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Antimicrobial activity and HR-LCMS analysis of methanolic extract of *Calotropis gigantea*

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Abstract
Calotropis gigantea is very unique plant which belongs to family Apocynaceae. According to Shivpuran the flowers are very much liked by lord Shiva even it is also one of the major parts of nine astrological plants but it is found to have many medicinal values. In the present study, a sensitive, reliable and rapid ultra-performance high resolution liquid chromatography method was established for determination of Deoxy streptomycin, Protorifamycine-I, Citrulline, 4-Trimethylammoniobutanal, cystamine, 6-hydroxy-2-hexynoic acid, Carnitine, D-Arginine, Choline, Pantoic acid, Betaxol, Madecassic acid, Avocadynone acetate, 6-deoxyerythronolide-B, O-Acetylserine, 4,6-Diamino-5-formidopyrimidine, Dihydrorobinetin, Barbituric acid, 5-ethyl-5-(2-hydroxyethyl), Idebenone Metabolite isolated from the leaves extract of *Calotropis gigantea*. Chromatographic separation was accomplished on a C18 column with a multiple-step gradient elution using water and acetonitrile (95: 5) with 30 minutes acquisition time as mobile phase. Antifungal activities were estimated by the food poison technique whereas, agar disc diffusion method was used for antibacterial activity. The results showed that phytoconstituents were separated and identified by HR-LCMS from the leaves of *Calotropis gigantean* revealed antimicrobial activities against *Fusarium oxysporum, Colletotrichum capsici* and *Xanthomonus axonopodis.*

Keywords: *Calotropis gigantea*, HR-LCMS, *Fusarium oxysporum, Colletotrichum capsici* and *Xanthomonas axonopodis*

1. Introduction
Medicinal plants are very important for cure various diseases can be traced back over thousands of years in India. due to presence of various chemical constituents. Plants are used medicinally in different countries and are a source of many potent and powerful drugs. Srivastava *et al*., (1996) [10] Green plants are an important source of various chemical compounds which are used for mankind for thousands of years to cure diseases. It contain many chemical compounds such as alkaloids, glycosides, phenols, flavonoids, saponins, tannins resins, steroids, and volatile oils which were deposited in their specific parts such as fruits, flowers, leaves, roots, seeds and bark etc. Tonthubthimthong *et al*., (2001) [12]. The roots of *Calotropis gigantea* have been used in leprosy, eczema, syphilis, elephantiasis, ulceration, and cough in the Indian system of traditional medicine. It contains alkaloids, tannins, phenols and resins Evans and Saunders (2002) [3]. Dandekar *et al*., (2015) [2] also evaluated the alcohol extract of *Epiphyllum oxypetalum* contains secondary compounds like Megastigmatrienone Cyclocta- 1,3,6 –triene, 2, 3, 5,5, 8, 8,-hexamethyl; 4-((1E)- 3- Hydroxy-1 -progeny)-2-methoxyphenol; 2, 5-Dihydroxy- 4-isopropy l-2, 4, 6-cycloheptatrien-1-one, by GC-MS analysis. Yong-Chun Jin *et al*., (2011) reported that 63 components were separated and identified by GC/MS from the varieties of bamboo leaves. *cis*-3-Hexenol, whose content in cv. *Pubescens*, *Gracilis*, *Heterocycla* and *Ph. kwangsiensis* was 27.11%, 24.62%, 30.51% and 34.65%, respectively, which having Antioxidant and Antimicrobial Activities. Seniya *et al*. (2011) [9] studied that aqueous latex extract of *Calotropis gigantea* contains alkaloids, tannins, saponins and glycosides. But it don't showed antimicrobial activity. The present investigation was revealed to identify new plant based compounds against *Fusarium oxysporum* and *Colletotrichum capsici* using *C. gigantean* root extract. In this regard, Primary screening was carried out to find the Antimicrobial activity of the plant extract. Further, based on the primary phytochemical screening, high resolution liquid chromatography and mass spectrometry (HR-LCMS) was performed to separation and identification of the phytoconstituents based on their retention time and data base difference from the crude extracts showing good antimicrobial activity. HR-LCMS techniques allow accurate determination of chemical compounds with known and unknown structures, it also offers excellent sensitivity and attain high-quality data within minimum acquisition time. In particular, these analytical techniques greatly used identification and highly sensitive quantification of natural products at trace concentrations in complex matrices Sauvage *et al.,* (2006) [7].

