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TO DISCERN AND DERACINATE THE ACTIVE CONGLOMERATE FROM HERBS IN ANTAGONISM TO FOOD POISON EFFECTUATE *S. AUREUS* - AN *IN-VITRO* PHYTOTHERAPY

V. Prasanth Kumar, R. S. Priyengha, Y. Sam Alexander and S. Umamaheswari *

Department of Biotechnology, Manonmaniam Sundaranar University, Tirunelveli - 627012, Tamil Nadu, India.

Keywords:

Glycyrrhiza glabra, Antibacterial activity, *Staphylococcus aureus*, TLC, HPLC

Correspondence to Author:
Prof. S. Umamaheswari

Professor & Head,
Department of Biotechnology,
Manonmaniam Sundaranar University, Tirunelveli - 627012,
Tamil Nadu, India.

E-mail: umamsu@gmail.com

ABSTRACT: Medicinal plants are widely used to treat disease in non-industrialized societies for not being far cheaper than modern medicines. In this study, five types of medicinal plants *viz*., *Glycyrrhiza glabra*, *Aegle marmelos, Centella asiatica, Cinnamomum verum, Cynodon dactylon* were collected from Alwarkurichi, Tirunelveli District, Tamil Nadu, and extracts were prepared using various solvents such as methanol, ethanol, chloroform, diethyl ether, benzene, and water. These extracts were checked for antibacterial activity against *Staphylococcus aureus* isolated from a hospital environment. On the basis of antibacterial activity, three plants viz., *Glycyrrhiza glabra*, *Aegle marmelos*, and *Centella asiatica* were chosen for further research in which the ethanolic extract of *Glycyrrhiza glabra* rendered higher activity against *Staphylococcus aureus*. Thin Layer Chromatography of the plant extract of *Glycyrrhiza glabra* was carried out, which showed the development of four bands of which the first three bands rendered higher sensitivity against *Staphylococcus aureus*. The eluted bands from TLC were visualized under the UV-Spectrophotometer to monitor the absorbance of the eluted three bands in the wavelength spectrum ranging from 350-380 nm. Further confirmation of the separated compound was done by treating the band with Iodine and Ninhydrin spray for effective visualization of the isolated compound. The selected three bands from *Glycyrrhiza glabra* were taken for HPLC to quantify each component in the samples. Two to three clear peaks were obtained from the bands in HPLC indicates the presence of antibacterial activity against *S. aureus*.

INTRODUCTION: With the perception of emerging side effects and resistance by pathogenic microorganisms to antibiotics, much attention has been focused on extracting biologically active compounds from plant species. Medicinal plants may offer a natural and new source of antibacterial agents [1].

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Glycyrrhiza glabra is a legume native to Southern Europe, India, and parts of Asia [2]. The roots are stoloniferous with an impressive list of well-documented uses.

Aegle marmelos, commonly known as Vilvam Tamil quince, golden apple, stone apple, wood apple, is a species of tree native to India [3]. Research has found the essential oil of the Bael tree to be effective against 21 types of bacteria [4]. *Centella asiatica* is a perennial grass forming thick mats by means of stolon and rhizomes, which is used for indigestion and treating wounds according to old Vedic tradition [5]. *Cinnamomum verum* also called a true cinnamon tree or Ceylon cinnamon tree is a

small evergreen tree belonging to the family Lauraceae, native to Sri Lanka [6]. *Cinnamomum verum* is also cultivated on a commercial scale in Seychelles and Madagascar. The presence of these bioactive constituents such as alkaloids, flavonoids, steroids, terpenoids, phenols, and carbohydrates which is related to the antibacterial activity of the plant [7]. Although numerous antibiotic classes have been discovered with different modes of action, bacteria evolve and adopt numerous strategies to counteract the action of antibiotics. Antibiotic resistance was observed soon after the discovery of antibiotics [8]. It is a natural consequence of drug exposure and results from both the use and overuse of antimicrobial agents [9]. To overcome the problem of antibiotic resistance in bacteria, many medicinal plants have been studied extensively as an alternative way to treat and prevent infection [10]. Coagulase Positive *S. aureus* involved in a massive outbreak on staphylococcal scalded skin syndrome was reported. Nasal *Staphylococcus aureus* carriage, affecting about 20% of the population, has been identified as a risk factor for the pathogenesis of community-acquired and nosocomial infections [11]. Some strains of *S. aureus* can cause food poisoning and other diseases [12]. The present work focuses on the antibacterial activity of different solvent extracts from the selected five medicinal plants.

