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Isolation, Characterisation and Anticancer Activity of Stigmasterol from Leaf Extract of Ficus Hispida

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ABSTRACT

The study aim was to investigate the potential use of*Ficus hispida*leaf extracts as an alternative treatment for breast cancer by identifying phytochemical constituents and evaluating its antioxidant and anticancer activities on MCF-7 breast cancer cells.The leaf extract was subjected to Soxhlet extraction using different organic solvents and phytochemical screening was done using different biochemical analyses. Methanol extract was selectedfor isolation and identification of bioactive compound using extensive spectral analysis such as GC-MS, FT-IR, and NMR. Stigmasterol was identified as the bioactive compound with molecular formula $C_{29}H_{30}O$ and mass of 412. The antioxidant activity of Stigmasterol was found to be effective by inhibiting DPPH, H_2O_2, andNO^-potential with IC_{50} of 66.3µg/ml, 46.7µg/ml and 44.5µg/ml, respectively.Moreover, Stigmasterol demonstrated significant anticancer activity against MCF-7 cells in a dose-dependent manner at 24-hours, with morphological changes confirming the inhibition of cell proliferation. These finding suggest thatStigmasterol from leaf extract of *Ficus hispida* leaf extract has potential as an alternative candidate for breast cancer treatment due to rich antioxidant and anticancer properties. However, further research is necessary to assess its therapeutic purpose.

Keywords: *Ficus hispida*; Stigmasterol; Spectroscopy;Antioxidant;MTT assay; Breast cancer

INTRODUCTION

Cancer is a significant global health burden, withapproximately 10 million deaths worldwide in 2020. Recently, female breast cancer was diagnosed as most common cancer, with 4.4 million women dying of cancer every year, 25% of which were due to breast cancer.[WHO 2022] Despite the increased availability of various cancer treatments, the effective of these therapies remains limited by drug resistance and a range of side effects, such as fatigue, thrombocytopenia, anemia, appetite loss, and cardiovascular issues.[Sathiyamoorthy et al., 2017; Wang et al., 2019]Therefore, there is an urgent need for alternative cancer treatment that both safe and effective, with minimal side effects.

Plants have been a valuable resource for human medicine since ancient times, and their use in traditional medicineis still prevalen today.Plant extracts are commonly used as a form of folk medicine for various health conditions. [Umadevi*et al.*,2013] Plants are an inexhaustible source of active ingredient with

great potential for the management of human ailments. [Shariff 2001; Suresh et al., 2008] The importance of plants as a resource of bioactive compounds with therapeutic properties has received more attention in recent years. According to the World Health Organization (WHO), more than 80% of the people worldwide rely on folk medicine for primary healthcare treatment and medicinal plants are a crucial compound of their practice. [Vines, 2004; Bandaranayake WM 2006] The uses of medicinal plants are more effective and safer due to their minimal side effects, low costs, and easy availability. [Ekor, 2013; Bandaranayake WM 2006] Around 25% prescribed medicines are from plant basis, highlighting the importance of screening phytochemicals and pharmacological properties to identify a more efficient therapeutic agents for treating diseases. [Khaliq*et al*., 2017; Sathiyamoorthy*et al*., 2017]

India has anextendedrecord of plant derivative usage for medicinal purposes, and it is the twelve mega biodiversity centres in the world. Ayurveda, a conventional medical system, originated in India and form an alternative system of medicine. [Shapiro, 2006]Currently, about 28% of current medications are derived from medicinal plants. [Samuelsson 2004; Chin *et al*., 2006]Medicinal plant contain various active compounds that play a vital role in prevention of various disease namely cancer, diabetes, cardiovascular diseases, and inflammatory. [RohitkumarBargah, 2015] Various techniques like morphological, phytochemical, pharmacological and various chemical screening are employed for evaluation of phytochemical constituents due to their defensive mechanism and their unique pharmacophore features. [Newman DJ et al., 2007]India has the most valuable medicinal plants which has various pharmacological features.However, still medicinal values of several plant species need to be investigated. [Umadevi M et al., 2013]

The genus Ficus, belongs to the Moraceae Family,*Ficus hispida* Linn.has the greatest medical value which known as peyatti in Tamil, dumoor in Bengali and gobla in Hindi.has a significant.*Ficus hispida*Linn.is widespread in India, China, Srilanka, Myanmar, and Australia. Traditionally, all the parts of *Ficus hispida*includes fruits, leaves, stem, park, root and latex were used for various aliments of diseases, leaves are prioritised from a medicinal point of view. [Ali and Chaudhary, 2011 &Nadkarni 1976]

Phytochemical screening studies were reported the presence of flavonoids, alkaloids, terpenoids, sterols, phenols which exhibit biological characteristics such as anticancer, antioxidant and antimicrobialproperties. [Salvi*et al*., 2013] Antioxidants are the substances that inhibit the oxidation of other substances, has a crucial role in defense mechanism. [Miguel, 2010]Free-radical scavenging compounds includes polyphenolic, flavonoids, vitamins, steroid, terpenoidscompounds and other metabolites possess significant antioxidant propertiesby various mechanisms. [Zheng et al., 2001] Phytochemicals act as anticancer agents, selectively destroying rapidly dividing cells and removing oxidative stress by quenching free radicals or converting it to less reactive oxygen species. [Mallikaharajuna PB et al., 2007]

The phytosterol compounds exhibit antioxidant properties because of their redox features, which allow them to act as hydrogen donors, reducing agents, and singlet oxygen quenchers.It has a crucial role in the structure and stability of the cell membrane which consist C28 and C29 steroid alcohols. [Grattan BJ Jr, 2013] So far, nearly 300 varieties of phytosterol found in nature, namely beta-sitosterol, stigmasterol and campesterol which are present in most of the plants. [Grattan BJ Jr, 2013] There is increasing scientific evidence supporting the idea that phytosterols and their derivatives have multiple pharmacological properties, including human-wellness-promoting abilities. Scientific evidence is mounting in supporting that phytosterolspossess a range of pharmacological properties, including the capable to enhance human wellness. One of these health benefits is the great potential to reduce total and low-density lipoprotein (LDL) cholesterol levels, which lowers the risk of developing a number of diseases.[Plat et al., 2019]Moreover, phytosterolhave greatest effect of antioxidant, antiulcer, immunomodulatory, antibacterial, and antifungal characteristics. In addition, it has the ability to prevent platelet aggregation inhibition and aid in wound healing. [Salehi 2021] Further, it has been documented that phytosterol has a range of activities against several diseases include cardiovascular ailments, diabetes, hepatic toxicity renal disorder, and cancer. [Grattan BJ Jr, 2013]

Stigmasterol (stigmasta-5,22-dien-3-ol, $C_{29}H_{48}O$), is a major constituent found in various plants including *Ficus hispida,* and has various applications in the pharmaceutical industry. It is used as a precursor in the production of synthetic progesterone which plays an important physiological role in regulatory and rebuilding tissues to estrogen effects, and inter linked in the biosynthesis of androgens, estrogens, and corticoids. It is serves as a precursor of vitamin D3 as well. [Kametani and Furuyama, 1987; Muthukrishnan*et al.,* 2015; Sundararaman and Djerassi, 1977] Stigmasterolhas been found to havesignificant anti-tumor potential by inhibiting cell proliferation and inhibiting cell proliferation in several cancers such as lung,liver, gallbladder, gastric, and ovarian cancer.[Shuo Zhang 2008; Qingyong 2012; Pandey 2019; Kangsamaksin 2017; Zhao H 2021; Li 2018; Bae 2020; Dong *et al.*, 2021]. It activates apoptotic genes/proteins, molecular signaling pathways involved in various cancers, repressed chemoresistance, and improves cell inhibitory activity, which makes it a promising candidate for pharmaceutical therapy against cancerous disease. [Bakrim*et al.,* 2022] Moreover, stigmasterol has a crucial role in pharmaceutical industry, utilised to manufacture synthetic and semi-synthetic compounds. [Navpreet*et al.,* 2011]

The present study aimed to identify the bioactive compound from the leaf extract of *Ficus hispida*through various spectral analysis include GC-MS, FT-IR and NMR. Further,the study was to evaluated the antioxidant and anticancer activity of the identified compound using breast cancer MCF-7 cells, considering the plant medical benefits.

