Antimicrobial activity confirmed in phytoconstituent, Gossypin extracted from *Hibiscus vitifolius Linn.*

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Abstract

In this research, an attempt has been made to find the potential of selected medicinal plant towards their utility as a pharmaceutical aid. *Hibiscus vitifolius Linn.* was identified, authenticated and utilized for further research work.Extraction of plant material was completed, Pharmacognostic as well as phytochemical parameters were studied, Qualitative phytochemical analysis of the extract was performed. During the research various Physico- chemical Characteristics for the isolated extract were determined. Quality control safety parameters of the isolated extract were studied as per WHO and ICH Guidelines. Herbal plant reports therapeutic as well synergistic effect that has been recognized in medicine. Analytical studies of the Extract were also completed and results obtained. A large number of synthetic antimicrobial agents are available for antibacterial use; however, many limitations are associated with it. Even though preferred mode of application is topical, but long term administration may elicit systemic side effects. In addition, development of resistance is another major hurdle which keeps on demanding new drugs periodically. Unfortunately rate of mutations among these pathogens is more than pace of development of drug. This in turn makes the research and development a priority but costly affair. These concerns suggest the development of potent, economical, patient friendly and time tested treatment with reduced rate of development of resistance which tempted to undertake this project.

The objective of this study was to develop extract from the *Hibiscus vitifolius Linn.* for the delivery of the active constituents present in plant. The plant extract of *Hibiscus vitifolius Linn.* was utilized for antibacterial activity.

From the significant results obtained in this study, it is concluded that extract of *Hibiscus vitifolius Linn.* possesses excellent antibacterial properties. This proves that the selected plant and their parts are excellent sources of pharmaceutical aids. The antimicrobial activity of prepared Extract showed significant effect against various pathogens.

1. Introduction:

Ayurveda is an ancient healthcare system that evolved in India about 5000 years ago. As per the ancient literature on Ayurveda, it was practised in India during the Vedic era. In the first millennium BC, the Charaka Samhita, and the Sushruta Samhita each described about 700 plants. This sort of alternative medicines is widely used throughout the world. The Indian Ayurvedic System focuses on maintaining, promoting, and sustaining good health by preventing diseases through adopting healthy lifestyle habits. Hence, the "Science of life" is an Ayurveda in its literal sense [1]. In the 21st century, natural products represent more than 50% of all drugs in clinical use. Up to 50% of approved herbal medicines in the last 3 decades are derived directly or indirectly from natural products including plants, fungi, microorganisms and animals.

However, India has made progress in popularising the Traditional Medical System of AYUSH (Ayurveda, Yoga, Unani, Siddha and Homeopathy) in the healthcare sector through global networks [2] (Fig. 1). [3].

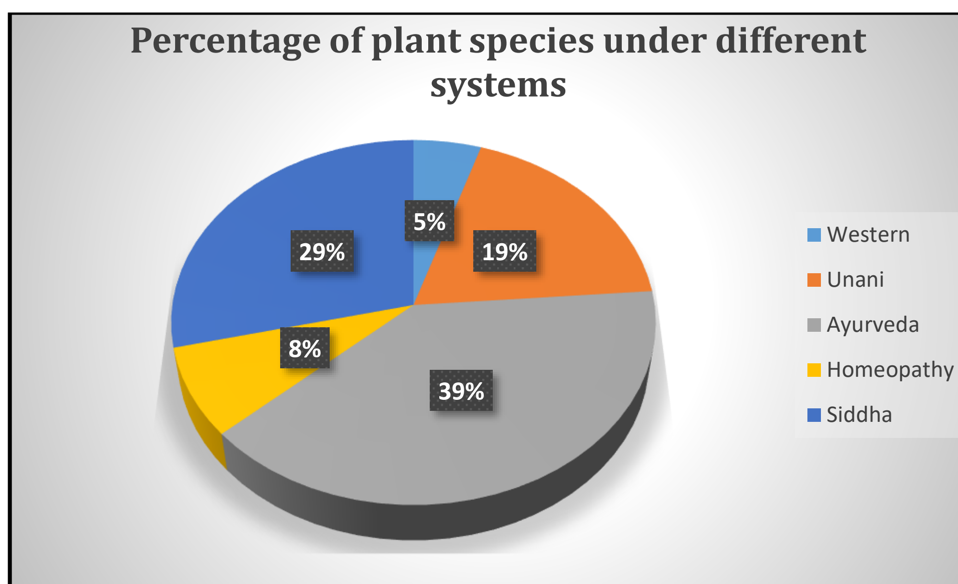


Fig. 1: Percentages of plant species under different systems of medicines (Own creation)

Since tribal medicine is regarded as the origin of indigenous medical systems, it is crucial to study the medicinal flora for scientific and commercial purposes [4]. Chittoor district is rich in forest area and the hilly areas of that forest are home to various tribal peoples. The Seshachalam hills of Tirupati are the major mountain ranges of the district which are ecologically sensitive

and rich in medicinal plants forests [5]. In the forests of Andhra Pradesh Chittoor district, ethnobotanical studies on the usage of medicinal plants were extensively conducted. Many undiscovered uses of plants are discovered which include 62 species and 32 Families, to treat various conditions include mouth ulcers, wounds, toothaches, asthma, eye diseases, stomach aches, rheumatism, antidotes, burning micturition, fevers, intestinal worms, foot cracks, general debility, gout, paralysis, cough, diarrhoea, headaches, colds, asthma, eye diseases, indigestion, piles, cuts and wounds, abscesses [6], [7]. Many medicinal plant species are available to treat skin diseases and dyspepsia.