2. Materials and Methods
2.1. Collection of plant Material
The fresh and Healthy Roots of *Calotropis gigantea* were collected from Belora district Jalna, during August 2018. The identification is done with the help of standard floras (flora of Marathwada by V.N. Nail *et al.,* 1998). The Roots were shade dried, powdered and stored in airtight container for further study.

2.2. Preparation of plant extract
About 30 gm of root powder were subjected to Soxhlet extraction with 300 ml of the methanol for 12 to 14 hrs (60-70 ^{0}c**)**, root powder were successively extracted. then the extracts were filtered through muslin cloth and then finally

through Whatman filters paper no 1. Solvent was evaporated at 40-50 ^{0}C by using Rotary evaporator. The collected powder was weight and dissolved in Dimethyl sulfoxide (DMSO) with 10% concentration. The extracts were preserved in sterile glass bottles at 4 ^{0}C temperature for further study Subramanian *et al*., (2016) [11].

3. Antifungal activity of plant extract

The food poisoned technique Schmitz, (1930) was used to test the antifungal activity of the extracts. The antifungal activity was observed on basis of mycelial growth that was compared with standard fungicides (Propacanozole). 5% Extract was added with Potato Dextrose Agar & poured in sterile Petri plates. Fungal disc of 4 mm of 7 days old culture were used for inoculated aseptically on Potato Dextrose Agar plates were incubated at 37 ^{0} ± 2 ^{0}c for 24 hours and the diameter of zone of inhibition of fungal growth was measured in mm.

4. Antibacterial Activity of Plant Extract

The assessment of antibacterial activity, agar disc diffusion method was used Islam *et al.,* (2014) [4]. Bacterial inoculums were prepared by inoculating a loop full of target bacterial colony (24 hours old culture) in 5 ml nutrient broth and incubated at 27 ± 2 ^{0}C for 10 hours till a moderate turbidity was developed. For comparison the antibiotic Streptomycin (1000 ppm) was used. All the plates were incubated at 37 ± 2 ^{0}C for 48 hours test was carried out in triplicate and results were recorded in terms of diameter of the zone of inhibition in mili-meters.

5. High Resolution Liquid Chromatography and Mass Spectrometry (HR-LCMS) analysis

The extract was prepared in methanol and then subjected to HR-LCMS analysis. The HR-LCMS of sample was carried out in Sophisticated Analytical Instrument Facility (SAIF), IIT Bombay, Powai, and Mumbai. Chemical finger prints of selected medicinal plant extracts were prepared by Agilent high resolution liquid chromatography and mass spectrometry model- G6550A with 0.01% mass resolution. The acquisition method was set to be MS- minimum range 50 *(M/Z)* and maximum 1000 Dalton *(M/Z)* with scanning rate each spectrum per second. Gas chromatography had maintained at 250 ^{0}C with gas flow 13 psi/minute. Chromatographic separations were performed on column18 (100 × 1.0 mm, particle size 1.8 µm; Waters); 100 µl/minute, ejection speed with flush out factor 5µl and 8µl injection volume.

5.1 Solvent was use for HR-LCMS

1. A- 100% Water
2. B- 100% Acetonitrile

6. Identification of components

Interpretation on mass spectrum HR-LCMS was conducted using the database of Sophisticated Analytical Instrument Facility (IIT Bombay) having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the SAIF library. The name, molecular weight and structure of the components of the test materials were ascertained.