1. MATERIALS AND METHODS

1.1. PLANT MATERIAL COLLECTION

The leaves of *Ficus hispida* Linn. (Moraceae) was collected from ABS research conservation research and training centre, Karripatti, Salem and authenticated by Director of the institution Dr. A. Balasubramanian which bear a code AUT/MCS/2120.

1.2. EXTRACTION AND ISOLATION

The dried and pulverised leaves of *Ficus hispida*(1.5 Kg) were extracted by maceration at 24°C. The extract was subjected to fractionation with various solvents such as Methanol, Chloroform, Ethyl acetate, and n-Hexane, three time each by using Soxhlet apparatus. All fractions were dried using rotary evaporator, stored in a freezer for further phytochemical and spectral analysis.

1.3. PHYTOCHEMICAL ANALYSIS

The highest yield observed with methanol fraction of *Ficus hispida* was selected for qualitative and qualitative phytochemical screening todetermine the metabolites and identification of bioactive compound.

1.3.1. Qualitative analysis

The bioactive compounds of *Ficus hispida* were analysed by qualitative test by standard procedures. The compounds were observed in UV-light as per the standardised procedure.

1.3.1.1. Test for alkaloids

Two ml of *Ficushispida* leaf extract, two ml of conc. HCl was added and then added few drops of Mayer's reagents. The presence of alkaloids confirmed by formation of green color or white precipitate.

1.3.1.2. Test for phenol

With one ml of *Ficushispida*leave extract added two ml of distilled water and following few drops of10% $FeCl_3$will show blue or green color which indicates the presence of phenols.

1.3.1.3. Test for flavonoids

Two ml of the leaf extract of*Ficushispida*, one ml of 2N NaOHadded and mixed will which turn to yellow color conform flavonoids presence.

1.3.1.4. Test for steroid

Salkowski Response:A few drops of concentrated $H_2S0_4$was added with two ml of leave extract of *Ficus hispida*shows a reddish color which indicates the presence of phytosteroid.[Victor *et al.,* 2009]

Liebermann-Burchard test:A few drops of $H_2SO_4$was added to the leave extract of *Ficus hispida*followed by the addition of 2-3 drops of $C_4H_6O_3$which turned green by Liebermann-Burchard reaction method. [Victor *et al.*, 2009]

1.3.1.5. Test for tannins

One ml of *Ficushispida*leaf extract and added with two ml of 5% ferric chloride solution which turn intodark blue/greenish black confirms the presence of tannins.

1.3.1.6. Test for glycosides

Two ml of leaf extract of *Ficushispida*and added with three ml of chloroform following few drops of 10% ammonia appears pink color which indicated the presence of glycosides.

1.3.1.7. Test for saponins

Two ml of leaf extract of *Ficushispida*were mixed with two ml of distilled water and the start shaking shows the foam formation which indicates the presence of saponins.

1.3.2. Quantitative analysis

1.3.2.1. Estimation of total phenols

The total amount of phenolicscompounds in leaf extract of *Ficus hispida*was done by Folin-ciocalteumethod. 200 µl of leaf extract of *Ficus hispida*were taken, added one ml of folin-ciocalteureagent and mixed well with 800µlof 7.5% sodium carbonate. Then,it kept in incubator at 37°C for 30 minutes. Further, the absorbance was read at765 nm using gallic acid as standard.

1.3.2.2. Estimationof total flavonoids

Total flavonoid content from leaf extract of *Ficus hispida*was determined by a modified colorimetricmethod. [Bao*et al.*, 2005]For this one ml of distilled water and mixed with 75µl of 5% $NaNO_2$ incubated for 5 minutes at room temperature. Then,500 µlof 1M NaOH wasadded and kept for 15 minutes. After that the absorbance was recorded at 510nm using quercetinas standard.

1.3.2.3. Estimation of total sterols

In this sample *Ficus hispida*, total steroids were assessed according to themethod of Trease and Evans. [Trease and Evans, 1996]For this, two ml of 4N sulphuricacid, two ml of 0.5% ferric chloride and 500 µl of 0.5% potassium hexacyanoferratewere added with one ml of leave extract of *Ficus hispida*and then incubated in water bath at 70-90°C for 30 minutes. After incubation, it was diluted up to 5 ml with distilled water,following absorbance was recorded at 780 nm using β-Sitosterol as standard.

1.4. EVALUATION OF ANTI-OXIDANT SCREENING

1.4.1. DPPH radical scavenging activity

DPPH (1, 1- Diphenyl-2-Picrylhydrazyl radical) was used to assess free-radical scavenging activity of methanol extract *Ficus hispida*at various concentration (20, 40, 60, 80, 100 µg/ml) followed with some modifications of Hatano*et al.* [Hatano*et al.*, 1989] The leave extracts were prepared at 1 µg/ml with DMSO solution, made uniformity and then 100-500 µg/ml as working solutions. Further, 0.004% (W/V) solution of DPPHwas added in methanol and incubated in the dark at 24°C for one hour. The absorbance was measured at 517 nm against control. All the tests were performed in triplicate.The antioxidant activity was calculated using

$$\text{Inhibition (\%)} = (\text{Absorbance of control} - \text{Absorbance of leaf extract}) / \text{Absorbance of control} \times 100$$

The antioxidant activity of leave extract of *Ficus hispida*(µg/ml) was expressed as IC_{50} (inhibits the formation of scavenging activity by 50%).

1.4.2. Hydrogen Peroxide (H_2O_2) radical scavenging activity

Free-radical scavenge activity of leave extract of *Ficus hispida* was evaluated usingH_2O_2method. [Ruche*t al.,* 1989]Dose increasingconcentrations (20, 40, 60, 80, 100 µg/ml) of samplewere prepared and 40 mMhydrogen peroxide (H_2O_2)solutionadded which kept for 10minutes. After thatabsorbance were taken at 230 nm using control which containing the saline of phosphate buffer without H_2O_2 and usingascorbic acid. It was calculated as below.

Radical scavenging activity (%) = (Absorbance of control - Absorbance of sample)

/ Absorbance of control X 100

1.4.3. Nitric Oxide (NO^-) radical scavenging activity

Free-radical scavenge activity of leave extract of *Ficus hispida* was evaluated by NO^-method. 3 ml of sample (20, 40, 60, 80, 100 µg/ml) mixed with 5mM of $Na_2[Fe(CN)_5(NO)$in phosphate-buffered saline and then incubated at 25°C for 2 hours 30 minutes. The leaf extract of *Ficus hispida* reacted with Greiss reagent which consist 1% sulphanilamide, 2% $H_3PO_4$and 0.1% napthylethylenediaminedihydrochloride and measured at 546 nm using ascorbic acid standard.