MATERIALS AND METHODS:

Sample Collection: The medicinal plants for the research work were collected around the Tirunelveli District and were identified as *Glycyrrhiza glabra, Aegle marmelos, Cynodon dactylon, Centella asiatica, Cinnamomum verum* based on the morphology and stored.

Preparation of Plant Extract: Plant extract was prepared using a modified method [13]. Fresh parts of the chosen plants were dried in an oven and ground to a fine powder with a mechanical grinder. Ten grams of each plant part were macerated in 100 ml of absolute ethanol and kept for 72 h, covered using aluminum foil, and labeled. After 72 h of extraction, it was filtered using Whatman filter paper, and the filtrate was evaporated to dryness at room temperature, stored in the refrigerator.

Test Microbes: Four different *S. aureus* samples were isolated in Mannitol salt broth, incubated at 37 °C for 24 h. The growth of S*taphylococcus aureus* in Mannitol salt agar was confirmed by visualizing the color change of the medium from pink to yellow, and the cultures were named as 04, 07, sink, dialysis respectively and subsequently streaked on MSA agar to obtain pure colonies and the cultures were stored at 4 °C for further work.

Antibiotic Susceptibility Test: The Antimicrobial sensitivity testing for *S. aureus* was conducted using a procedure by Kirby-Bauer [14] with commonly used antibiotics in the hospital, which includes Discs with the following Antibiotic potency. The susceptibility assay was performed on Muller Hinton Agar [15] plates using 9 different Antibiotics including Vancomycin 30 mcg, Penicillin 10 unit, Methicillin 5 mcg, Ampicillin 25 mcg, Cefotaxime 30 mcg, Ciprofloxacin 5 mcg, Amikacin 30 mg, Erythromycin 15 mg, Clinda-mycin 2 mg, Gentamicin 10 mcg, Nitrofurantoin 300 mcg, Kanamycine 30 mcg, Norfloxacin 10 mcg, Oxacillin 1 mcg, Tetracycline 3 mcg, quality control for susceptibility testing was done using *S. aureus*.

Thin Layer Chromatography: The TLC plate was prepared by the ratio 2:3 as per normal protocol. The prepared plant extracts were applied on pre-coated TLC plates by using capillary tubes and developed in a TLC chamber using a suitable mobile phase. The developed TLC plates were air-dried and observed under UV at 254 and 366 nm, respectively. The mobile phase was prepared; the standard volume of solvents was prepared and allowed for separation for 4 h. The compound separation could be confirmed by visualizing under UV trans-illuminator, and the compounds were scarped and transferred into a centrifuge tube. The movement of the analysis was expressed by its retention factor (R_f), which was calculated as per the equation.

Iodine Vapour: A chamber may be assembled as follows: To 100 ml wide mouth jar with the cap a piece of filter paper was added with few crystals of iodine. Iodine has a high vapor pressure for solid, and the chamber will rapidly become saturated with iodine vapor. The TLC plate was inserted and allowed to remain within the chamber until the development of light brown color over the entire plate.

Commonly, if a compound has an affinity for iodine, it will appear as a dark brown spot on a lighter brown background. Carefully the TLC plate was removed and the spots were noted.

Antibacterial Assay: Overnight grown cultures of the isolated *Staphylococcus aureus* were swabbed [16] on Mueller Hinton Agar plates into which 6mm diameter wells were bored with the help of a well puncture. The test plant extracts (100 µl) were loaded into the wells. Plates were incubated at 37 ± 0.1 °C for 20-24 h. The zone of inhibition around the wells was recorded. Control samples were loaded into the wells with respective solvents.

HPLC for Active Compounds: HPLC is an analytical chemistry technique that combines the physical separation capabilities of Liquid Chromatography with the mass analysis capabilities of Mass spectrometry. 2 mg of sample was prepared by dissolving in 2 ml methanol in a volumetric flask, and the solution was filtered. Stock solution 1mg/ml was stored in the refrigerator at 4 ºC. HPLC for various layers of bands was performed.

