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Antimicrobial Activity of Stigmasterol from the Leaf Extract of *Ficus hispida*

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Abstract

Antimicrobial resistance is a serious global threat to human health which necessitated to search safe and effective therapeutic. The present study investigated antimicrobial activity of Stigmasterol, isolated from the leaf extract of *Ficus hispida*. Different concentration of Stigmasterol was subjected to antimicrobial activity using well diffusion method against gram-positive and gram-negative bacterial species, with Ciprofloxacin as standard. The susceptibility results showed that the compound stigmasterol (100 µg/ml) inhibited the growth of all the test organisms with mean zone of inhibition range from 20.3 to 38.5 mm. Stigmasterol exhibited antibacterial activity over to Ciprofloxacin (10 µg/ml) against specially on *Streptococcus faecalis* and *Pseudomonas fluorescens* and moderate effect with other bacterial species includes *Bacillus subtilis, Bacillus cereus, Streptococcus faecalis* and *Escherichia coli.* The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of stigmasterol range from 3.12 to 100 µg/ml. Stigmasterol, a potent antibacterial similar the standard drug which could be safe and effective alternative and serve as lead compound in the development of novel antimicrobial drugs.

***Key words*:** *Ficus hispida*, Bioactive compound, Stigmasterol, Ciprofloxacin, Antibacterial activity

The incidence of microbial infections has grown over the past few decades worldwide. In developing countries, up to one-half of deaths are due to infectious diseases [1]. According to the Centres for Disease Control and Prevention, at least two million people suffer from serious infections every year caused by bacterial resistance and at least 23,000 people may die [2]. According to the World Health Organization (WHO), these resistant microorganisms (such as bacteria, fungi, viruses, and parasites) may combat attack by antimicrobial medications, which results in inefficient treatment that causes diseases to remain and spread [3]. Treating microbial infections, particularly in immunosuppressed patients remains challenge due to antimicrobial resistance. The unavailability of an adequate antibiotic pipeline has resulted in the emergence of many types of infections that are very difficult to treat, owing to the paucity of viable alternative antibiotic alternatives [1].
The mystery of multidrug resistance and side effects of most antibiotics commercially available necessitate the continuous search for new antimicrobial agents [4]. The plant-derived substances as alternative for synthetic antibiotics against the diseases has impact on metabolic, genetic and physiological fronts and serves as a barrier for destroying the progression of resistant microbes [5]. WHO recognizes that medicinal plants are the major sources of new drug varieties [6]. Further, the researchers are actively involved in search of plant derived substances using various techniques. Though the interest of the pharmaceutical companies in natural products has dwindled, there is an urge to tackle multidrug resistance and other emerging disease has fueled the rebirth of natural product drug discovery [7].

The plant-based drugs used in the traditional systems continue to play a significant role in health care and it has been projected by the WHO 80% of the world's inhabitants rely mainly on traditional medicines for their primary health care and remaining 20% of the populations residing in developed countries the plant products also play an important role in the health care systems [8]. Extraction, isolation, identification and biological properties of chemical substances from the various medicinal plants have now framed the significant field of study in biochemical and pharmaceutical sciences. This may be due to the presence of wide range phytochemical constituents present in the plant. Thus, keeping the importance of plants is being screened for newer and effective chemotherapeutic agents. Higher plants can serve as potential anti-infective and source of new antimicrobial agents [9].

Ficus is one of the largest genera in the plant kingdom that belongs to the Moraceae family. There are more than 800 species of *Ficus* that have been discovered, generally known as figs or fig trees. Theses has shown promising results in treatment of parasitic infection and in addition it has a broad

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spectrum of activity against pathogenic bacteria and fungi [10. Among *all the species Ficus hispida* (Tamil Name: Kodi Atthi) considered as a valuable plant due to its various pharmacological activies. *Ficus hispida* is a hairy shrub, moderate sized tree and has figs which hangs on long stems found throughout the year and growing in ever green forest. This plant is often cultivated in villages for shade and its edible fruits in India, Sri Lanka and Myanmar [10]. As per the ancient and recent literatures, different parts of the plant have been used ethnomedicine for the treatment of a number of diseases including in the treatment of anemia, ulcers, dysentery, psoriasis, piles, jaundice, vitiligo, hemorrhage, convulsion, hepatitis, diabetes and biliousness [11].