NO^- scavenging activity (%) = (Absorbance of control – Absorbance of sample)

/Absorbance of control X 100

1.5. Anti-cancer Activity of *Ficus hispida*Extract

Fort this study, breast cancer cell line MCF-7 was purchased from National Centre for Cell Science, Pune, India and cell culture was maintained using DMEM (Dulbecco's modified Eagle's medium) consisted with 15% fetal bovine serum and streptomycin.

Leaf extract of *Ficushispida*using various solvents was subjected to cytotoxicity analysis using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide) method. MCF-7 cells were seeded in 96-well plates, incubated overnight in 5%CO_2 at room temperature. After incubation, various concentrations of solventsextract i.e., 0, 25, 50, 75, 100, 150 and 200 µg/ml were tested using MCF-7 breast cancer cells. Totally,500 µg/ml was prepared for each concentration with 0.01% DMSO, untreated considered as control. The procedure was repeated in triplicate and viable cells were calculated using hemocytometer.

To detect the cell viability, 20µl of MTT solution in phosphate buffered saline was added to each culture well after completion of 24 hours' incubation. The colour was allowed to develop for an additional 4 hours of incubation. In order to block this reaction from solubilize the crystals, an equal volume of DMSO was added. Absorbance was absorbed using ultraviolet visible spectrophotometer at 570 nm. Further, the effect of extract of *Ficus hispida* was determined using

Cell viability (%) = Test OD/ Control OD X 100

1.6. ISOLATION OF BIOACTIVE COMPOUNDS

The methanol fraction of *Ficus hispida*was selected to column chromatography on a silica gel 60 with mesh size of 70-230 and pore size of 0.035-0.070 mm. The mobile phase of organic solvents was used to pack and elute the column.Then, the collected fractions were concentrated using a rotary evaporator to get semi-purified sub-fractions.Single compound was isolated over silica gel with particle size of 0.04-0.036 mm, 230-400 mesh using mobile phase chloroform-methanol 4.2:4 (v/v) solvent system and then subjected to Gas chromatography-mass spectrometry (GC-MS) to confirm the purity of isolated bioactive compound.

1.6.1. SPECTROSCOPIC ANALYSES

The purity and mass of the isolated *Ficus hispida* compound were validated using a mass spectrometer and comparison to published data.

1.6.1.1. GC-MS analysis

The content of *Ficushispida* extract was analyzed by GC-MS (Agilent Technologies, USA) was used along with electrospray-ionization which connected to autosampler injection system. In this, helium gas used as carrier, assessed with temperature range from 40°C to 340°C with a run of 28 minutes. The phytochemical constituents of leaf extract of *Ficushispida*were identified and evaluated using National Institute of Standards and Technology (NIST) as reference which is the library mass spectra.[Dilshad*et al.*, 2022]

1.6.1.2. FT-IR analysis

The functional group (chemical bonds) identification was performed using Fourier-transform infrared (FT-IR) spectroscopy through infrared ray absorption. The absorption bands were coated in wave numbers (cm^{-1}) at frequency of 400-4000 cm^{-1}.

1.6.1.3. NMR analysis

The isolated compound, 5-10 mg was dissolved in 3ml of DMSO for nuclear magnetic resonance. The peak position measured using δ scale, using an internal standard. Nuclear magnetic resonance (NMR) spectrum data, Proton and Carbon spectra including the two-dimensional spectra i.e., ^{1}H and ^{13}C NMR was carried on Bruker AVANCE 600, Germany at 600MHz at 30°C using chloroform as a solvent with internal standard, Tetramethylsilane (TMS).

1.7. BIOLOGICAL CHARACTERISTIC OF BIOACTIVE COMPOUND

1.7.1. Antioxidant activity of stigmasterol by various methods

Antioxidant activity of isolated compound Stigmasterol was evaluated by various methods such as DPPH, H_2O_2radical scavenging and NO^-scavenging activity as described previously. Various concentrations i.e., 3.12, 6.25, 12.5, 25.0, 50.0, 100 µM/µL of Stigmasterol was assessed against Ascorbic acid standard. The absorbance of sample was recorded using an ultraviolet-visible (UV) spectrophotometer.

1.7.2. Anti-cancer Activity of Stigmasterol

The isolated compound was subjected to cytotoxicity by MTT assay using MCF-7 cells. Various concentrations (3.12, 6.25, 12.5, 25.0, 50.0, 100 µM/µL) of isolated compounds was tested using Tamoxifen (5µM//µL) as positive control. The absorbance of sample was recorded at 570 nm using UV spectrophotometer. [Kakrani*et al.*, 2019]

1.8. Statistical Analysis

The findings were shown as a mean and standard deviation (SD). To ensure that the test results were accurate, each experimental sample was performed in triplicate. Data with $p<0.05$ was considered significance.

2. RESULTS

2.1. Preparation of Leaf Extracts and Yield

The leaves of *Ficus hispida* powder (400 g) was subjected to various solvent extraction includes Methanol, Ethyl acetate, Chloroform and n-Hexane using Soxhlet extractor. The colour of the extracts was ranged from dark green to light yellow. The yield of extract was obtained highest with methanol (5.6%) followed by ethyl acetate (2.7%), n-hexane (2.4%) and chloroform (1.9%) due to the more polar substance presence in leaf of *Ficus hispida*(Table 1).

Table 1: Yield of extracts of *Ficus hispida*and its consistency

Name of the Solvent	Initial Weight of the Powder (gm)	Yield of Extract (%)	Colour of the Extract and Consistency

Methanol	100	5.6	Dark Green - Powder
Ethyl acetate	100	2.7	Light Yellow - Powder
Chloroform	100	1.9	Dark Yellow - Powder
n-Hexane	100	2.4	Light Yellow - Paste

2.2. Phytochemical Screening of *Ficus hispida*

2.2.1. Qualitative analysis of leaf extract using various solvents

In the study, preliminary phytochemical screening of *Ficus hispida* was carried out using various solvents such as Methanol, Chloroform, Ethyl acetate, and n-Hexane. All the phytochemicals presence was highly observed with methanol followed by ethyl acetate, chloroform and n-hexane. The study highly observed with steroidscomponent and also presence of other bioactive constituents includesalkaloids, steroids, flavonoids, tannins, phenols, saponins, terpenoids and glycosides (Table 2).

Table 2: Phytochemical screening of different solvents extract of *Ficus hispida*

Groups	Methanol	Ethyl acetate	Chloroform	n-Hexane
Alkaloids				
Mayers Test	+	+	-	+
Phenolics				
$FeCl_2$Test	++	+	+	+
Flavonoids				
NaOHTest	+	+	+	-
Steroids				
Salkowski'sTest	+++	+++	+	+
Liebermann-Burchard Test	+++	++	++	+
Tannins				
$FeCl_2$Test	+	+	-	-
Saponins				
VigorousShakingTest	+	-	+	-
Glycosides				
Keller-KilianiTest	+	+	+	-

+++: Abundant presence ++: Moderate presence +: Little presence - : Absent

2.2.2. Quantitative analysis of leaf extract using various solvents

Quantitative analysis of phytochemicals such as alkaloids, flavonoids, phenols, steroid, tannins, saponins and Glycosideswas done using different solvent extracts. The presence of phytochemicals found in the range of 0.12 mg/g to 14.52 mg/g. Of all these components, steroidpresence highly found with methanol extract followed by ethyl acetate, chloroform and n-hexane(Table 3).