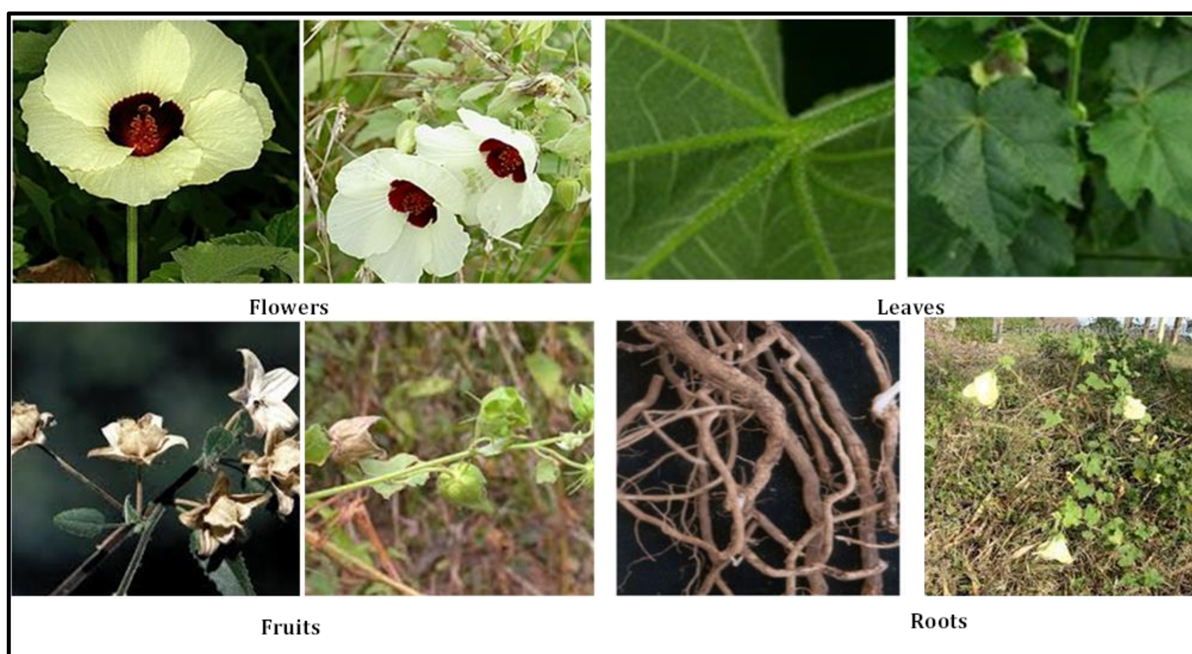


Fig. 2: Images of Flowers, leaves, fruits and roots [8]–[10]

Flavonoids and alkaloids present in *Hibiscus. Vitifolius Linn.*

As mentioned above, the plant *H. vitifolius* composed of different phytoconstituents, including gossypin, flavonoid, alkaloid, vitiquinolone, kaempferol, stigmasterol, n-octacosanol, xanthyletin, tannin, carbohydrates, steroids, phloba-tannin, saponin, cardiac glycoside, anthocyanin, anthraquinone, protein, and glycoside phenols. From all phytoconstituents, flavonoids, alkaloids, and phenolic acids are thought to be the major classes of compounds in the genus Hibiscus. The flavonol biocide 3,4,6,8-tetrahydroxy flavonol-5-methyl ether 7-O-neohesperidoside isolated from the flowers of *H. vitifolius* showed significant hypoglycemic activity compared to glibenclamide in Raghunathan's research. A bioflavonoid isolated from *H. vitifolius* flowers, has shown a protective effect and increase in capillary permeability of rat intestine on X-ray exposure as studied by Parmar and Ghosh. Phytochemical analysis of the

roots of *H. vitifolius* have revealed the presence of bioflavonoid Gossypin. It is also reported to have oral anti-oxidant, anti-hypercholesterolemic, and anti-nociceptive properties. Gossypin suppresses beta-amyloid induction, and protects against carbon tetrachloride-induced toxicity. It also inhibits cell proliferation in K562, L929 and HT29 tumour cell lines [11]–[13]. In other investigations conducted by Ramasamy et al., the phytochemical analysis of the powdered root of *H. vitifolius* Linn. (Malvaceae) were extracted sequentially with n-hexane and chloroform. Analysis of the n-hexane extract by GC-MS identified nearly twenty-six components by comparing their mass spectra with GC-MS library data. which yielded eight known compounds viz. β-amyrin acetate, n-octacosanol, β-amyrin, stigmasterol, xanthyletin, alloxanthoxyletin, xanthoxyletin and betulinic acid were isolated from chloroform extract by column chromatography over silica gel. Hence, the study reported that, quinolone alkaloid is also present in *H. vitifolius* root [14].

Structure of Gossypin

Gossypin [**2-(3,4-Dihydroxyphenyl)-3,5,7-trihydroxy-4-oxo-4H-chromen-8-yl b-D-gluco-pyranoside]** is a flavanol glucoside (Fig. 3) with molecular formula $C_{21}H_{20}O_{13}$. It is primarily found in the roots, flowers of several species of Hibiscus like *Hibiscus vitifolius, Gossypium indicum*, and *Hibiscus esculentus*. It is functionally related to a gossypetin. The presence of different groups especially glucose moiety in the basic chemical structure affects solubility i.e., presence of glucose moiety makes it soluble in water, whereas sparingly soluble in alcohol and other solvents. Moreover, it also lessens the toxicity of dermally induced lipid peroxidation and delays the onset of cataract formation in rats [15], [16].

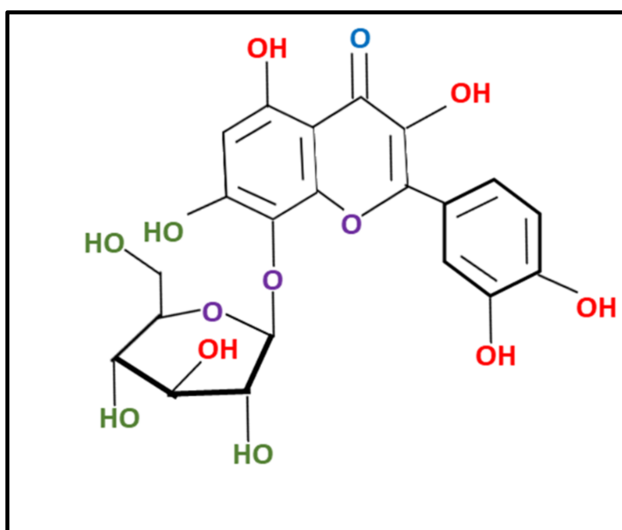


Fig. 3: Basic structure of gossypin (Own creation)

2. Materials and methods

2.1 Materials

2.1.1 Collection and Identification of medicinal plants

The plant is a shrub, a wild species found at higher altitudes of the hills, which was collected from the southern region of India in July 2021. For the identification and authentication of the plant material, the prepared herbarium and plant specimen was identified and confirmed by authenticated plant taxonomist (IAAT: 337), Dr K. Madhava Cheety, M.Sc. M.Ed., M.Phill. Ph.D., PG DPD, Assistant Professor, Department of Botany, Shri Venkateshwara University, Tirupati, Andhra Pradesh, India. The herbarium of the plant was deposited under voucher specimen no. 0606. Botanist Mr Dinesh Shirodkar, Botanical Survey of India, Pune, Maharashtra, India in December 2021, also authenticated it.