Phytochemical screening of *Ficus hispida* using various solvents such as hexane, ethylacetate, and ethanol extracts reported the presence of different secondary metabolites including tannins, phenols, alkaloids, terpenoids, glycosides, steroids, and flavonoid [12]. The search for the healing potential of natural products is an idea from ancient times that is once again being pursued. Several studies revealed that *Ficus hispida* highly consist with phytosterols compound, naturally occurring compounds found in plant cell membranes plays unique role for human health [13]. Stigmasterol is one of the most essential steroid compounds that belongs to the group of phytosterols of the plant. Stigmasterol natural agent was found to be the major ingredient isolated from the leaf extract of *Aegle marmelos, Annona muricata* and *Acacia nilotica* from India and *Ophiopogon japonicas* (Maidong in Chinese). In addition, from the extracts of *Plectranthus scutellarioides*, *Albizia gummifera*, *Combretum hypopilinum*, *Salvadora persica* and *Neocarya macrophylla* [14]. It has different biological properties and considered a popular medicinal compound throughout the world. Stigmasterol has established and emerging health benefits including anti-inflammatory activity, immunomodulatory effect, antimicrobial, anticancer effect, hepatoprotective, antioxidant, antidiabetic activities [15]. Therefore, this steroid derivative can be used as a potential drug in treatment of various diseases.

Some reports exist in the literature on the antimicrobial properties and different extracts of *Ficus hispida*. Recently, isolation of stigmasterol and its characterization from the stem bark of *Nepenthes macrophylla* was reported. The antimicrobial activity of stigmasterol which was isolated from the stem bark of *Nepenthes macrophylla* was investigated against representative gram-negative gram-positive bacteria and two fungi are also reported. Stigmasterol was subjected to antimicrobial screening against methicillin-resistant *Staphylococcus aureus*, vancomycin resistant *Staphylococcus aureus*, *Streptococcus faecalis, Escherichia coli, Salmonella typhimurium, Pseudomonas fluorescens, Klebsiella pneumonia, Candida albicans,* and *Candida krusei* using agar diffusion and broth dilution methods. Results showed Stigmasterol acts as an inhibitory compound, possess potent and broad spectrum antibacterial and antifungal properties and may be applied as a lead compound in the progress of novel antimicrobial drugs [16]. Antimicrobial activity of medicinal plant used in Ayurveda and traditional medicinal system for treatment of manifestations caused by microorganisms has to be analyzed deeper [17].

Stigmasterol was identified from the leaf extract of *Ficus hispida* using GC-MS and other spectral analysis previously. Further, different concentration of stigmasterol was subjected for antibacterial activity using selected bacterial strain includes against gram-positive and gram-negative bacteria using agar well diffusion method.

MATERIALS AND METHODS

Isolation and identification of stigmasterol

Stigmasterol (Fig 1) was isolated from methanol fraction of the leaf of *Ficus hispida* as a white crystalline solid substance by chromatographic techniques. Further, structure was established using nuclear magnetic resonance (NMR) analysis by comparison of data obtained with those reported in the literature [18].

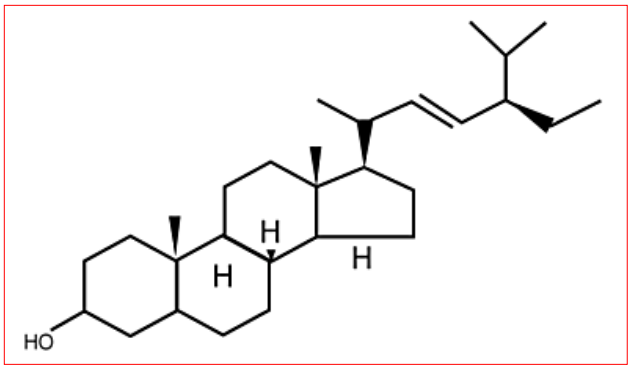

Fig 1 Structure of the isolated compound stigmasterol (Molecular Formula: $C_{29}H_{48}O$) from the leaf extract of *Ficus hispida*

Antimicrobial activity of stigmasterol

Preparation of isolates

The American Type Culture Collection (ATCC) pure isolates were obtained from the department of Microbiology, Muthayammal arts and science college, Salem, Tamil Nadu, India. All bacterial cultures were contamination free, sub-cultured frequently and maintained in stocks. The selected bacterial isolates include *Bacillus subtilis* (ATCC 6051a), *Bacillus cereus* (ATCC 14579), *Staphylococcus aureus* (ATCC 25923), *Streptococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), and *Pseudomonas fluorescens* (ATCC 27853). From the stock of each organism, an entire loop was taken, inoculated into 5 ml sterile nutrient broth and subsequently incubated for 18-24 hours at 37°C [16].