Table 3: Quantitative estimation of phytochemicals from *Ficus hispida*

Phytochemical constituents	Methanol (mg/g)	Ethyl acetate (mg/g)	Chloroform (mg/g)	n-Hexane (mg/g)

Alkaloids	3.92	1.58	-	-
Phenolics	5.82	5.04	5.62	1.35
Flavonoids	13.23	3.86	2.15	0.27
Steroids	14.52	12.36	1.94	4.32
Tannins	5.06	3.42	0.14	-
Saponins	4.72	3.21	1.28	0.12
Glycosides	3.65	1.54	2.32	-

Date were represented as Mean ± Standard deviation (n=3)

2.3. Biological characteristic of *Ficus hispida* using various solvents

2.3.1. Antioxidant activities

Antioxidant activity of *Ficus hispida* was done using methods such as DPPH, H_2O_2 and NO^- scavenging using methanol, n-hexane, chloroform and ethyl acetate (Table 4 a, b c). All the methods exhibited with significant antioxidant activity of methanol extract against ascorbic acid as standard. The methanol leaf extract exhibited high scavenging activity with a maximum DPPH inhibition of 92.46% at a concentration of 100 µg/ml and 33.45% of inhibition was noticed at a concentration of 20 µg/ml with IC_{50} of 66.3µg/ml.

Table 4a: Comparison of DPPH scavenging activity of *Ficus hispida*

Concentration(µg/ml)	Ascorbic acid (%)	Methanol (%)	IC_{50}	Ethyl acetate (%)	IC_{50}	Chloroform(%)	IC_{50}	n-Hexane (%)	IC_{50}
20	41.10	33.45	66.3	26.50	52.0	23.71	46.5	15.40	39.1
40	47.11	45.95		49.69		33.02		22.91	
60	53.57	62.12		51.77		44.60		45.30	
80	56.58	75.98		69.79		53.55		55.63	
100	70.90	92.46		77.41		68.56		57.83	

Data were represented as Mean ± Standard deviation (n=3)

Table 4b: Assessment of H_2O_2 radical scavenging activity of *Ficus hispida*

Concentration(µg/ml)	Standard/ Ascorbic acid (%)	Methanol (%)	IC_{50}	Ethyl acetate (%)	IC_{50}	Chloroform(%)	IC_{50}	n-Hexane (%)	IC_{50}
20	38.81	33.0	46.7	20.81	40.7	26.0	34.4	10.81	25.7
40	46.60	40.55		36.60		22.60		16.60	
60	52.43	59.20		42.43		34.43		22.43	
80	66.82	65.90		56.82		46.82		26.82	
100	72.95	70.65		62.95		58.70		32.95	

Data were represented as Mean ± Standard deviation (n=3)

Table 4c: Assessment of nitric oxide scavenging activity for the leaf extracts of*Ficus hispida*

Concentration(µg/ml)	Standard / Ascorbic acid (%)	Methanol (%)	IC_{50}	Ethyl acetate (%)	IC_{50}	Chloroform(%)	IC_{50}	n-Hexane (%)	IC_{50}
20	17.3	12.06	44.5	6.15	22.9	11.51	37.0	8.29	24.3
40	21.74	29.23		7.70		24.65		16.51	
60	42.5	50.76		20.7		40.23		25.43	
80	46.72	58.46		34.61		46.15		35.7	
100	52.63	60.0		43.01		48.26		46.81	

Data were represented as Mean ± Standard deviation (n=3)

2.3.2. Anti-cancer activity

Figure 1shows the anticancer activity as percentage inhibition among four different solvents used. Of all, methanol extract of *Ficus hispida*inhibited the proliferation of MCF-7 cancer cells with than other solvents. Increase in the concentration of methanol extract (25 – 200µg/ml) increased the anti-proliferation of cancer cell lines.

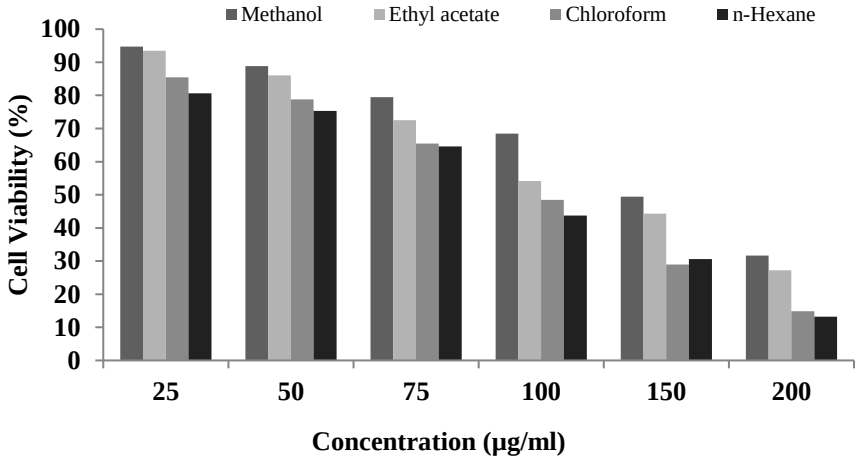

Fig. 1 Cytotoxicity effect of different solvent extract of *Ficus hispida* on Breast cancer cells (MCF-7 cell line). The data were expressed as mean ± SD (n=3).

2.4. Isolation and identification of bioactive compound from *Ficus hispida*

The methanol fraction of *Ficus hispida* was purified by column chromatography followed by GC-MS analysis and verified using TLC in chloroform-methanol 4.2:4 (v/v) solvent system.

2.4.1. GC-MS analysis

GC-MS analysis was conducted to determine the major and minor bioactive compounds in methanol extract of *Ficus hispida* extract showed various peaks in chromatogram (Fig. 2). The total 22 tentative compounds were identified and the major compounds were recognized by their peak percentage area which was determined by the total area of peaks.