RESULTS:

Collection & Identification of Cultures: The samples for the study were collected from a nearby children hospital in Tirunelveli district, South India. Four different *S. aureus* samples were collected in Mannitol salt broth, incubated at 37 ± 0.1 °C for 20-24 h. Confirmation of the growth of *Staphylococcus aureus* was through visualization of the color change of the medium from pink to yellow. The yellow color was formed due to the fermentation of mannitol by *S. aureus* for their growth **Fig. 1**. The cultures were named as PC6704, PC6705 (sink), PC6706 (Dialysis), PC6707 and subsequently streaked on MSA agar to get pure colonies, which were stored at 4 °C for further work. Staining and Biochemical characteristics of the identified *S. aureus* was noted in **Table 1**.

TABLE 1: BIOCHEMICAL CHARACTERS OF *S. AUREUS*

S. no.	Test	Test Culture
1	Gram staining	Gram-positive cocci cluster
2	Catalase	Positive
3	Oxidase	Negative
4	Coagulase	Positive
5	Mannitol Fermentation	Positive

PC6704 PC6705 PC6706 PC6707

STAINING AND BIOCHEMICAL CHARACTERISTICS OF THE CULTURE

FIG. 1: ISOLATION OF *STAPHYLOCOCUS AUREUS*

Antibacterial Susceptibility of Plant Extracts against *Staphylococcus aureus:* The type and level of biological activity exhibited by any plant materials depend on many factors such as plant parts, geographical sources, soil conditions, harvesting time, moisture content, drying methods, storage conditions, and post-harvest processing. For example, the relatively high temperatures that can be generated during tissue grinding can denature chemical constituents, and the extraction solvent, time period, and temperature can affect the level and composition of secondary metabolites extracted from plant tissues. The antibacterial susceptibility test for the various extracts against four different *S. aureus* samples was noted in the given **Table 2, 3, 4, 5**. Based on the results given, ethanolic extract of *Glycyrrhiza glabra* expressed high activity.

Antibacterial Activity against PC6704 Culture:

TABLE 2: ANTIBACTERIAL ACTIVITY AGAINST PC6704 CULTURE

	M	E	D.E	B	Ch	W
A. marmelos	4 ± 1.12	3 ± 1.14	3 ± 1.12	4 ± 2.11	5 ± 1.10	4 ± 1.21
G. glabra	8 ± 1.12	7 ± 2.14	3 ± 2.09	2 ± 1.21	2 ± 1.13	-

B. verum	3 ± 1.13	2 ± 1.17	3 ± 2.16	2 ± 1.18	2 ± 1.28	-
C. dactylon	1 ± 2.50	-	-	-	-	-
D. asiatica	5 ± 2.12	3 ± 1.24	3 ± 1.15	-	-	-

Measurement in millimeters; M: Methanol, E: Ethanol, D.E: Diethyl ether, B: Benzene, Ch: Chloroform, W: Water

Antibacterial Activity against PC6707 Culture:

TABLE 3: ANTIBACTERIAL ACTIVITY AGAINST PC6707 CULTURE

	M	E	D.E	B	Ch	W
A. marmelos	5 ± 1.12	5 ± 1.21	3 ± 2.18	3 ± 1.17	4 ± 1.20	4 ± 2.19
G. glabra	4 ± 2.23	6 ± 1.18	5 ± 2.13	4 ± 1.29	5 ± 1.52	4 ± 2.11
B. verum	5 ± 1.21	-	-	3 ± 2.17	3 ± 1.22	-
C. dactylon	5 ± 1.19	5 ± 2.10	2 ± 1.12	3 ± 1.14	3 ± 2.18	3 ± 1.18
D. asiatica	-	-	-	5 ± 1.16	5 ± 1.23	3 ± 1.21

Measurement in millimeters; M: Methanol, E: Ethanol, D.E: Diethyl ether, B: Benzene, Ch: Chloroform, W: Water

Antibacterial Activity against PC6705 Culture:

TABLE 4: ANTIBACTERIAL ACTIVITY AGAINST PC6705 CULTURE

	M	E	D.E	B	Ch	W
A. marmelos	5 ± 1.21	3 ± 2.18	-	5 ± 1.40	-	-
G. glabra	4 ± 1.53	6 ± 1.71	3 ± 1.62	4 ± 2.09	4 ± 1.11	-
B. verum	3 ± 2.15	-	-	-	-	-
C. dactylon	3 ± 1.28	4 ± 1.97	3 ± 2.00	4 ± 1.25	4 ± 1.59	-
D. asiatica	-	-	-	-	-	-