Antibacterial screening

The antibacterial assay of the stigmasterol was carried out by agar well diffusion method as per previous protocol [19]. The Muller Hinton Agar media was inoculated with 100 µl of the inoculum (1.5×10^8 CFU/ml) and poured in to petriplates. In this method, 4 mm well was prepared in the plate using a cork borer and added 50 µl of the sample at 50 µg/ml and 100 µg/ml concentration using dimethylsulfoxide (DMSO). The plates were incubated overnight at 37 °C and antibacterial activity was determined by measuring the diameter of the zone of inhibition surrounding bacterial growth. For each bacterial strain, Ciprofloxacin control was added instead of the sample. The study was carried out in triplicates.

Minimum inhibitory concentrations assay

Minimum inhibitory concentrations (MIC) of the Stigmasterol isolated from leaf extract of *Ficus hispida* was studied as per previous protocol [20]. Different concentration of Stigmasterol (3.12, 6.25, 12.5, 25, 50 and 100 µg/ml) were added and incubated at 37 °C for 24 hours to determine the lowest concentration of Stigmasterol inhibiting visible growth of each bacterial strain on the MHA plate.

Minimum bactericidal concentrations assay

Minimum bactericidal concentration (MBC) was carried out to assess whether all the selected bacteria were killed completely or only their growth was inhibited. MBC evaluated by sub-culturing no visible growth from minimal inhibition

concentration unto neutral sterile plates. MHA broth was prepared, sterilised at 121°C for 15 minutes and transferred into sterile petri dishes to cool and solidify. The bacteria were spread evenly over the surface of a prepared media and different concentration of Stigmasterol added 3.12, 6.25, 12.5, 25, 50 and 100 µg/ml, respectively. All the plates were incubated at 37 °C for 24 hours and observed for colony growth. The MBC was the plate with the lowest concentration of the Stigmasterol sample in serial dilution without colony growth. All experiments were carried out in triplicate.

Statistical analysis

The data were shown as a mean and standard deviation (SD). To ensure that the test results were accurate, each experimental sample was performed in triplicate.

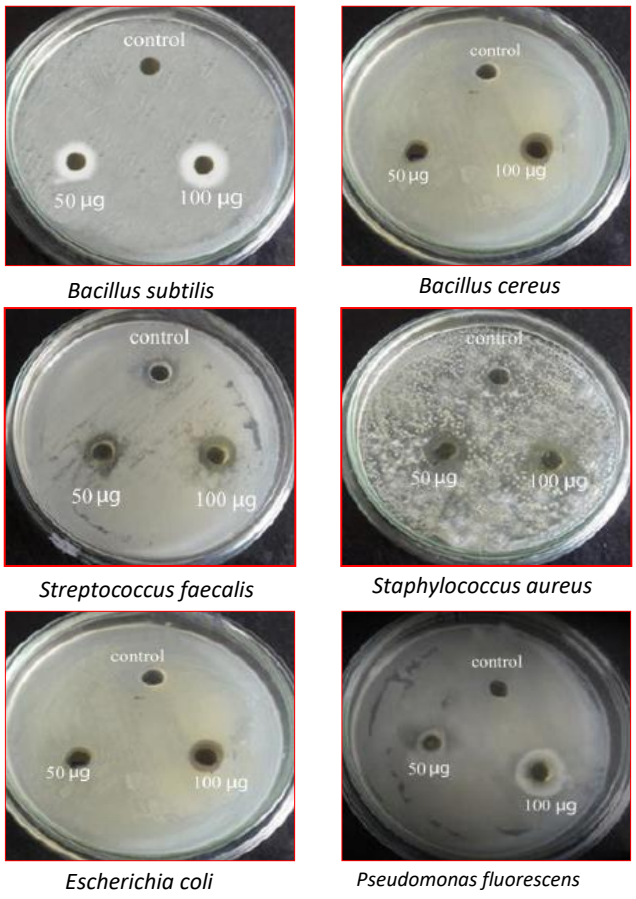

Fig 2 Antibacterial activity of Stigmasterol against test bacterial strains

RESULTS AND DISCUSSION

The antimicrobial activity of Stigmasterol, isolated from the leaf extract of *Ficus hispida* investigated against bacterial strains compared with Ciprofloxacin were shown in (Fig 2). Different concentration of Stigmasterol exhibited antimicrobial activity against all the selected bacterial species includes *Bacillus subtilis*, *Bacillus cereus*, *Staphylococcus aureus*, *Streptococcus faecalis*, *Escherichia coli*, and *Pseudomonas fluorescens* ranging from 20.3 to 38.5 mm. The compound showed higher sensitivity against *Staphylococcus aureus* (38.5 mm) and the least sensitive organism was *Escherichia coli* (20.3 mm). The standard antibacterial drug, Ciprofloxacin (10 µg/ml) had antibacterial effect against all the test organisms with zone of inhibition of 13.3 to 26.2 mm (Table 1).