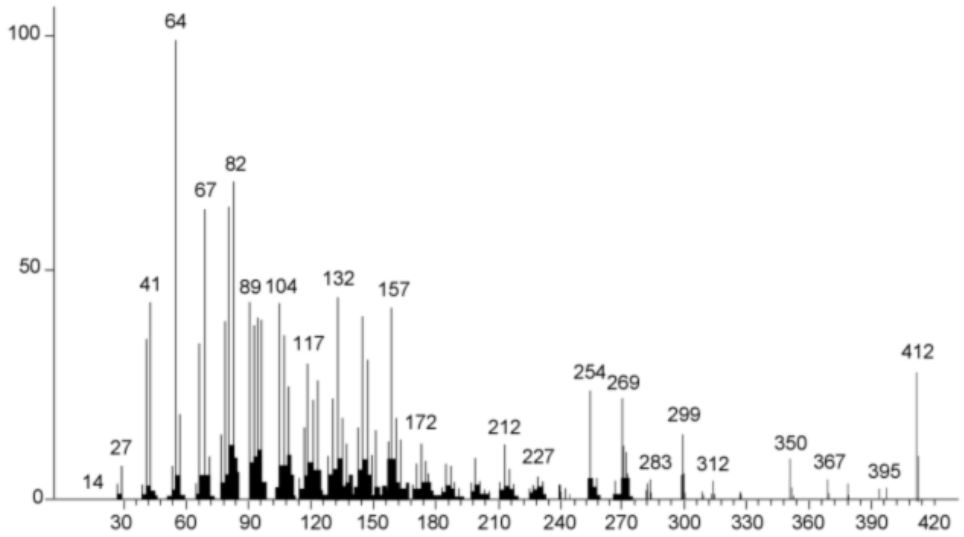

Fig. 2 GC-MS spectrum of isolated compound from *Ficus hispida.*

Further, the isolated compound was found as white crystalline powder with a melting point of 170-174°C, and its mass spectral data confirmed that molecular ions [M+] peak at m/z 412 correspond to molecular formula $C_{29}H_{30}O$ from its GC-MS spectral data. Other ion peaks appeared in mass spectra at m/z 395, 367, 350, 312, 299, 283, 269, 254, 227, 212, 172, 157, 132, 117, 104, 89, 82, 67, 64, 41, 27, 14. Further, spectroscopic data matched with the previous literature. [Habib et al., 2007] Thus, the isolated compound proposed as Stigmasterol (Fig. 3).

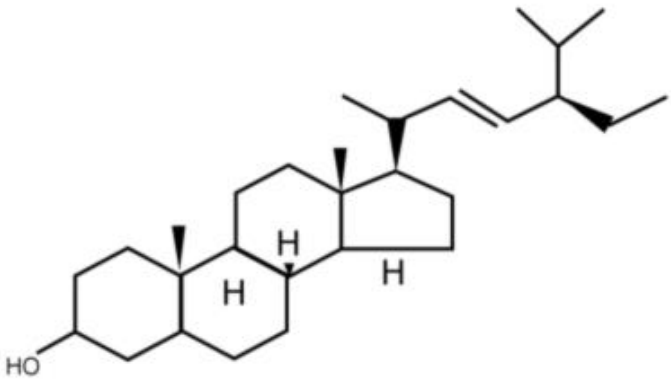

Fig. 3 Structure of Stigmasterol

2.4.2. FT-IR analysis

FT-IR spectrum of isolated compound showed a broad absorption at higher wavelength 3380.17 cm^{-1} which is attributable to the -OH bond vibration of a hydroxyl group. The moderate intense peak at 2894.03 cm^{-1} and 2810.40 cm^{-1}showed the stretching and bending vibration of the methyl group in the structure. The vibration of C-H stretching of a methyl moiety was found around 2122.3 cm^{-1} as absorption

of medium intensity. The corresponding out of pane C-C vibration was shown around 1247.13 cm^{-1} as weakly intense band. The corresponding C=C vibration was shown as weak intense band at 749.62 cm^{-1} which confirm the structure of Stigmasterol (Fig. 4).

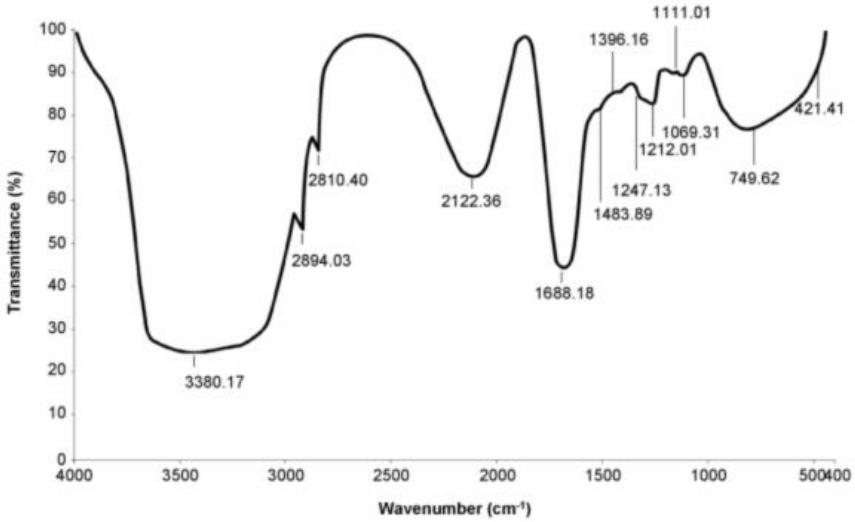

Fig. 4 Fourier-transform infrared spectrum (FT-IR) analysis.

2.4.3. NMR analysis

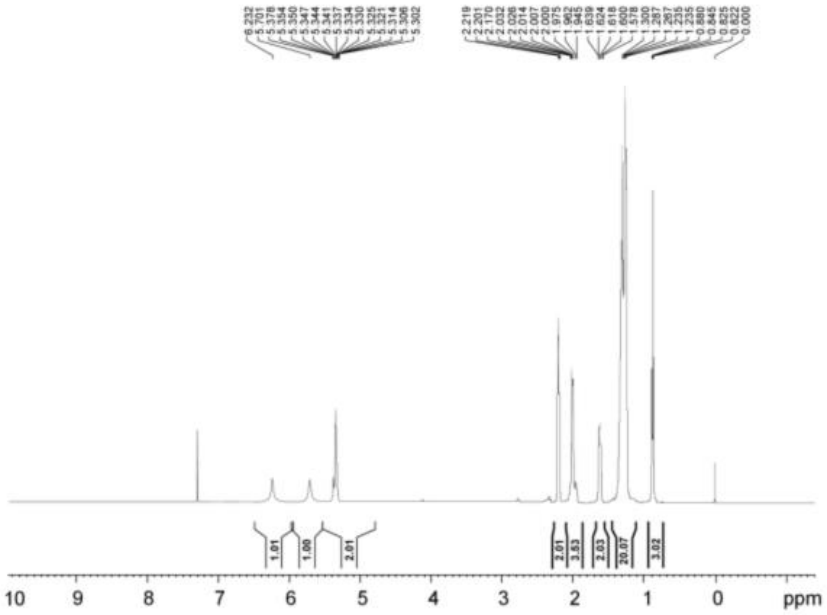

Fig. 5 ^{1}H-NMR spectrum.

In the ^{1}H-NMR spectrum of isolated compound, H-3 proton appeared as a multiplet of a doublet doublet (tdd) at δ3.54 (J=4.5 and 1.1 MHz) and H-6 olefinic proton showed a multiplet at δ5.36. Two olefenic protons appeared downfield δ5.22 and δ4.71 which were identical with the chemical shift of H-

22 and H-23. Six methyl protons at δ 1.23(s, 3H), δ 1.19(s, 3H), δ 1.06(s, 3H), δ 1.00, δ 0.98 (s, 3H) and δ 0.91 (s, 3H); [(3H each, s, CH3)] confirms the structure of stigmasterol (Fig. 5).