2.1.2 Drugs and Chemicals

All materials used in this formulation were of an analytical and cosmetic grade (Table 2.1).

Table 2.1: List of materials used along with the suppliers

S. No.	Drugs and chemicals	Supplier
1.	Gossypin standard	Sigma Aldrich (Bengaluru, India)
2.	Methanol, Ethyl acetate, Trifluoroacetic acid, acetonitrile	Merck Ltd. (Mumbai, India)
3.	HPLC grade water	Thermo Fisher Scientific India Pvt. Ltd, Pune, India

2.1.3 Used Microorganisms

The microorganism used in this research including *Staphylococcus epidermidis,* and *Staphylococcus aureus.* were procured from National Collection of Industrial Microorganisms (NCIM) CSIR- National Chemical Laboratory, Pune, 411008 Maharashtra, INDIA.

2.2.1 Preparation of crude material for further analysis

The root of the selected plant was isolated from the *Hibiscus vitifolius Linn.* plant. The separated material was processed for size reduction using a cutter mill (Portable mixer) and dried in the shade. The crushed material was run through a 40 # sieve (coarse powder) for uniform particle size, resulting in an effective extraction and extract yield. The technique was chosen due to its efficiency and simplicity in separating phytochemical constituents based on solvent polarity. [17].

2.2.2 Extraction of a crude sample with different solvents

Extraction is suitably dissolving the desired constituent in a suitable solvent. Extraction is the process of extracting soluble material from an insoluble residue, which might be liquid or solid, by employing a solvent. Figure 2.2 indicates the mechanism of extraction [18].

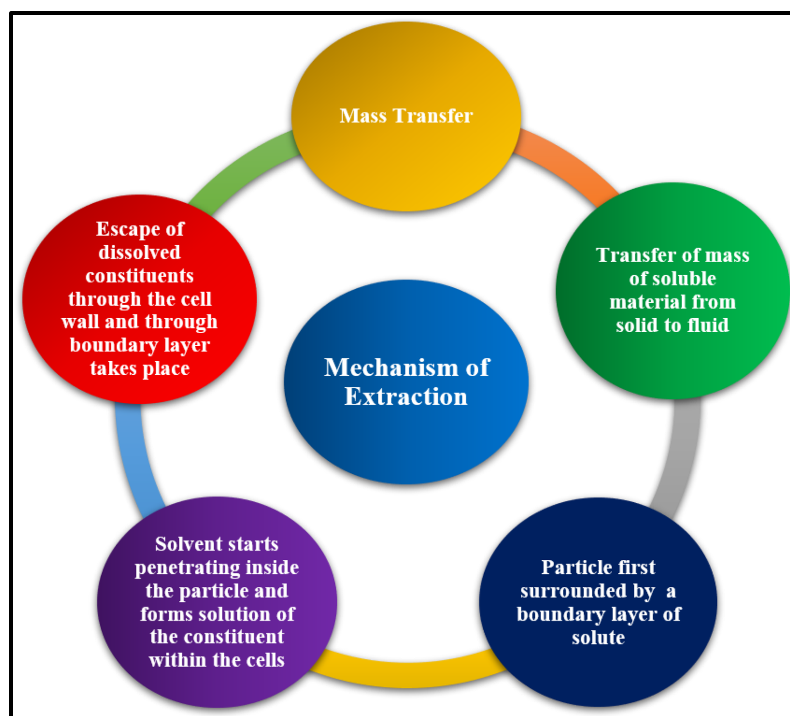


Figure 2.2: Mechanism of extraction (Own creation)

Precisely weighed 500 mg of dried plant roots that were transferred into 50 ml volumetric flasks. Further, the sample was dissolved in 10 mL of HPLC-grade Ethyl acetae and deionized water (10 mL). The extract was sonicated for 20 minutes. This procedure is known as the *ultra-sonication-assisted* extraction technique. To facilitate the entire release of essential components from root extracts, the sample was left at room temperature for 24 hours. After 24 Hours, the sample was heated at 50°C for 20 minutes to accelerate the extraction rate and to

avoid phytochemical degradation. After filtering the root extract through 0.20 µ nylon filters, samples were gathered for additional analysis. [19].

2.3 Evaluation of quality control parameters *Hibiscus vitifolius Linn.* root powder

The combination of all elements that directly or indirectly affect the safety, efficacy, and acceptability of product constitutes the quality of herbal medicines. Therefore, the quality control evaluation was carried out several parameters such as loss on drying at ash values (acid insoluble, water soluble), and extractive values (water soluble and Ethyl acetate soluble). The values are presented in Table 2.3.1.

Table 2.3.1: Quality control evaluations of *Hibiscus vitifolius* root

S. No.	Quality control parameters	Results (%)
1.	LOD	10.50 ± 0.05
2.	Total ash value	5.42 ± 0.02
3.	Acid insoluble ash value	0.08 ± 0.03
4.	Water soluble ash value	2.41 ± 0.04
5.	Water soluble extractive value	9.07 ± 0.09
6.	Ethyl acetate soluble extractive value	3.72 ± 0.07

Quantitative standards revealed that excessive or deficient moisture content can adversely impact the physical properties of the herbs. Low moisture content in sample 10.50% suggested minimal microbial growth and higher stability. The purpose of ash content determination was to remove all traces of organic matter otherwise they may be interfering with analytical characterization. The ash content of the herbal plant was 5.42%, indicating that there was very little acid-insoluble siliceous matter present. Hence, it implies the purity of crude sample. The water-soluble ash concentration was used to check for inorganic content. Extractive values play a vital role for evaluation of crude drug. Less extractive value indicates addition of exhausted material, adulteration, inappropriate processing during drying, formulation, or storage. The water-extractive value was higher than Ethyl acetate extractive value indicated more amounts of water-soluble Phytoconstituents present in plants root. Sugar, acids, and inorganic substances were present in the sample at a 9.07% water-soluble extractive value. The extract contained both polar and non-polar secondary metabolites, as indicated by the alcohol-soluble

extractive value of 3.72%. This value ensured that the quality control test was effective and that it was considered.