Deoxy streptomycin, protorifamycine-I, Citrulline, 4-Trimethylammoniobutanal, cyst amine, 6-hydroxy-2-hexynoic acid, Carnitine, D-Arginine, Choline, Pantoic acid, Betaxol, Madecassic acid, Avocady none acetate, 6-deoxyerythronolide-B, O-Acetylserine, 4,6-Diamino-5-formidopyrimidine, Dihydrorobinetin, Barbituric acid, 5-ethyl-5-(2- hydroxyethyl), Idebenone Metabolite (Benzenebutanoic acid, 2,5- dihydroxy-3,4-dimethoxy-6-methyl-) and Hexadecanedioic acid these important compounds were isolated by *Calotropis gigantea* showed highly antimicrobial activity.

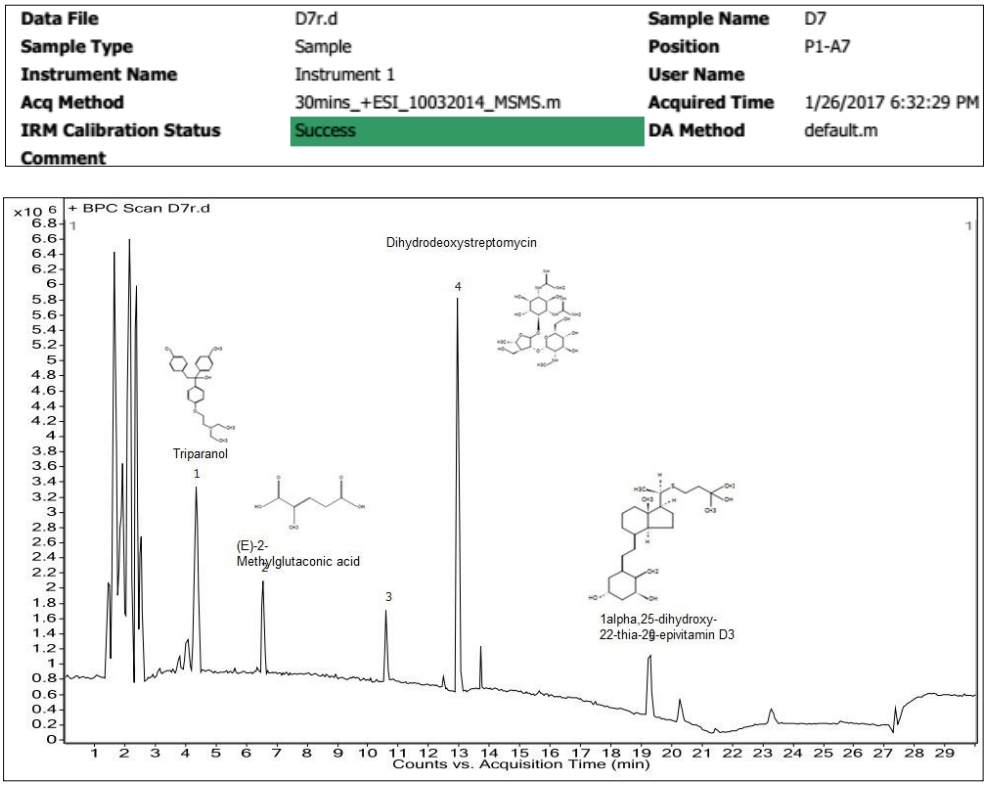


Fig 1: Chromatogram of methanolic root extract of *Calotropis gigantean*

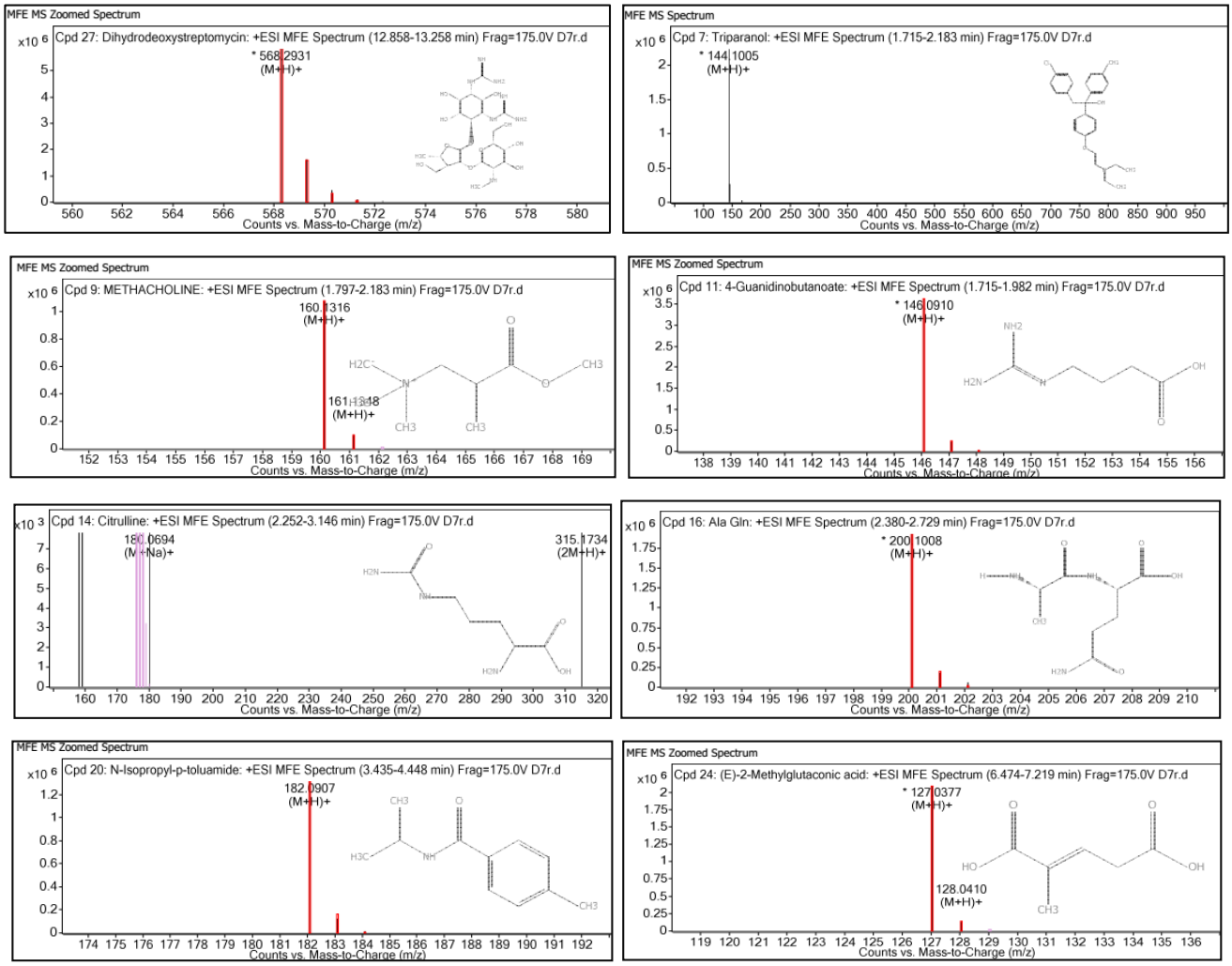


Fig 2: HR-LCMS MFE MS Zoomed Spectrum of purified compound isolated from methanol root extract of *Calotropis gigantea.*

Table 1: Bioactive compounds identified in methanol extract of *Calotropis gigantea* root by HR-LCMS.