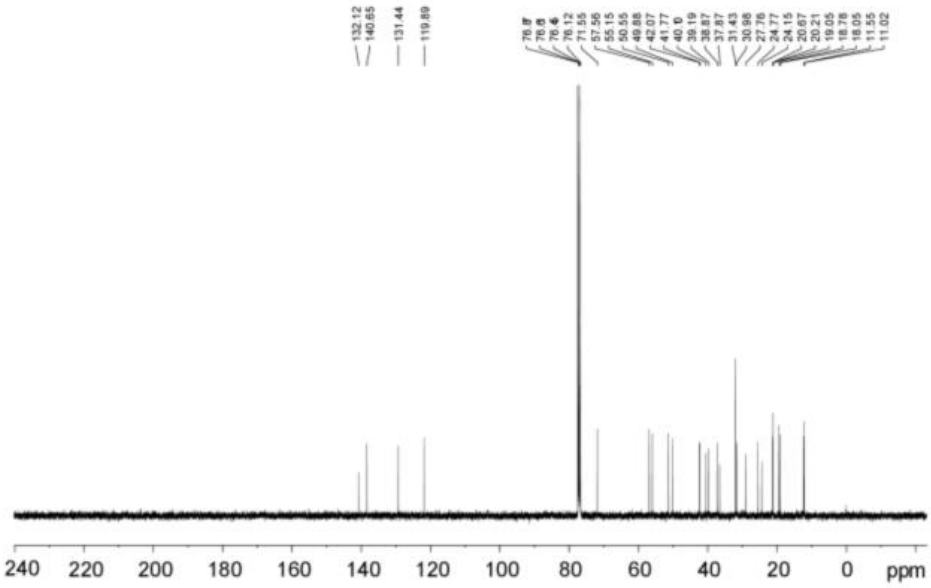

Fig. 6 ^{1}C-NMR spectrum

The ^{13}C-NMR spectra of the isolated compound revealed the presence of 29 carbons, the chemical shift at δ76.7 (C-1) were assigned for -OH bearing carbon of the molecular structure (Fig. 6). The chemical shift recognizable signals at 140.9 and 121.7 ppm which is assignable to the double bond at C-5 and C-6 which similar with the data in the literature of stigmasterol (Table 5). [Li *et al*., 2006 &Habib*et al*., 2007]

Table 5: ^{1}H-NMR and ^{13}C-NMR data of isolated compound from methanol fraction of *Ficus hispida*

Position	Observed isolated crystal		Literature values [Li *et al*., 2006' Habib*et al*., 2007]	
	δC	δH	δC	δH
1	37.5		37.3	
2	32.2		31.5	
3	71.9	3.54 (1H, tdd)	71.8	3.53 (tdd, J=4.5 MHz)
4	42.9		42.3	
5	140.9		140.8	
6	121.7	5.36 (1H, m)	121.7	5.14 (1H, m)
7	29.1		31.8	
8	31.9		31.9	
9	50.2		51.2	
10	36.7		36.5	
11	21.5		21.1	
12	39.9		39.7	
13	42.5		42.3	
14	56.8		56.9	
15	24.4		24.4	
16	29.3		28.4	
17	56.2		56.1	
18	12.6		11	
19	21.3		21.2	
20	40.7		40.5	
21	25.5		21.2	
22	138.5	5.22 (1H, m)	138.3	4.62 (1H, m)
23	129.5	4.71 (1H, m)	129.3	4.61 (1H, m)
24	46.1		51.2	
25	29.3		31.9	
26	20.8		21.2	
27	19.8		19	
28	26.1		25.4	
29	12.5		12.1	

δ_C(ppm) - Chemical shift values in ^{13}C-NMR spectrum; δ_H(ppm) - Chemical shift values in ^{1}H-NMR spectrum.

Further, the molecular formula of isolated molecule stigmasterol is $C_{29}H_{48}O$ that is confirmed by FT-IR and NMR data.

2.5. *In vitro* biological characteristic of purified bioactive compound from *Ficus hispida*

2.5.1. Antioxidant activity of Stigmasterol

Antioxidant potential of Stigmasterol isolated from *Ficus hispida* was carried out by various method includes DPPH, H_2O_2 and NO^- radical free-scavenging activity.The different concentration (3.12, 6.25, 12.5, 25, 50, 100 µM) of bioactive compound stigmasterol was assessed.The stigmasterol showed maximum radical scavenging activity in DPPH assay in dose-dependent manner (Fig. 7a, 7b &7c).

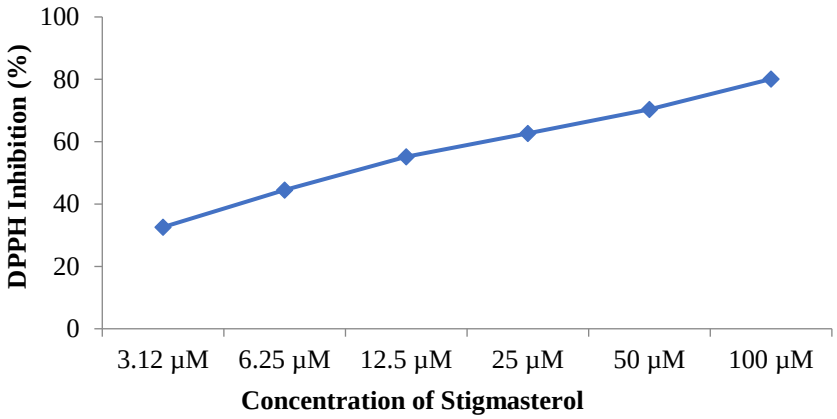

Fig. 7aDPPH radical scavenging activity of Stigmasterol.Data represent mean± SD (n=3)

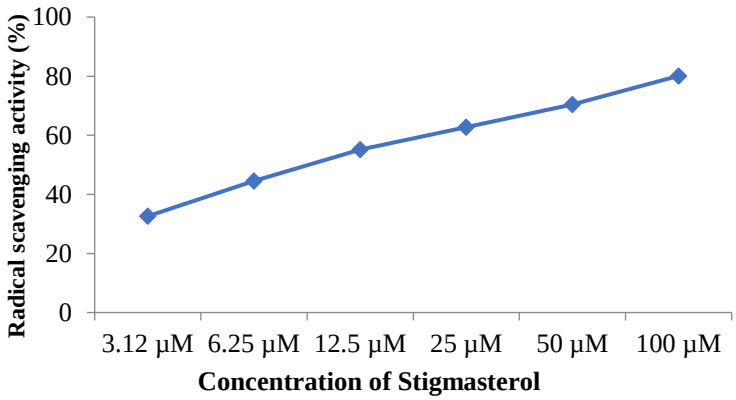

Fig. 7bH_2O_2 radical scavenging activity of Stigmasterol.Data represent mean± SD (n=3)

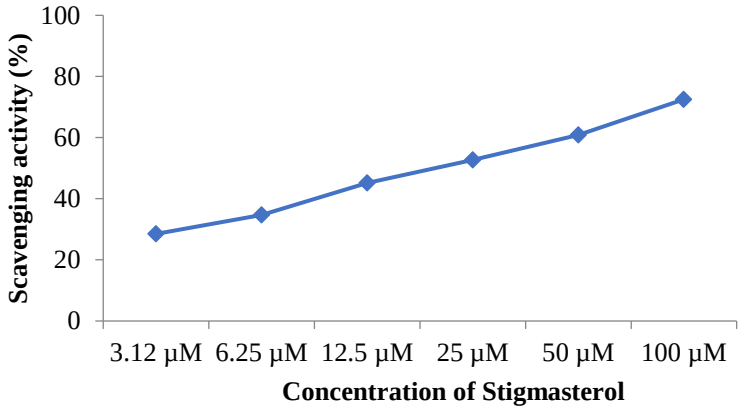

Fig. 7cNO^- radical scavenging activity of Stigmasterol.Data represent mean± SD (n=3)

2.5.2. Anti-cancer activity of Stigmasterol

The isolated bioactive compound stigmasterol showed a cell proliferation inhibition effect against MCF-7 breast cancer cell line in dose-dependent manner at 24 hours (Fig. 8). Further, the morphological cytotoxic activity wasexpressed as percentage of cell viability (Fig. 9). Furthermore, decreasing cell proliferationwas revealed that treatment of stigmasterol compared to control (Tamoxifen) which has the ability to induce apoptosis in breast cancer.