2.3.1 Preliminary phytochemical studies

Phytochemical screening is the primary step to assure the quality of herbal plants or extracts. It not only helps to reveal the constituents of the plant extracts and the ones that predominates over the others but also helps search for bioactive agents that can be used in synthesizing the valuable drugs. The preliminary phytochemical evaluation of extract of *Hibiscus vitifolius Linn.* root were performed for identification of phytoconstituents by using a different qualitative chemical test as mentioned below (Table 2.3.2) [18], [20]–[27].

Table 2.3.2: Test for Identification of Phytoconstituents

Test	Procedure	Observation
Carbohydrates		
Molisch Test	2 mL filtrate + few drops of α- napthol solution + shaken + 2 mL conc. sulphuric acid from the sides of the test tube	Appearance of brown ring at the junction of two liquids
Barfoed's test	1mL filtrate + 1mL Barfoed's reagent + heated for 2 min	Brick red ppt
Benedict's test	1mL filtrate + 1mL Benedict's reagent + heated for 2 min	Brown ppt
Proteins		
Millon's Test	3 mL filtrate + 5 mL Million's reagent	White ppt
Precipitation Test	3 mL filtrate + few drops of absolute alcohol + 5% copper sulphate solution + 5% lead acetate + 5% ammonium sulphate	White colloidal ppt
Xanthoprotein test	2 mL filtrate + conc. nitric acid	Yellow ppt
Sterols		

Salkowski Test	Plant extract filtrate + 2.0 mL of chloroform + 2.0 mL of conc. sulphuric acid + shaken for few min	Red colour
Libermann Test	Filtrate + few mL of acetic anhydride + heated gently + added few drops of conc. sulphuric acid	Blue colour
Flavonoids		
Shinoda test	A small quantity of the extract + 5mL of ethanol (95% v/v) + few drops of conc. hydrochloric acid + 0.5 g of Magnesium turnings	Pink to crimson colour
Ferric-chloride test	Filtrate + few drops of ferric chloride	Intense green colour
Zinc-HCl reduction test	Filtrate + zinc dust + few drops of hydrochloric acid	Magenta red colour
Lead acetate test	Filtrate + few drops of lead acetate	White ppt
Alkaloids Few mL of extract + 5mL of 1.5% v/v hydrochloric acid + filtered. Filtrate was then subjected to following test:		
Dragendorff's Test	Few mL filtrate + Dragendorff's reagent	Orange-red colour
Hager's test	Few mL filtrate + Hager's reagent	Yellow ppt
Mayer's Test	2-3 mL filtrate + few drops Mayer's reagent	Creamy white ppt
Wagner's test	2-3 ml filtrate + few drops Wagner's reagent	Reddish brown ppt
Saponins		
Foam test	20mL water in measuring cylinder + 50gm extract (vigorously shaken for 15 min.)	A foam layer on the top of the test tube
Tannins		
Ferric chloride test	5 mL of extract + 1 mL of 5% ferric chloride solution	Greenish black colour

Dilute nitric acid test	2 mL of extract + few drops of dilute HNO_3 solution	Reddish to yellow colour
Glycosides		
Bromine water test	Filtrate dissolved in Bromine water	Yellow ppt
Legal's test	Filtrate + pyridine	Pink to red colour
Borntrager's test	Boil the powdered drug with dilute sulfuric acid + filter + chloroform to the filtrate + Shake + collect the organic layer + Add a few drops of strong ammonia solution + shake slightly + keep the test tube aside for few minutes.	The lower ammoniacal layer takes on a pink or red colour

An extract of a selected plant root contained alkaloids, carbohydrates, glycosides, flavonoids, tannins, and phenolic compounds, according to preliminary phytochemical research. Ethyl acetate root extracts indicated presence of all phytoconstituents whereas, this extract revealed that it was rich in flavonoids and hence, confirmed by Lead acetate and Shinoda tests. The existence of these Phytoconstituents has physiological and therapeutic effects.

2.3.2 Identification of Ethyl Acetate extract

HPLC method and FTIR was utilized to identify the phytoconstituents present in extract. Root extract i.e., ethyl acetate was independently re-equilibrated in mobile phase methanol-acetonitrile (50:50 v/v) up to 5 ml and then were sonicated for 5 minutes. Furthermore, before the analysis, extract was filtered through 0.45 μ nylon filters, and then precisely 20μL of the sample was analyzed using HPLC-DAD instrument [28]. The Phytoconstituents were identified based on the retention time.

The liquid chromatography is used to qualitatively or quantitatively estimate the specific composition of samples obtained from natural sources. The results of the qualitative analysis are evaluated based on the consistency of the retention time of the reference standard and the compounds in the analyzed sample [29].

Also, analysed retention time, separation, and tailing factor. The graph obtained from extract is depicted in Figure 2.3.