Retention time (min)	Name of compound	Molecular weight	Molecular Formula	Db diff (ppm)
1.768	Betaine	118.0857	C5 H12 N O2	9.6
1.808	Triparanol	143.0934	C7 H13 N O2	8.55
1.883	Methacholine	160.1322	C8 H18 N O2	9.92
2.149	4- Guanidinobutanoate	145.0838	C5 H11 N3 O2	9.13
2.348	4- Trimethylammoniobutanal	130.1231	C7 H16 N O	0.82
2.731	Hydroxylysine	162.0994	C6 H14 N2 O3	6.42
4.354	Triparanol	143.0934	C7 H13 N O2	8.64
4.355	2-Amino-3-methyl-1-butano	103.0997	C5 H13 N O	0.1
4.356	2S-aminoheptanoic acid	145.1097	C7 H15 N O2	4.24
4.406	2-Amino-3-methyl-1- butanol	103.0995	C5 H13 N O	1.8
6.559	E)-2-Methylglutaconic acid	144.041	C6 H8 O4	8.8
7.033	Leucine	131.0936	C6 H13 N O2	7.73
9.449	Bergenin	328.0779	C14 H16 O9	4.69
11.303	Convallatoxin	550.2743	C29 H42 O10	6.39
11.848	Dihydrogambogic acid	630.3179	C38 H46 O8	2.13
12.494	C16 Sphinganine	273.2646	C16 H35 N O2	8.08
12.914	Dihydrodeoxystreptomycin	567.2851	C21H41N7 O11	2.22
12.962	Dihydrodeoxystreptomycin	567.2861	C21 H41 N7 O11	0.59
13.385	Proto-rifamycin I	639.3036	C35 H45 N O10	1.12
13.953	Hexadecanedioic acid	286.2119	C16 H30 O4	8.8
14.221	Deoxystreptomycin	565.2693	C21H39N7 O11	2.49
14.318	Clovanediol diacetate	322.2139	C19 H30 O4	1.44
14.898	Gedunin	482.2271	C28 H34 O7	6.97
14.952	Avocadynone acetate	324.2294	C19 H32 O4	1.97
15.976	Oxandrolone	306.2192	C19 H30 O3	1.01
19.547	2- hydro xyhexadecanoic acid	272.2348	C16 H32 O3	1.37
20.774	Avocadene	286.2489	C17 H34 O3	6.57
24.838	Coproporphyrin II	654.2631	C36 H38 N4 O8	9.03

Table 2: Antifungal activity of *Calotropis gigantean*

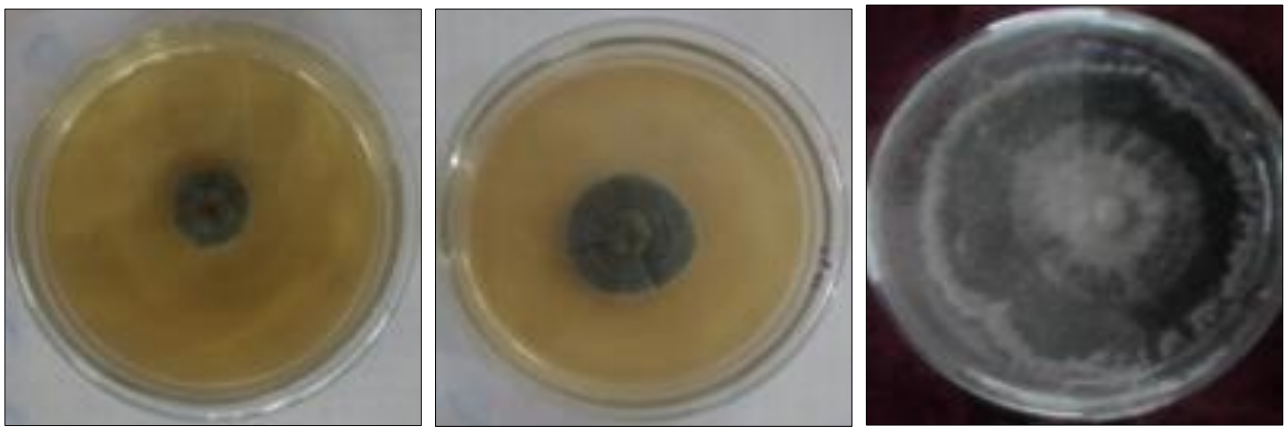
Fungal pathogen	7 days growth of mycelium against *C.gigantea* Extract(triplicate)			Mean	7days growth of mycelium against Fungicide (Propacanozole) (triplicate)			Mean	Control Mean of triplicate
Fusarium oxysporum	43	44	42	42	63	63	63	63	90
Colletotrichum capsici	20	19	21	20	31	30	32	31	84

Table 3: Antibacterial activity of *Calotropis gigantea*. Values expressed in mean ± S.D. of triplicate.