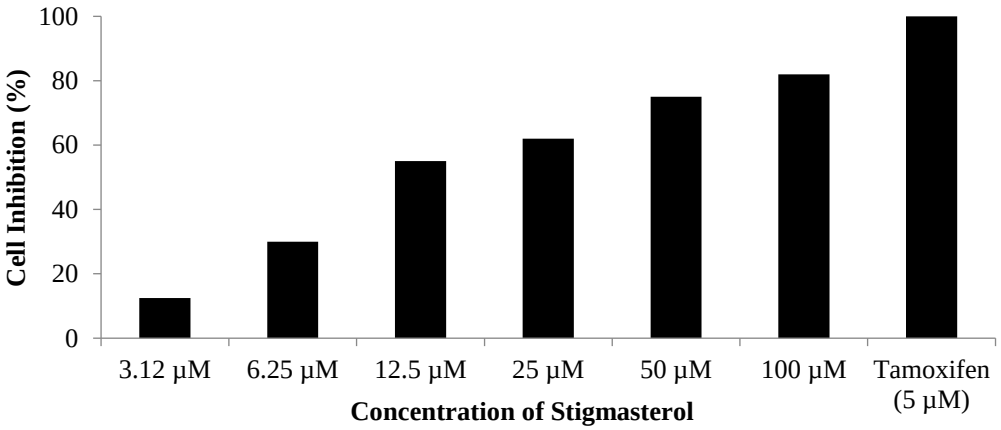

Fig. 8 Anti-proliferative effect of Stigmasterol on the viability of MCF-7 breast cancer cells for 24 hours. Data represent mean± SD (n=3)

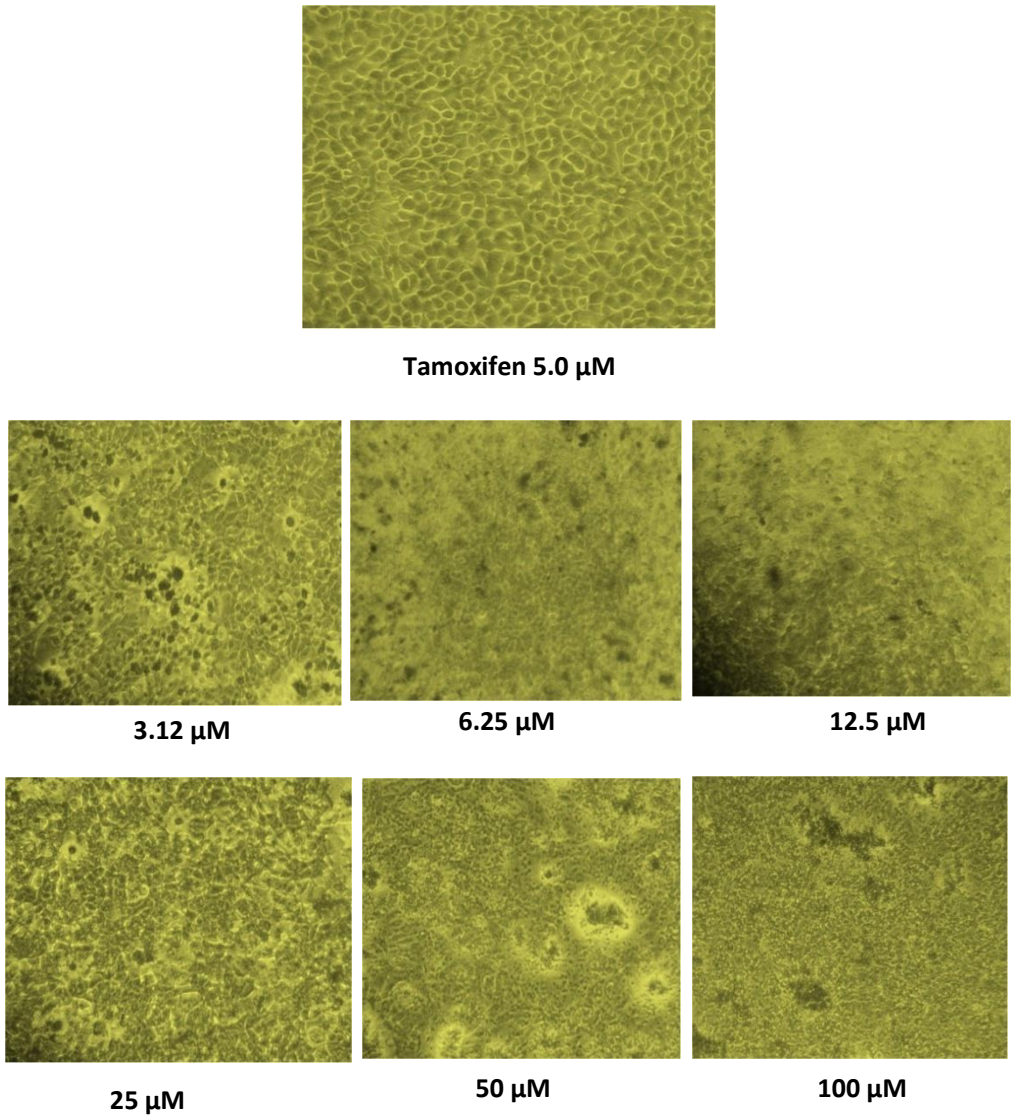

Fig. 9The morphology of MCF-7 cells exposed to Stigmasterol isolated from *Ficus hispida*at each concentration at 24 hours

3. DISCUSSION

Plants are main source of natural products. Search of new bioactive compounds from medicinal plants is a never-ending phenomenon, to meet the perpetual demand for novel biomolecules with medicinal properties to fight against various human ailments. Secondary metabolites have its own medicinal value which produce physiological effect on mammalian system. [Edeoga*et al.*, 2005]To driven the bioactive potential of plants and to promote their use as herbal medicines, it is necessary to intensify the search on medicinal plants that find place in folklore. [Ali *et al.*, 2001] Thus, assessing anticancer activities of plants in a scientific way which will definitely be helpful in the treatment of cancer.