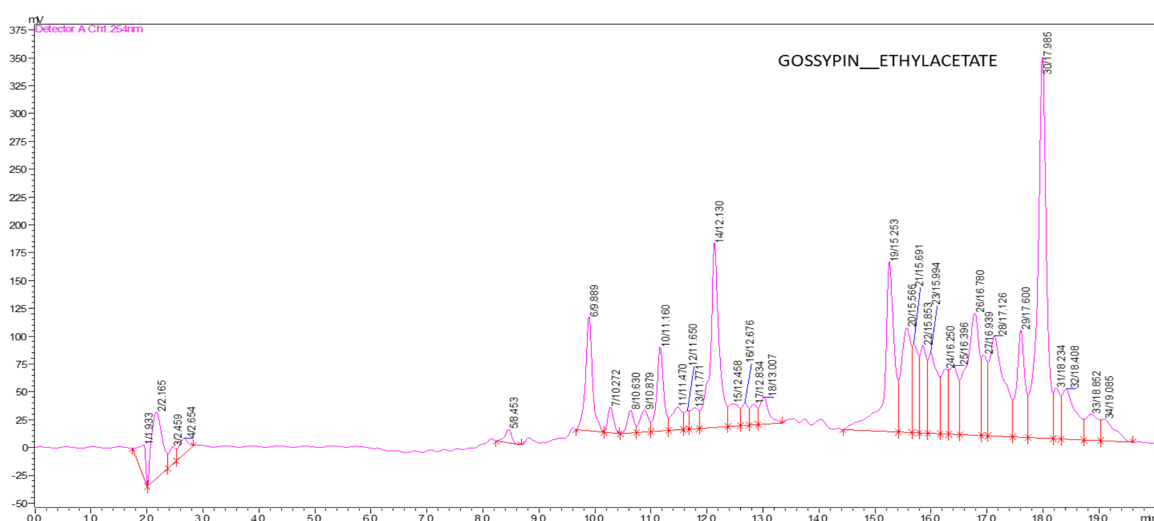


Figure 2.3: HPLC graph of EA extract

2.3.3 Analytical characterization

Herbal plants contain several secondary metabolites which possess therapeutic activities in chronic and acute disease conditions. But the biggest challenge for working on secondary metabolites is closely associated with using the predicted methods to isolate the expected compound and analyze the spectroscopic data to elucidate and characterize the structure. Thus, analytical techniques are used to identify, isolate, or quantify chemicals, materials, or phytoconstituents and characterize their physical properties. Analytical methods such as photometric analysis (UV, IR, MS, and NMR), thin layer chromatography (TLC), high-performance liquid chromatography (HPLC), and gas chromatography (GC) can be employed to establish the constant composition of herbal preparations. These techniques can also be employed for standardizations of herbs [30], [31].

2.3.4 Identification of Gossypin

I. Fourier transform-infrared spectroscopy

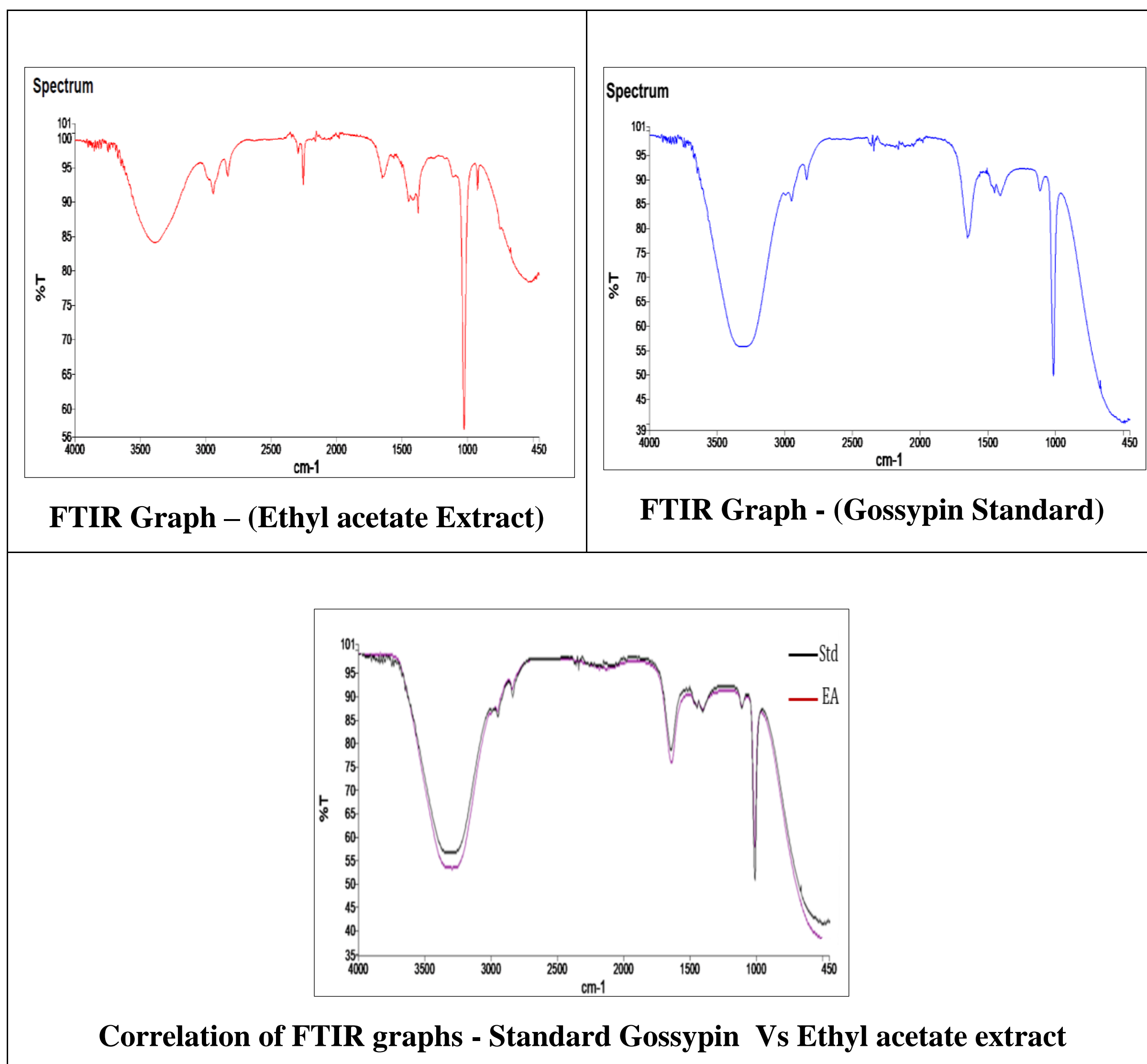
The utilization of Fourier Transform Infrared Spectroscopy (FTIR) for the identification of active compounds in plants, fruits, vegetables, and microorganisms have grown in recent years. It has also become a potent analytical tool for investigating active compounds found in samples. Because it is quick, easy to prepare samples, requires small sample sizes, and does not use solvents, which is more cost-effective, FTIR has gained popularity. The identity of the molecules under the FTIR spectrum is indicated by the different peaks shown due to the

wavenumber shift of various molecules with the same functional group within their wavenumber range; thus, they are used for the fingerprint of the molecules. The correlation shows between the infrared band positions and chemical structure becomes the fundamental information to identify the unknown molecules or even structural identification [23]. Hence, this technique was used for identification of flavonoids. The FTIR (99798, PerkinElmer, Spectrum Two) was employed to discover the pure drug by detecting its functional groups. The samples of standard Gossypin and Ethyl Acetate extract were used for detection by FTIR. The scanning was done at 450 to 4000 cm-1, and peaks were observed. The sample extracted from the selected plant was compared with those of a sample of pure Gossypin [32].

