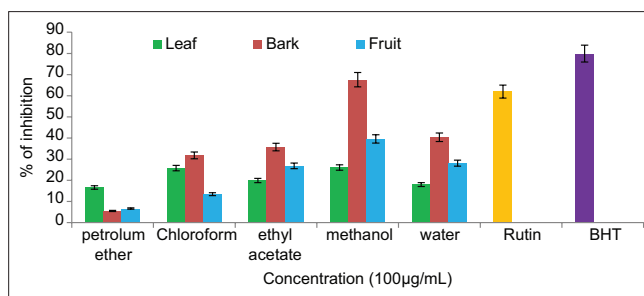
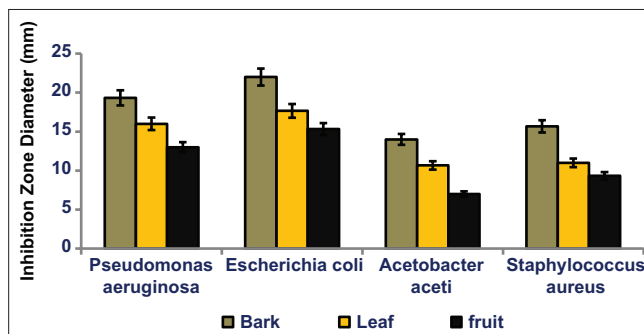
(AAE – Ascorbic acid equivalent)

**Fig. 1: Determination of total alkaloid content****Fig. 2: Determination of total saponin content****Fig. 3: (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) radical cation scavenging activities of *Ficus tsjahela***

obtained in *E. coli* and in *P. aeruginosa* with diameter 22±1 mm and 19.33±1.5 mm, respectively (Table 3). Similarly, fruit methanol extract (7±1 mm) showed minimum activity.

DISCUSSION

F. tsjahela is commonly consumed as a vegetable and used as folk medicine by the traditional community for the control of various diseases. A very few investigations are available on the biochemical components and potential medical effects of this plant. This study report has added credits to the antimicrobial activity and chemical components of the plant parts. In general, plants possess a wide range of compounds to protect themselves from external harmful factors. In such a way, phytochemicals are considered as the potential supplement in

**Fig. 4: Superoxide radical scavenging activity of *Ficus tsjahela*****Fig. 5: Antimicrobial activity of methanol extracts of *Ficus tsjahela* plant parts**

pharmaceutical industries. Plants and their bioactive substances are the only effective resource for the replacement of synthetic drugs [23].

In the present study, leaves, bark, and fruits of *F. tsjahela* were extracted with non-polar to polar solvents. The phytochemical analysis of plant extracts recorded the presence of alkaloids, saponins, glycosides, and flavonol glycosides. Estimation of saponins, alkaloids, and *in vitro* antioxidants studies on *F. tsjahela* leaves, bark, and fruits was investigated for ABTS radical cation scavenging activity, phosphomolybdenum, and superoxide radical scavenging activity. Results have shown a higher response to methanol bark extract when compared to petroleum ether, chloroform, ethyl acetate, methanol, and aqueous extracts. It has also proven the effectiveness of the extracts in comparison to that of the reference standard antioxidants. Superoxide anion damage on biomolecules is directly or indirectly influenced by the formation of H_2O_2 , OH, peroxy nitrite, or singlet oxygen during aging and other physiological events. The action of superoxide may also directly involve in lipid peroxidation. The superoxide scavenging activity of the plant extracts was found to be increased with the increase in concentrations. Methanol bark extract showed potent superoxide radical scavenging activity. Thus, higher inhibitory effects of methanol extract of *F. tsjahela* bark on superoxide anion formation showed promising antioxidants [24]. ABTS+ diammonium salt (ABTS) cation, a more drastic radical, chemically produced and is frequently used for screening complex antioxidant mixtures such as plant extracts, beverages, and biological fluids. The ability in both the organic and aqueous media and their stability in a wide pH range raised the interest in the use of ABTS for the estimation of antioxidant activity [25]. It has been found methanol extract of bark of *F. tsjahela* as an effective scavenger of ABTS radical and showed higher activity relative to Trolox standard. Proton radical scavenging is a significant characteristic of antioxidants. Hence, ABTS, a protonated radical, has a distinguishing absorbance maximum at 734 nm which decreases with the scavenging of the proton radical [26]. Antioxidant and antimicrobial properties of various plant extracts are of immense interest in several industries, since their probable use as a natural alternative instead of synthetic antioxidants [27].

Table 3: The zone of inhibition produced by *Ficus tsihela* methanol extracts against pathogenic bacteria

Microorganism	Zone of inhibition			Standard	Control
	Bark	Leaf	Fruit	Streptomycin	DMSO
<i>Pseudomonas aeruginosa</i>	19.33±1.5	16±2.6	13±1	28.24±0.45	0
<i>Escherichia coli</i>	22±1	17.67±1.5	15.33±1.5	30.45±1.02	0
<i>Acetobacter aceti</i>	14±1	10.67±1.52	7±1	20.53±0.13	0
<i>Staphylococcus aureus</i>	15.67±0.57	11±2.6	9.33±1.52	21.34±1.03	0

Values were expressed as mean±SEM and determined by one-way analysis of variance (ANOVA) followed by Dennett's t-test; *p<0.05

Antioxidants can protect our body from the formation of free radicals and help to scavenge them. Phytochemicals are probably rich in antioxidant properties. The methanol extract of *F. tsihela* was more effective in inhibiting the growth of pathogens by the good diffusion method. The leaf, bark, and fruit methanol extracts were tested for antibacterial activity and showed significantly ($p<0.05$) results against *S. aureus*, *E. coli*, *A. aceti*, and *P. aeruginosa* bacterial strains. Plants were considered as the live treasure of nature producing a wide collection of phytochemicals which significantly roles in antimicrobial activities as well as metabolic activities and serves as a relevant resource [28]. This diverse existence of rich phytochemicals has become popular due to its enhanced defense mechanisms alongside a variety of microorganisms, nematodes, insects, and other plants [29]. Consequently, the exploration for novel products with antimicrobial properties is a very lively segment of research and here plays the importance of natural resources [30]. Comparatively, herbal preparations are cheaper and safe compared to synthetic products which are harmful to more side effects, in such a way, antimicrobial studies were carried out. The plant *F. tsihela* is having a high potential value both ethnobotanically and scientifically, hence, it can be referred to as a safer medicine against pathogenic resistant bacteria.

CONCLUSION

Phytochemicals are bioactive chemicals of plant origin and regarded as secondary metabolites. They are naturally synthesized in all parts of the plant part such as bark, leaves, stem, root, flower, fruits, and seeds as active components. Preliminary screening of this active component is most important in the pharmaceutical industry. Our study has shown that the extracts of *F. tsihela* leaf, bark, and fruit contain effective phytocomponents and showed antibacterial activity. Analysis of plant extract showed the presence of glycosides, saponins, steroids, terpenoids, flavonoids, alkaloids, and reducing sugar. The sensitivities of the test organisms toward the plant extracts were indicated by a clear zone of inhibition around the holes containing the plant extracts and the diameter of the clear zone was taken as an index of the degree of sensitivity. The results revealed that the methanolic extracts of the selected plant are efficiently suppressing the growth of food pathogens and spoilage microorganisms with variable potency.

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AUTHORS' CONTRIBUTIONS

Thamaraikani V and Amala Divya S have performed data collection, drafting of manuscript, and graphical representation of statistical data, Sekar T performed the study design and editing of the manuscript.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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