Measurement in millimeters; M: Methanol, E: Ethanol, D.E: Diethyl ether, B: Benzene, Ch: Chloroform, W: Water

Antibacterial Activity against PC6706 Culture:

TABLE 5: ANTIBACTERIAL ACTIVITY AGAINST PC6706 CULTURE

	M	E	D.E	B	Ch	W
A. marmelos	6 ± 2.42	5 ± 1.13	5 ± 2.17	3 ± 1.09	4 ± 2.07	-
G. glabra	5 ± 2.21	6 ± 1.23	5 ± 1.29	8 ± 1.33	8 ± 1.79	-
B. verum	-	9 ± 1.20	-	7 ± 1.37	-	-
C. dactylon	-	-	-	-	-	-
D. asiatica	-	-	-	-	-	-

Measurement in millimeters; M: Methanol, E: Ethanol, D.E: Diethyl ether, B: Benzene, Ch: Chloroform, W: Water

Thin Layer Chromatography: *Glycyrrhiza glabra* ethanol extract was run using Thin Layer Chromatography technique with the ratio of the combination of solvent Methanol: Chloroform: water (36:65:3.5) ml. The mobile phase was prepared for 100 ml. The developed TLC plates were air-dried and observed under UV at 254 and 366 nm, respectively **Fig. 2**.

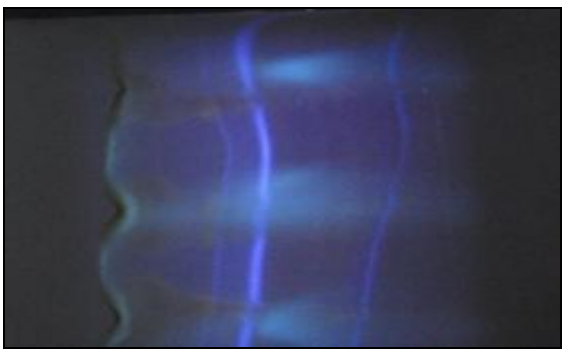


FIG 2: TLC OF COMPOUND AGAINST *S. AUREUS* IMAGE

The four fluorescent compounds were named as S1, S2, S3, and S4 and were separated and diluted in the same methanol solvent. The R_f value for each bands was noted in **Table 6**. This sample was further carried out for an antibacterial study.

R_f = Distance travelled by the solute / Distance travelled by the solvent.

TABLE 6: R_f VALUE FOR THE COMPOUNDS SEPARATED

Layer	R_f value
First Band (S1)	0.52
Second Band (S2)	0.76
Third Band (S3)	0.88
Fourth Band (S4)	0.97

Antibacterial Assay: Overnight growth cultures of four different strains of *Staphylococcus aureus* were swabbed on Muller Hinton plate in which 6mm diameter wells were made in each plate with the help of a well puncture. The scrapped

compound was centrifuged, and the supernatant was added into the well, and the plates incubated at 37 ± 0.1 °C for 20 - 24 h. The zone of inhibition around the charged wells was recorded after incubation. Control samples were maintained with respective solvents in the wells. The experiment was carried out in triplicates **Fig. 4** plates were inoculated.

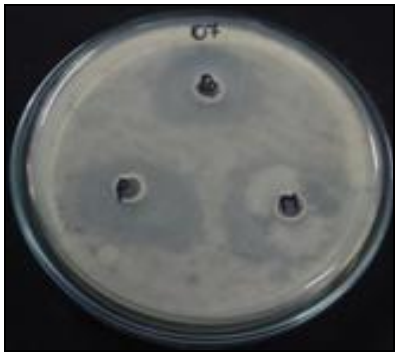
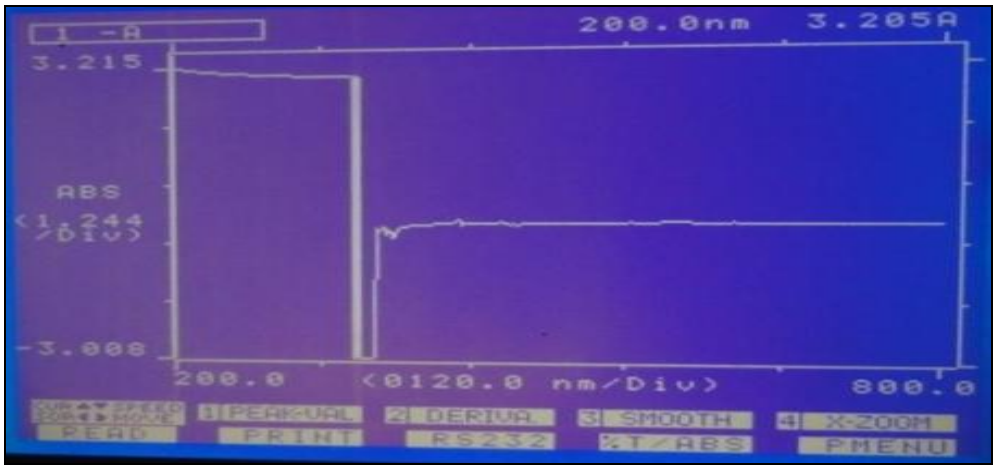