FTIR spectrum is used as an analytical technique to qualify or estimate pure drug samples. It is a label-free, non-destructive analytical technique that can be used widely to study a wide variety of different molecules in a range of different conditions. The FTIR spectrum and overlay graph of standard and root extract was recorded between 450-4000 cm^{-1} (Figure 2.4). The corresponding peaks of samples are depicted in Table 2.3.4.1 below. The reference sample Gossypin indicated the functional groups O-H, C=O, C-C, SP^3 C-H, and SP^2 C-H at 3646, 1641, 1493, 3084, and 2845 cm^{-1} respectively. And the characteristics peaks of Ethyl Acetate extract found at 3645, 1638, 1498, 3079, and 1220 cm^{-1} which indicated the functional groups alc. O-H, C=O, C-C, C-H, and aromatic ring.

Table 2.3.4.1: FTIR of standard and selected root extract

S. No.	Samples	Peak (cm^{-1})	Functional group
1.	Standard Gossypin	3646	Alc. O-H
		1641	C=O
		1493	C-C
		3084 2845	SP^3 C-H SP^2 C-H
		1223	Aromatic ring
2.	EA extract	3645	Alc. O-H
		1638	C=O
		1498	C-C
		3079	C-H
		1220	Aromatic ring



FTIR Graph – (Ethyl acetate Extract)

FTIR Graph - (Gossypin Standard)

Correlation of FTIR graphs - Standard Gossypin Vs Ethyl acetate extract

Figure 2.4: FTIR Graphs

2.3.5 Assay of Extract

I. By using HPLC

In order to detect the possibility of coelution of other substance(s), the purity of the analyte peak should also be determined. For instance, the HPLC-DAD can be used to determine 'the purity' of the analyte peak. The purity and identity of the analyte peak can be proved by comparing to the standard [33]. A standard Gossypin and Ethyl Acetate extract of 10 ppm was made by dissolving the samples each separately re-equilibrated in 5 mL of methanol-acetonitrile (50:50 v/v). The samples were sonicated for 10 min. Additionally, prior to analysis,

all samples were filtered through nylon filters 0.20 to 0.45 μ, and then an exact 20 μL sample was analyzed using the HPLC-DAD instrument. Further the purity of the HPLC peak can be demonstrated by calculating below formula.

Assay % = (AS/AT) X (CS/SC) X (P/LC) X 100

Where,

AS = Average area/ Absorbance of sample solution

AT = Average area/ Absorbance of standard solution

SC = Conc. of standard in ppm

CS = Conc. of sample in ppm

P = Potency of standard

LC = label claim standard

Percentage assay was carried out using HPLC method of Ethyl Acetate extract by comparing with standard % of extracts was carried out.

Ten ppm extract was used to determine the purity of different extract. Samples were run for 20 min using HPLC and calculated area, height and % purity. The results obtained from this analysis are depicted in Table 2.3.5.1.

Table 2.3.5.1: Parameters obtained by HPLC

Parameters	Ethyl Acetate Extract
AS	1875245
AT	6357891
CS	100
SC	100
P	95.6
LC	89.5

Assay %	31.50

Based on the information presented in Table 2.3.5.1, the marker compounds Gossypin are clearly observed the retention time for Ethyl Acetate extracts was 10.678 min. Additionally, extracts' purity was estimated at 31.50 %. thereby, it was concluded that the phytoconstituents (Gossypin) was present in root extract and reflected tR values under acceptance criteria. So, for reliable and accurate results, the secondary metabolites in plants/extracts are estimated using the HPLC technique.