Bacteria	Zone of inhibition (mm) of Methanolic extract			Zone of inhibition (mm) of Antibiotic		
C. gigantea on *X. axonopodis*	16	16	16	12	13	13
			Mean- 16			Mean- 12.6

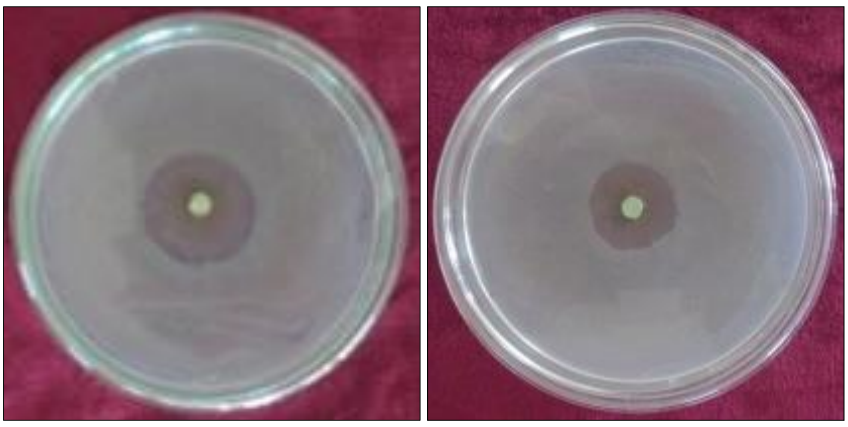
C. gigantean Propiconazole Control

Plate 1: Antifungal activity against Fusarium oxysporum



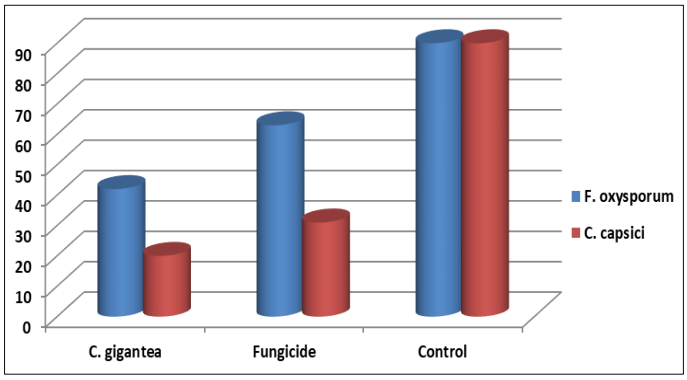
C. gigantean Propiconazole Control

Plate 2: Antifungal activities against *Colletotrichum capsici*



C. gigantean Streptomycin

Plate 3: Antibacterial activities against *Xanthomonus axonopodis*



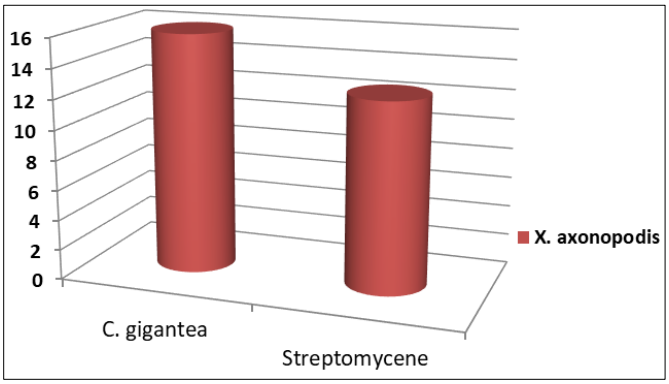


Chart 1 & 2: Antifungal and Antibacterial activity of *Calotropis gigantean*

7. Phytochemical screening

Methanolic Extract of *Annona squamosa* recorded the presence of tannins, alkaloids, saponins, glycosides, steroids And flavonoids through the qualitative analysis (Yadav and Agarwal, 2011) [13].