FIG. 3: ANTIBACTERIAL ASSAY OF FLUORESCENT COMPOUND ISOLATED FROM *GLYCYRRHIZA GLABRA*

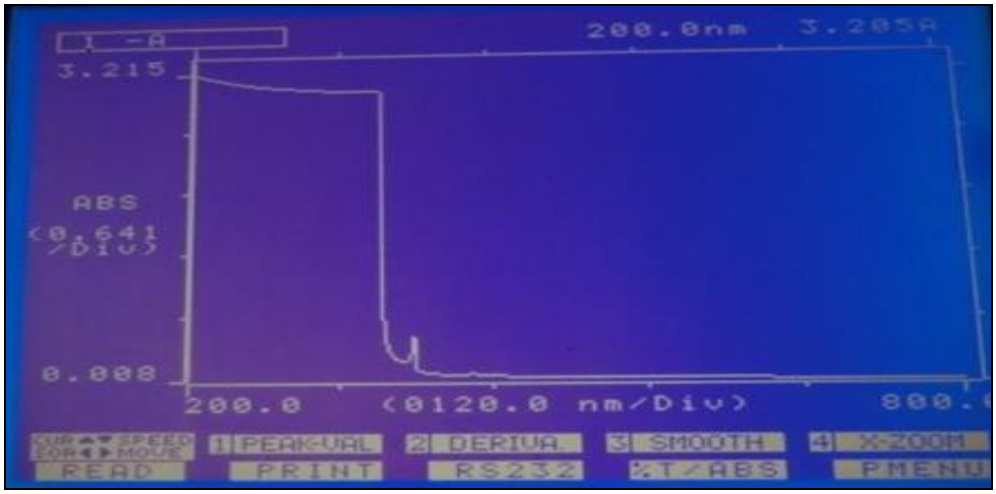
The first three bands separated from *Glycyrrizha glabra,* such as S1, S2, and S3 showed activity against all the four strains of *S. aureus.*

Spectrophotometer Analysis of Extracts: The extracts were subjected to spectrophotometry analysis to identify the wavelength with maximum absorbance. Sample 1 exhibited the maximum absorbance at the wavelength of 378.8 than that of other samples. Sample 2 exhibited a wavelength of 358.4, and Sample 3 shows the absorbance rate of 370.4. The wavelength spectrum of three samples was plotted as a graph **Graph 1, 2, 3**. All samples shows absorbance at the range of 350-370 nm. The absorbance readings were noted in **Table 7**. There are earlier reports regarding the absorbance characteristics as flavanols in the range of 350-370 nm [17]. Hence, it is confirmed as flavanols, which may be responsible for the antibacterial activity.