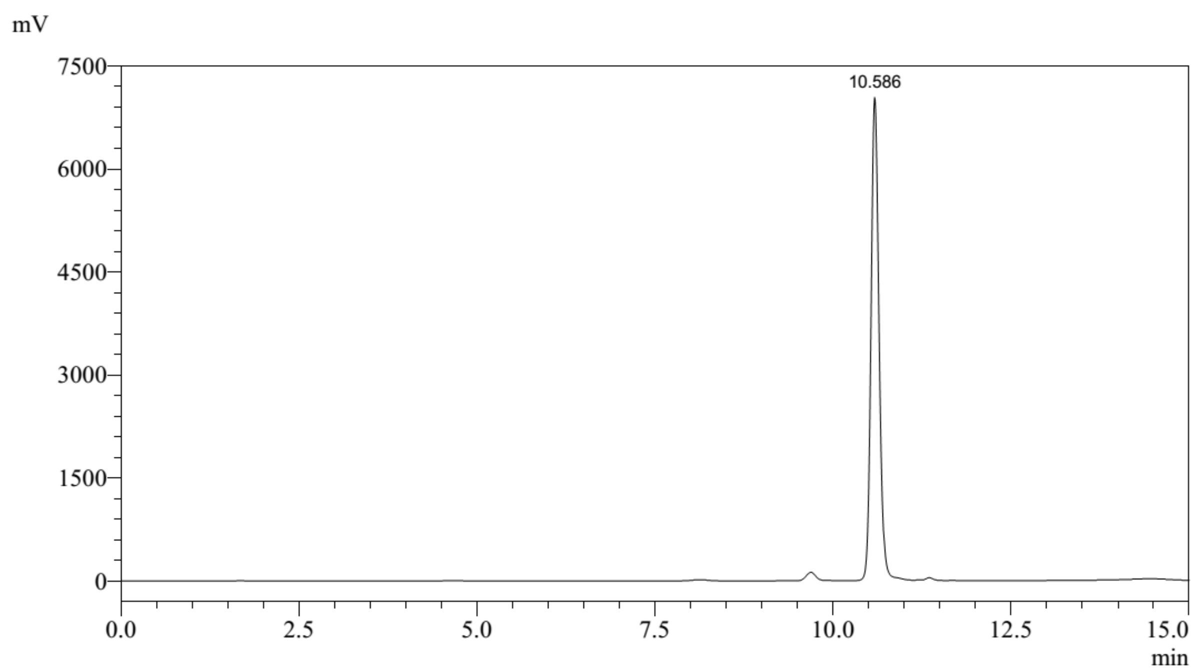


Figure 2.5: HPLC graph of Standard Gossypin

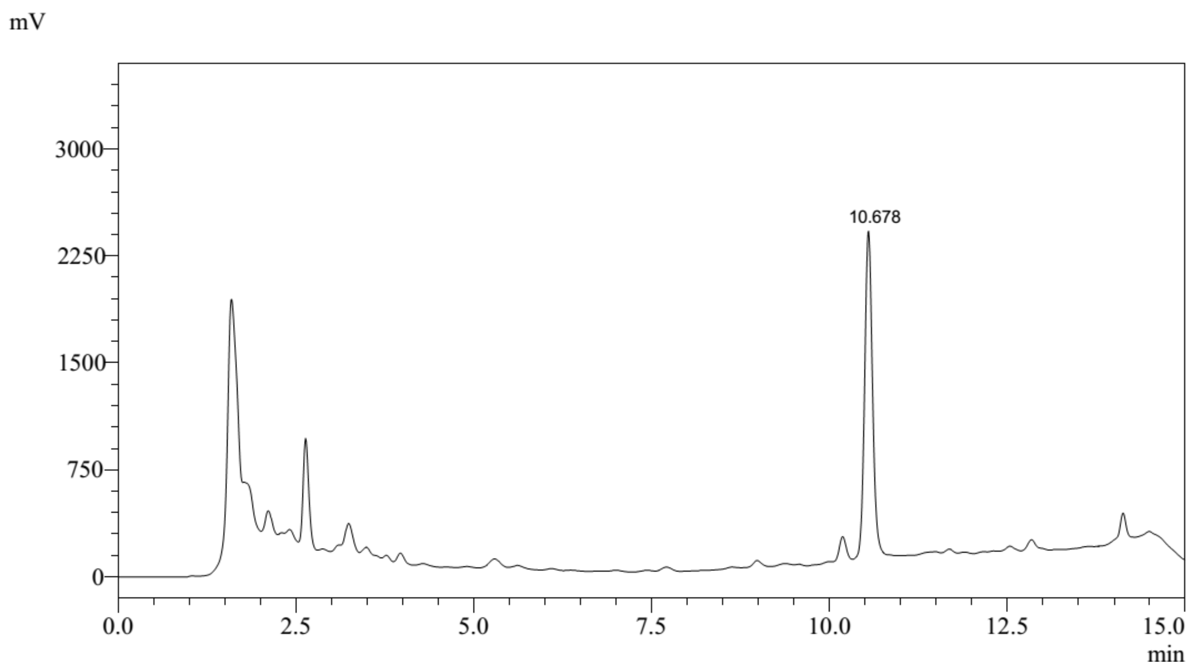


Figure 2.6: HPLC graphs of Ethyl Acetate extract

2.3.6 Microbiological study

Antibiotics are used for hypersensitivity testing, drug discovery, epidemiology, and prediction of therapeutic effect. Various laboratory methods are used to evaluate or investigate the in vitro antimicrobial activity of extracts or pure compounds. The most known and basic methods are the disk-diffusion and broth or agar dilution methods [34].

Staphylococcus aureus, Staphylococcus epidermidis, strains were used for this investigation. The culture conditions were mentioned in Table 2.3.6.1. The disk diffusion method was used for the microbial evaluation. Autoclave suitable paper discs (Filter Papers No. 740-E, 12.7 mm diameter). An extract solution was prepared. Thorough mixing was done on a vortex mixer to obtain a homogeneous suspension. 0.1 ml (about 3 drops) of an extract solution was saturated on the disk surface. Two discs were made for two organisms. Prepared soybean casein digest agar plates and nutrient broth tubes were incubated at 30-350C for 24 hours. Contaminated plates and tubes were discarded after incubation. 100 to 200 microliters of each culture (Staphylococcus aureus (ATCC 6538); and *Staphylococcus epidermidis* (ATCC 12228) were aseptically inoculated into 10 ml of sterile nutrient broth. Tubes were incubated at 30–350°C. 1 ml of seed nutrient broth of each culture was aseptically added to soybean casein digest agar medium tubes and immediately mixed on a vortex mixer. Soybean casein digest agar medium tubes were plated on 24-hour old soybean casein digest agar medium plates. The plates were gently rotated clockwise and counter clockwise to ensure adequate mixing of the medium. Let

it become medium thick. The plates were not allowed to stack while pouring the agar or solidifying the agar. Filter paper discs saturated with the tested extract was placed on the middle surface of each of two inoculated soybean casein digest agar plates. All plates were kept overnight in the refrigerator to obtain proper dispersion of the tested extract. The prepared plates were incubated at 37^0 C for 24 hours. The diameter of zone of inhibition (in mm) was noticed with the help of a Vernier calliper. Extract's antibacterial activity was measured in triplicate and mean value was recorded.

In the present study, the antimicrobial activities of extract against the various microorganism were investigated. The presence or absence a of zone of inhibition and their potency were also checked. The results are depicted in Table 1.4.2, Figure 1.4.1 below.

Table 2.3.6.1: Micro-organisms with culture conditions

Name of culture	Strain No.	Incubation temp.	Incubation Period	Selective Media	Colony characters on selective media	Morphology/ Gram Character
Staphylococcus epidermidis	ATCC 12228	30-35°C	18-24 hrs	Mannitol Salt Agar	white colour raised, cohesive colonies	Positive Cocci
Staphylococcus aureus	ATCC 6538P	30° –35°C	18-24 hrs	Mannitol Salt Agar	Yellow colonies with yellow zone	Positive Cocci

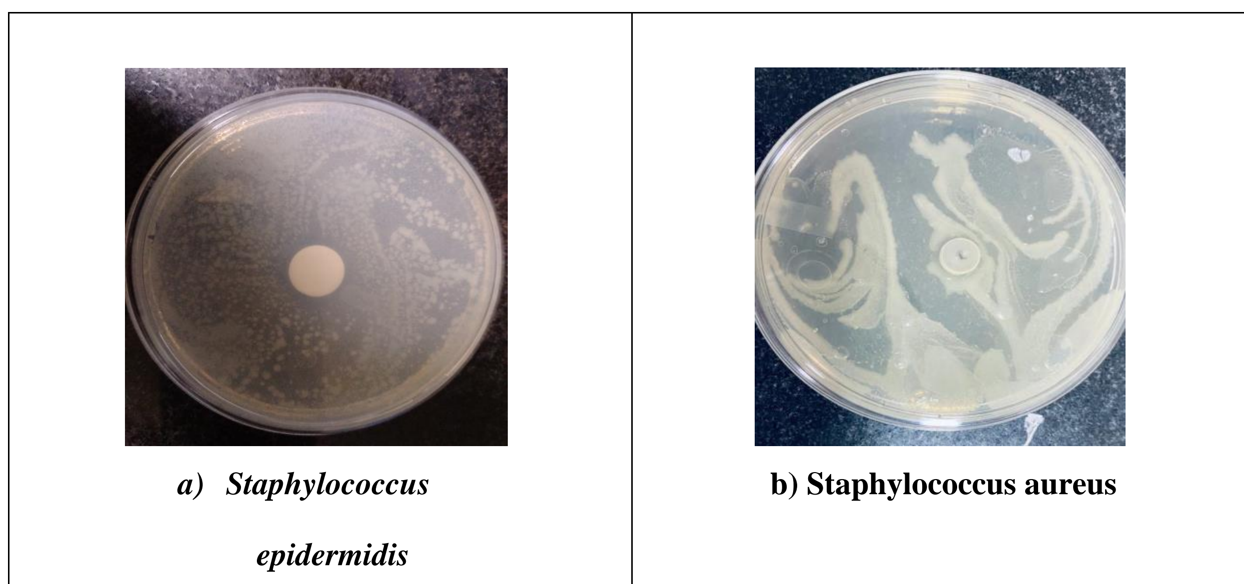
Agar well diffusion method was used for antimicrobial study as it offers various advantages. In the present study, the antimicrobial activities of extract against the various microorganism were investigated. [34]