Stigmasterol exhibited a broad-spectrum of comparable antibacterial activity against all the screened bacterial species using ciprofloxacin standard antibiotics. The zone of inhibition stigmasterol was observed in a dose-dependent manner. 100 µg/ml of Stigmasterol exhibited antibacterial activity over to the standard specially on *Streptococcus faecalis* and *Pseudomonas fluorescens* and moderate effect with other bacterial strain includes *Bacillus subtilis*, *Bacillus cereus*, *Streptococcus faecalis* and *Escherichia coli.*

The MIC (Table 2) and MBC (Table 3) showed that all the sensitive bacterial strain has MIC values at 12.5 µg/ml, while the MBC (50 µg/ml) were observed for the microbes at concentration higher than that of the MIC. MBC has wide range of antibacterial activity against all the bacterial strain. Thus, Stigmasterol can be used as an antibacterial agent in the treatment of bacteria related diseases.

Multiple resistances in human pathogenic microorganism have been increasing incidence in recent years, largely due to indiscriminate use of novel antimicrobial drugs commonly active in the treatment of infectious diseases [21]. Thus, natural compounds may still play a significant role in the development of new antimicrobial drugs [22].

Ficus hispida is one of the traditional and ecologically important plant species was chosen for an evaluation of antimicrobial activity particularly using leaf extracts. The well diffusion was used in this study since it was found to be better than the disc diffusion method. Although several compounds were identified from *Ficus hispida*, literature reported that there are two compounds i.e., Stigmasterol and β-sitosterol possess antimicrobial properties against various strains [23]. Stigmasterol is reported to exhibit a spectrum of pharmacological activities against various disease conditions. These include conditions such as arthritis, cardiovascular ailments, renal disorder, inflammation, diabetes, microbial infections, hepatic toxicity, and cancer [14]. Earlier reports by many investigators have proven that the biological effects of Stigmasterol to have anti-inflammatory, inhibiting cell proliferation [24]. Furthermore, literature has been reported that the stigmasterol possess the potent antioxidant, antimicrobial, hypoglycemic, anti-cancer cells [14].

Table 1 Zone of inhibition of Stigmasterol against bacterial strain

Test organisms	Zone of inhibition (mm)		
	Ciprofloxacin (10 µg/ml)	Stigmasterol (50 µg/ml)	Stigmasterol (100 µg/ml)
Gram-positive bacteria			
Bacillus subtilis	15.2 ± 0.90	27.4 ± 1.3	29.5 ± 1.1
Bacillus cereus	16.4 ± 0.40	28.2 ± 1.1	30.0 ± 2.5
Streptococcus faecalis	26.2 ± 0.50	32.2 ± 0.5	37.8 ± 0.5
Staphylococcus aureus	20.3 ± 0.6	37.5 ± 0.5	38.5 ± 0.9
Gram-negative bacteria			
Escherichia coli	13.3 ± 0.10	20.3 ± 0.6	27.6 ± 0.4
Pseudomonas fluorescens	24.4 ± 0.25	26 ± 0.7	35.0 ± 0.2

Data are presented as mean ± standard deviation (n=3)

Table 2 Minimum inhibitory concentration of Stigmasterol against bacterial strain

Test organisms	Concentration of Stigmasterol (µg/ml)					
	3.12	6.25	12.5	25	50	100
Gram-positive bacteria						
Bacillus subtilis	++	+	*	-	-	-
Bacillus cereus	++	+	*	-	-	-
Streptococcus faecalis	++	+	*	-	-	-
Staphylococcus aureus	+++	++	+	*	-	-
Gram-negative bacteria						
Escherichia coli	++	+	*	-	-	-
Pseudomonas fluorescens	+++	++	+	*	-	-

- No turbidity (no bacterial growth); *Minimum inhibitory concentration; + Light turbidity; ++ Moderate turbidity; +++ High turbidity