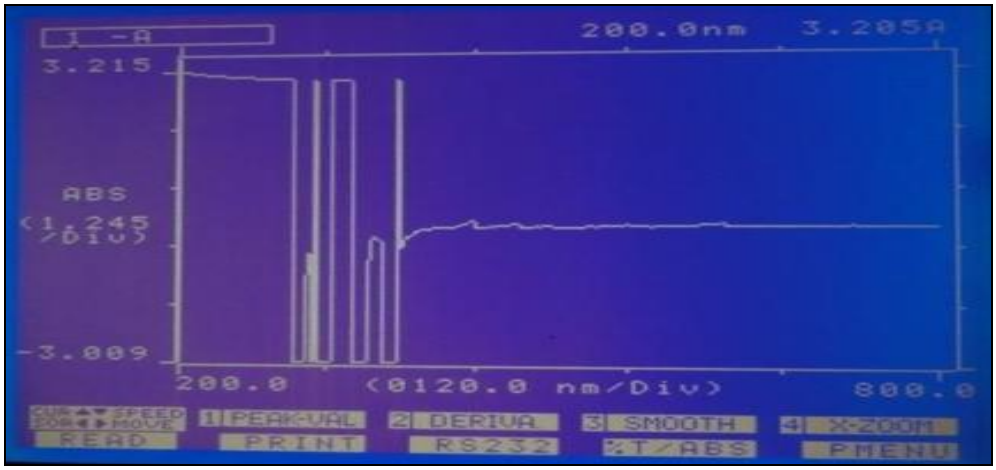
Wavelength Spectrum for S1, S2 & S3 Bands



GRAPH 1: GRAPH FROM THE FIRST BAND OF *GLYCYRRHIZA GLABRA*



GRAPH 2: GRAPH FROM THE SECOND BAND OF *GLYCYRRHIZA GLABRA*



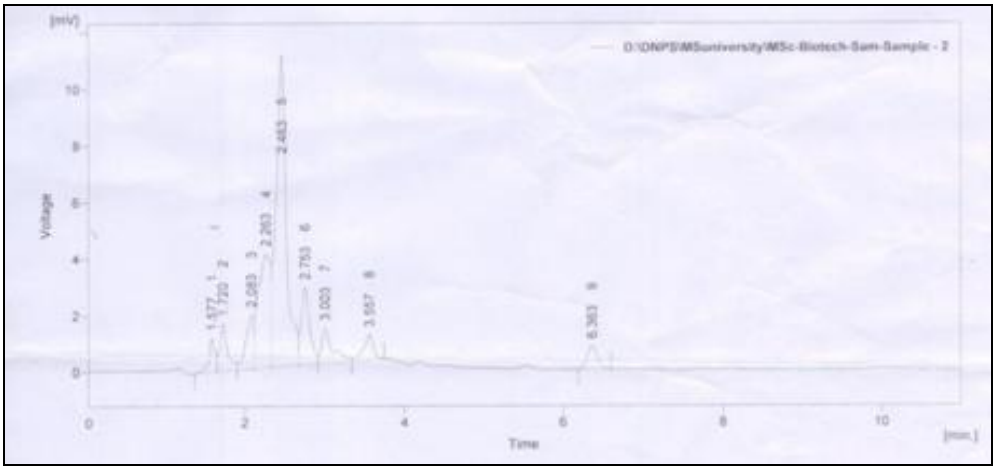
GRAPH 3: GRAPH FROM THE THIRD BAND OF *GLYCYRRHIZA GLABRA*

TABLE 7: SPECTROPHOTOMETRIC ANALYSIS OF THE SEPARATED COMPOUNDS

S. no.	Sample	Wavelength (nm)	Absorbance
1	Band 1	378.8	0.768
2	Band 2	358.4	0.343
3	Band 3	370.4	0.208

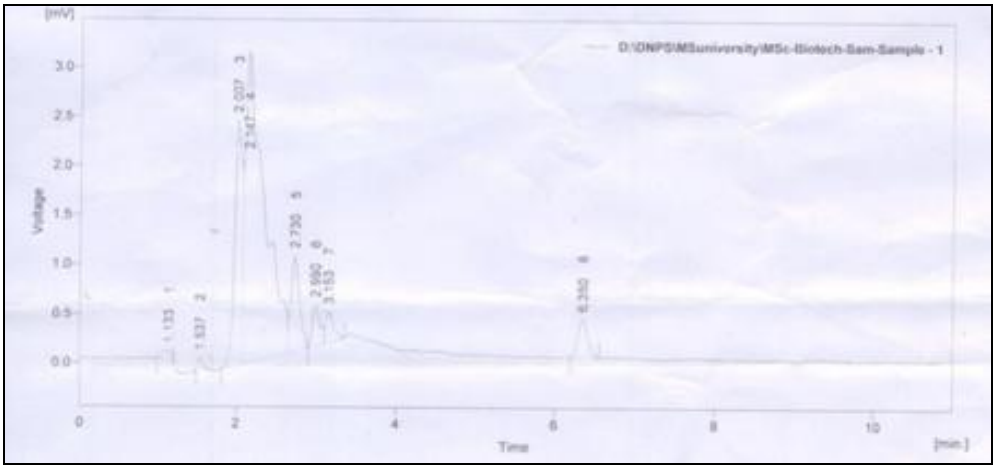
HPLC for Active Compounds: Clean and high peak was obtained from all the three samples **Graph 4, 5, 6**. In Band 1, 2, and 3 the compound shows a high area which is responsible for the Antibacterial activity **Table 8**.