Using a bacteriological loop inoculate a test tube of inoculum medium or nutrient broth with growth from a fresh agar slope & incubated at 37°C for 16-18 hours. Store at 4°C. 100 ml was sterilized and cooled to 45°C. Add 1 ml of inoculum of *Staphylococcus epidermidis* of required thickness. Mix gently but thoroughly.

Dispense 25 to 30 ml of the inoculated medium into sterile Petri plates. Allow media to be set and store in refrigerator still use. Plates should be used within one day or on the same day. A standard 6.0 mm diameter borer is taken. After immersing the borer in isopropyl alcohol and burning off the residual isopropyl alcohol in the borer, the borer is cooled properly and cups/wells are drilled in pre seeded agar plates. Bore three cups/wells per plates. Mark every cup (well) with proper dilution and add 100 µl of different dilution to different cups and keep the plates at low temperature (around 10°C) for 10-12 hours for diffusion. Incubate the plates at 30-37°C for overnight (18-24 hours). The diameter of each growth zone was measured from three different sides. Note down every reading.

Table 2.3.6.2: Zone of inhibitions for the selected micro-organisms

Bacteria used	Extract	Zone of Inhibitions (mm)
Staphylococcus epidermidis	Ethyl acetate	7.6
Staphylococcus aureus	Ethyl acetate	3.4



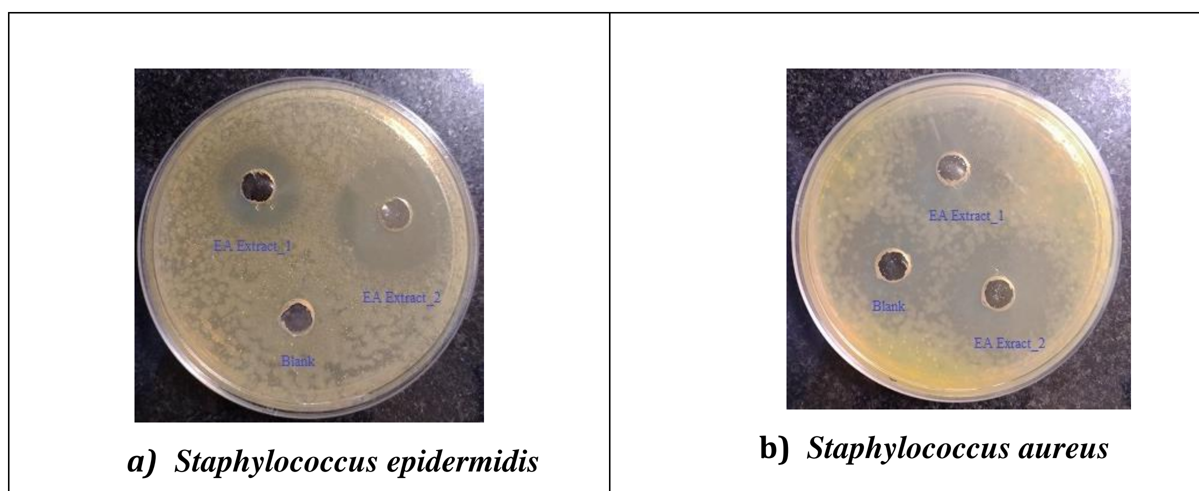
a) Staphylococcus epidermidis **b) Staphylococcus aureus**

Figure 2.7: Antimicrobial activity of *a) Staphylococcus epidermidis, b) Staphylococcus aureus*

Agar well diffusion method was used for antimicrobial study as it offers various advantages. In the present study, the antimicrobial activities of Ethyl Acetate Extract against the various microorganism were investigated. The presence or absence a of zone of inhibition and their potency were also checked. The results are depicted in Table 2.3.6.3, Figure 6.105 below.

Table 2.3.6.3: Zone of inhibitions for the selected micro-organisms

Bacteria used	Extract	Zone of Inhibitions (mm)
Staphylococcus epidermidis	Ethyl acetate	9.8
Staphylococcus aureus	Ethyl acetate	7.6



a) Staphylococcus epidermidis **b)** ***Staphylococcus aureus***

Figure 2.8: Antimicrobial activity of a) Staphylococcus aureus *b) Staphylococcus epidermidis*

Conclusion:

The present work reveals the excellent scope of herbal Extract for the use of antibacterial safely and effectively. Hence, it was concluded that the quality control test was successful and was adopted for the final Extract development. In the research work extracts of *Hibiscus vitifolius Linn.* was obtained from ethyl acetate solvent. Extracts was identified using HPLC-DAD

instruments. Among, gossypin was found to be more abundant in ethyl acetate extract. The gossypine flavonoid was analytically identified using FTIR and HPLC methods.

Extracted Gossypin showed very good antimicrobial activity. It was observed that the zone of inhibition was for *Staphylococcus aureus, Staphylococcus epidermidis* was within range. Based on these results, it was concluded that this extract shows significant activity against *Staphylococcus aureus, Staphylococcus epidermidis*. The presence of different functional groups at different positions of the flavonoid structure (gossypin) results in the antimicrobial activity of the extract against pathogens.

Hence, based on overall observations and results, it can be concluded that the herbal Extract containing Gossypin show anti-bacterial effect in safe and efficacious way.

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