Table 3 Minimum bacterial concentration of Stigmasterol against test organism

Test organisms	Concentration of Stigmasterol (µg/ml)					
	3.12	6.25	12.5	25	50	100
Gram-positive bacteria						
Bacillus subtilis	++	+	*	-	-	-
Bacillus cereus	++	++	+	*	-	-
Streptococcus faecalis	+++	++	+	*	-	-
Staphylococcus aureus	+++	++	+	+	*	-
Gram-negative bacteria						
Escherichia coli	+++	++	+	*	-	-
Pseudomonas fluorescens	+++	++	+	+	*	-

- No turbidity (no bacterial growth); *Minimum inhibitory concentration; + Light turbidity; ++ Moderate turbidity; +++ High turbidity

The antimicrobial activity of steroids majorly attributable by disturbing the membrane, it inhibits 'sortase' a participant in pathways involving secretion and anchoring of cell wall proteins [1]. Generally, the zones of inhibition of the microorganisms increased with the increasing concentration of the compound. The zones of inhibition of Stigmasterol concentration against different microorganisms increased with compound concentration. The inhibition zone of the compound against an organism depends on the initial population density of the organism, and the nature and diffusion rate of the antimicrobial agent [25].

The broad-spectrum antimicrobial activity of stigmasterol observed in this study is in good agreement with previous studies. Odibaa *et al*. [26] reported that Stigmasterol (100 µg/ml) showed 29 mm zone of inhibition against *Staphylococcus aureus*, 24 mm against *Escherichia coli*, and 25 mm against *Candida albicans*. Further, Stigmasterol (50 µg/ml) showed zones of inhibition of 21 mm against *Staphylococcus aureus*, 24 mm against *Bacillus subtilis*, 21 mm against *Escherichia coli*, and 21 mm against *Candida albicans* [25]. In the way of previous reports, Stigmasterol identified from *Ficus hispida* is a promising molecule in the development of drugs, the antimicrobial activity of Stigmasterol notified in this study is well comparable with the previous studies. Stigmasterol showed similar effect of Stigmasterol (50 µg/ml) compared to the commercial standard antibacterial drug ciprofloxacin (10 µg/ml) among *Streptococcus faecalis* and *Pseudomonas fluorescens* and moderate with other bacterial species includes *Bacillus subtilis, Bacillus cereus, Streptococcus faecalis* and *Escherichia coli.* This variability in antimicrobial activity observed for stigmasterol could be attributed to the difference in concentration of the compound used in the studies.

The minimum inhibitory concentration, considered as the lowest concentration of the sample that inhibits the visible growth of microorganisms, was determined by the well diffusion method in Mueller Hinton for antibacterial activity. In a previous study, Stigmasterol isolated from the stem bark of *Nepenthes macrophylla* displayed an improved inhibitory effect and lowerminimal inhibitory concentration when compared to the standard drug [25]. The low MIC (6.25-25 µg/ml) and MBC (12.5-50 µg/ml) indicated that the compound has good antimicrobial activity against the susceptible organisms considering the fact that compounds with minimal inhibition concentration less than 100 µg/ml are regarded as having strong antimicrobial property [27]. Stigmasterol also demonstrated a strong antibacterial effect with MIC ranging from 3.12 to 100 µg/ml against all the bacterial strains were tested. Particularly, the compound was the most active and highly inhibitory with a MIC value of 100 µg/ml against all the bacterial strain used. In the study, the isolated compound from this extract displayed an improved inhibitory effect and significantly lower minimal inhibition concentration at 12.5 µg/ml.

Alternative therapy with herbal medicinal products and supplements has continued to increase globally, with a significant proportion of the population in developing countries relying on herbal products for health care needs. A synthetic drug has their own limitations. Although Food and Drug Administration have licensed use of ciprofloxacin, United States restrictions to use due to its side effects. It is recommended only when other antibiotics have failed [28]. Stigmasterol a natural product does not have such toxic effects. It demonstrated broad spectrum antibacterial action, highlighting its potential as a contender in the development of novel antimicrobial drugs.

CONCLUSION

Overall, this study's findings are hopeful in terms of discovering new plant-based medicines. Thus, Stigmasterol from *Ficus hispida* may beneficial in treating bacterial diseases. Thus, the present scientific investigation work will enhance the scientific communities to do more work on this important medicinal plant in near future. However, Besides the findings, it would be more appropriate to enhance further researches are required for clinical applications to explore the exact mechanism of action and its pharmacological evaluation by using suitable animal models for improving the plant-based drug and the development of new medications of natural source.

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Conflicts of interest

No conflicts of interest.

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