Sample from First Band of *Glycyrrhiza glabra* (Athimadhuram):



GRAPH 4: HPLC OF COMPOUND FROM FIRST BAND

Sample from Second Band of *Glycyrrhiza glabra* (Athimadhuram):



GRAPH 5: HPLC OF COMPOUND FROM SECOND BAND

Sample Third Band of *Glycyrrhiza glabra* (Athimadhuram):

GRAPH 6: HPLC OF COMPOUND FROM THIRD BAND

TABLE 8: HPLC OF THE SEPARATED COMPOUNDS

S. no.	Sample	Retention Time (min)	Area (mV/min)	Height (mV)
1	Band 1	2.147	52.729	3.153
2	Band 2	2.463	98.437	11.005
3	Band3	1.870	331.618	22.848

DISCUSSION: The three bands eluted from Glycyrrhiza glabra in TLC were analyzed in UV-spectrophotometer at a wavelength of 350-380 nm. Studies on flavonoids by spectroscopy have revealed that most flavones and flavonols exhibit two major absorption bands: Band I (320-385 nm) represents the B ring absorption, while Band II (250-285 nm) corresponds to the A ring absorption. Functional groups attached to the flavonoid skeleton may cause a shift in absorption18 such as from 367 nm in kaempferol (3,5,7,4′-hydroxyl groups) to 371 nm in quercetin (3,5,7,3′,4′-hydroxyl groups) and to 374 nm in myricetin (3,5,7,3′,4′,5′-hydroxyl groups). Thus, the partially purified compound was identified as flavanols.

SUMMARY: Five types of medicinal plants *viz*., *Glycyrrhiza glabra, Aegle marmelos, Centella asiatica, Cinnamomum verum, Cynodon dactylon* collected from Alwarkurichi was subjected to extract preparation with various solvents like methanol, ethanol, chloroform, diethyl ether, benzene, and water. The antibacterial activity of the five plant extracts tested did not show proper activity for *Cinnamomum veru* and *Cynodon dactylon*. *Glycyrrhiza glabra* rendered higher activity against *Staphylococcus aureus*. TLC of the plant extract from *Glycyrrhiza glabra, Aegle marmelos,* and *Centella asiatica* were carried out and the TLC plates were visualized under UV Spectrophotometer for the appearance of four bands (F1, F2, F3, F4). The four bands were scrapped and subjected to centrifugation with methanol, and the supernatant of F1, F2, and F3 exhibited activity against *Staphylococcus aureus*. Of the three selected G*lycyrrhiza glabra, Aegle marmelos* and *Centella asiatica* plants, *Glycyrrhiza glabra* showed higher activity against *Staphylococcus aureus*. The confirmation of the separated compound was done by treating the band with Iodine and Ninhydrin spray for compound visualization. The first three bands from *Glycyrrhiza glabra* (Athimathuram) were taken to perform HPLC to quantify each component in the samples. Two to three clear peaks were obtained from F2 and F3 bands in HPLC, indicating the responsibility for the activity against *S. aureus*.

CONCLUSION: Antibiotics provide the main basis for the therapy of microbial infections. Since the discovery of these antibiotics and their uses as chemotherapeutic agents, there was a belief in the medical fraternity that this would lead to the eventual eradication of infectious diseases. However, overuse of antibiotics has become the major factor for the emergence and dissemination of multidrug-resistant strains of several groups of microorganisms.

Traditionally used medicinal plants produce a variety of compounds nurtured with therapeutic properties. They have anti-diabetic, anti-bacterial, anti-inflammatory, anti-pyretic activities, gastro-protective effects and many more important

medicinal properties. It is expected that plant extracts showing target sites other than those used by antibiotics will be bioactive against drug-resistant pathogens.

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CONFLICTS OF INTEREST: Authors report there is no conflict of interest in the present study.

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