Table 4: Phytochemical analysis of Methanolic Extract of *Calotropis gigantean*

Phytochemical	*Calotropis gigantean*
Tannins	+++
Alkaloids	+++
Saponins	++
Glycosides	-
Flavonoids	++
Steroids	++

+++ Strongly present, ++ present, + weekly present, - absent

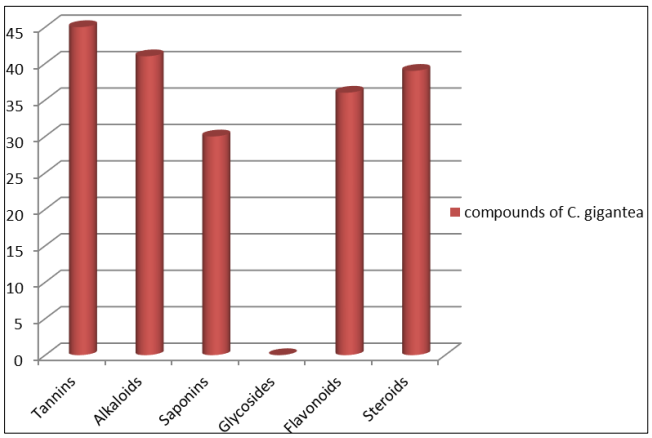


Chart 3: Phytochemical analysis of Methanolic Extract of *Calotropis gigantean*

8. Result and Discussion

HR-LCMS analysis of methanol extract of *Calotropis gigantean* roots showed respectively 1, 2, 3 and 4 major peaks indicating the presence of various phytochemical constituents. On comparison of the high resolution liquid chromatography and mass spectra of constituents with the main library all these compounds were characterised and probably identified. Identified compounds is Deoxy streptomycin, protorifamycine-I, Citrulline, 4-Trimethylammoniobutanal, cystamine, 6-hydroxy-2-hexynoic acid, Carnitine, D-Arginine, Choline, Pantoic acid, Betaxol, Madecassic acid, Avocady none acetate, 6-deoxyerythronolide-B, O-Acetylserine, 4,6-Diamino-5-formidopyrimidine, Dihydro robin tin, Barbituric acid, 5-ethyl-5-(2- hydroxyethyl), Idebenone Metabolite (Benzenebutanoic acid, 2,5- dihydroxy-3,4-dimethoxy-6-methyl-) Hexadecanedioic acid and Dihydro deoxy-streptomycin. Tannins, Alkaloids, Saponins, Flavonoids and Steroids were also reveals presence in root extract of *Calotropis gigantea* by simple phytochemical method.

Antifungal activity was carried out by food poison techniques, according the recorded antifungal and antibacterial assay (Table 2 & 3), the plant extract showed maximum inhibitory effect against both selected plant pathogenic fungi as well as selected Bacteria in comparison with fungicide (Propacanozole) and antibiotic (Streptomycin). But it Showed excellent result against *Colletotrichum capsici* even a very good result showed against *Fusarium oxysporum* than fungicide Through the study the results clearly reveals that the plant extract play the important role in controlling the plant diseases. Ramdas *et al*., (2006) [6] revealed that the phytochemical plays an important role in the treatment of diseases without any side effects, there is a need to search new drugs from natural sources. India is a home to a variety of traditional medicine system that relay to a very large extent on native plant species for new drug materials. Therefore now there is a need to look back towards traditional medicine which can serve a novel therapeutic agent Chitravadivu *et al*., (2009) [1]. The pharma cognostical evaluations also give valuable information which is essential to standardize the drug.

9. Conclusion

The result of HR-LCMS analysis specifies that the methanol extract of *Calotropis gigantea* roots contains various valuable secondary compounds which have a variety of medicinal properties that can be useful for the cure of various diseases. The study reveals the crucial roles of phytochemical which are released in the form of secondary metabolites in controlling the fungal and bacterial plant diseases without affecting the environment.

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