Globally, there has been rampant growth in Ayurveda due to the natural origin and minimal adverse effects in last few years. [Ekor, 2014] Based on folk use and some earlier reports focused highly in

leaf of plant unlike other parts of plant. Secondary metabolites derived from plant are effective alternative to synthetic drugs against various diseases. Screening of phytochemical compounds from the extract of plant and their pharmacological properties is an essential to identify a more efficient therapeutic agent to treat the diseases. [Sathiyamoorthy*et al.*,2017]

Secondary metabolites were extracted from the plant using specific solvents by various pharmaceutical extraction methods. Soxhlet extraction method is one of the user-friendlymethods to extract the metabolites which used for bulk extraction. [AmitaPandey and ShaliniTripathi, 2014] During this, the solvents will get diffused and solubilisebioactive compounds with similar polarity. [Iloki-Assanga SB *et al.*, 2015] The fact that methanol may rapidly enter cellular membranes and solubilize the intracellular components from plant cells may account for the large yield of bioactive metabolites from methanol extract. [Nguyen *et al.*, 2017; Vidhya and Umavandhana, 2016; Moyo*et al.*, 2013] Similarly, our resultsrevealed the presence of bioactive compounds high in methanol than others solvents.

The preliminary phytochemical screening depicts the confirmation of various components includephenols,steriods, saponins, alkaloid, glycosides and flavonoid etc. in various solvent. Methanol extract of *Ficushispida* is found to have a significant amount of phytosteriod compounds. [Muthukrishnan*et al.*, 2015]Phytosteroids are antioxidant with free-radical scavenging characteristics which perform as reducing agents like hydrogen donators, and singlet oxygen quenchers. Thus phytosteriods components may help contribute to the rich antioxidant and anticancer activities. [Tsao*et al.*, 2005] In line of previous studies, the present study revealed that methanol extract of *Ficushispida* consist high steroid content than other compounds which possess rich antioxidant.

Free radicals generated in the body, which responsible for various disease. Antioxidants is an essential to produce immunity, its play a key role to neutralize reactive oxygen species and prevent conversion to free radicals. The standard antioxidant compound, ascorbic acid and methanol solvent extracts was exhibited antioxidant potential by various methods.[Khanam*et al.*, 2015] Chatterjee*et al.*,reported that DPPH radical scavenging capacity of *Ficushispida* using various solvents. Methanol showed significant activity at the IC_{50} values of 160.80 µg/ml than others. [Chatterjee*et al.*, 2015] Moreover, the methanol extract was found to possess rich antioxidant activity. It may have very strong radical scavenging action and be dose-dependent due to free radicals' capacity to scavenge radicals. Previous reports also supported the results of the current study, methanol leaf extract of *Ficushispida* exhibited the anticancer activity against MCF-7 cell lines with the lowest IC_{50} concentration at 32.41 µg/ml. [Sriwatcharakul et al., 2016] In the present study, the methanol extract of *Ficushispida*shows highlevel of antioxidant activity by various methods. The leave extract of *Ficus hispida* exhibit scavenging activities against DPPH, $H_2O_2$and NO^-, has several physiological effects which also used in purpose. The IC_{50}value of DPPH, H_2O_2, and NO^- radical scavenging activity of methanol extract of *Ficus hispida*is 66.3µg/ml, 46.7µg/ml and 44.5µg/ ml, respectively. These observations confirm that methanol extract of *Ficus hispida*have potent antioxidant ability.

The cytotoxicity of methanol extract of *Ficushispida* was determined at 25-200 µg/ml.The results of this investigation support previous findings that methanol extract has stronger anticancer properties than solvent extracts made of ethyl acetate, chloroform, and n-hexane.A similar dose-dependent response of methanol fraction was reported against various breast cancer cells such as MDA-MB435, MCF7, SKBR3 and T47D. [Pratumvinit*et al.*, 2009] VijayarathnaandSasidharan also reported that methanol exhibited the best cytotoxicity than other solvents from *Ficushispida* extract against MCF-7 breast cancer cell line in dose-dependent manner. [Vijayarathna and Sasidharan, 2012]

One of the effect methods for determining the contents is GC-MS, which uses peak area, retention time, and molecular formulas. Volatile substances, long chain hydrocarbons, branched chain hydrocarbons, alcohols, acids, and esters were all frequently recognised using it. Ficushispida methanol

extract was treated to column and thin-layer chromatography for the isolation of bioactive chemicals, which resulted in the extraction of bioactive substance. Further, the isolated compound was characterized by various spectral analysis include GC-MS analysis and followed by FT-IR and NMR. As earlier studies, the current study also emphasized the importance of using a simple column technique approach for the purification of isolated compounds from leaf extract of *Ficushispida*.[Azam*et al.*, 2013 &Choudhury*et al.*, 2021]

FT-IR has shown to be an important approach for the characterisation and identification of functional groups present in the crude extract of plants. [Nisa*et al.*, 2022] Fig. 4 illustrated the isolated compound, stretchy peaks at 3380.17 cm^{-1} indicate the presence of alkane group and another peak at 2894.03 cm^{-1} corresponding to the presence of ester group. Further, a peak was also observed at 2810.40cm^{-1} which represent the presence of ester. Previous studies were also observed with similar results who isolated Stigmasterol from crude extract of plant. [Nisa*et al.*, 2022] Further, NMR results indicated the presence of 36 protons in isolated compound. The triplet at *d* 0.85 is specific for the terminal-carboxyl group which indicates structure of Stigmasterol.

Stigmasterol is a phytosterol identified from various herbal plants which received much attention due to its numerous health effects including anti-cancer, anti-diabetes, anti-inflammation, and lowering blood cholesterol. Previous studies reported a reduction of viability of breast cancer cells (MCF-7) and hepatocarcinomacells with increase of treatment dose and duration of stigmasterol ($p<0.005$). [Kim *et al.*, 2014]In the present study, MTT assay was used to evaluate the cell proliferation and viability of cells using different dose of Stigmasterol. The study showed that it has the ability to reduce and inhibit the cell proliferation of MCF-7 breast cancer cells in dose-dependent response at 24 hours of treatment. Further, morphological changes i.e., cell shrinkage and membrane blebbing which is the characteristics of apoptosis. Further, the results were consistent with previous studies, in which Stigmasterol have an inductive anti-cancer effect by promoting cell death pathway include apoptosis and autophagy, inhibiting cell proliferation,metastasis and invasion against cancer cells such as breast, prostate, lung and colon cancer cells. [Awad and Fink, 2000; Woyengo*et al.*, 2009; Grattan *et al.*, 2013; Rajeswari*et al.*, 2018] Thus, on the basis of these reports, Stigmasterol shows promise as a potential breast cancer treatment. Therefore, further studies suggested to evaluate the mechanism of action of Stigmasterol which might be a new therapeutic node for the cancer treatment specifically on breast cancer.

4. CONCLUSION

The present study has been concluded the presence of phytochemical constituents of *Ficus hispida*highly with methanol extract compared than ethyl acetate, chloroform and n-Hexane. Further, antioxidant and cytotoxicity using breast cancer MCF-7 cells of *Ficus hispida* also found high biological activity with methanol extract which subjected for spectral analysis. Phytochemical screening found higher phytosterol content and also spectroscopic analyses such as GC-MS, FTIR and NMR identified specific bioactive compound of Stigmasterol by comparing their properties reported in previous studies. Stigmasterol possess rich antioxidant and anticancer potential specifically on breast cancer MCF-7 in dose-dependent manner, morphological changes confirm the inhibition of cell proliferation. Thus, suggested further studies to evaluate efficacy of Stigmasterol at preclinical and clinical level for breast cancer.

CONFLICTS OF INTEREST

No conflicts of interest in this paper.

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