PHYTOCHEMICALS: EXTRACTION, ISOLATION AND IDENTIFICATION OF BIOACTIVE COMPOUNDS FROM CALOTROPIS GIGANTEA R.BR. (LINN) –ERUKKU

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Abstract : Natural products from medicinal plants, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity. Due to an increasing demand for chemical diversity in screening programs, seeking therapeutic drugs from natural products, interest particularly in edible, has grown throughout the world. Botanicals and herbal preparations for medicinal usage contain various types of bioactive compounds. Fractional analysis of these bioactive compounds have yielded several innovative compounds of pharmaceutical and biomedical importance. This study is focused on step-by-step visual demonstration of fractionation and isolation of biologically active plant secondary metabolites using column-chromatographic techniques. Isolation of bioactive compounds using column-chromatography involves: a) Preparation of sample; b) Packing of column; c) Pouring of sample into the column; d) Elution of fractions; and e) Analysis of each fractions using thin layer chromatography. The analysis of bioactive compounds present in the plant extracts involving the applications of common phytochemical screening assays, chromatographic techniques such as TLC as well as non-chromatographic techniques such as Immunoassay, Nuclear Magnetic Resonance (NMR) spectral analyses and Fourier Transform Infra Red (FTIR) analyses are discussed.

Key words **- Bioactive compound, Plant Extraction, Isolation, *Calotropis gigantea* Stem Methanolic Extract (CGSME).**

1. INTRODUCTION

Natural products, such as plants extract, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug discoveries because of the unmatched availability of chemical diversity (Cos et al., 2006). According to the World Health Organization (WHO), more than 80% of the world's population relies on traditional medicine for their primary healthcare needs. The use of herbal medicines in Asia represents a long history of human interactions with the environment. Plants used for traditional medicine contain a wide range of substances that can be used to treat chronic as well as infectious diseases (Duraipandiyan et al., 2006).

Isolation of pure, pharmacologically active constituents from plants remains a long and tedious process. It is necessary to have methods available for efficient separation from plant extracts, which are usually mixtures of thousands of different molecules (Peter, 2004). There are several ways to identify these molecules from these extracts. The common method is to set up a fractionation scheme and to screen the fractions for the presence of the desired bioactive properties. Active fractions are sub fractionated and tested, until the molecules responsible for the bioactive can be identified. Thin-layer chromatography (TLC) and column chromatography are the simplest and cheapest methods of detecting plant constituents because the method is easy to run, reproducible and requires little equipment (Jothy *et al*., 2011;Devi *et al*., 2010; Battu *et al*., 2009; Prachayasittikul *et al.*, 2009).

The premier steps to utilize the biologically active compound from plant resources are extraction, pharmacological screening, isolation and characterization of bioactive compound, toxicological evaluation and clinical evaluation. This has opened new doors in pharmacology, as pure, isolated chemicals, instead of extracts, are the standard for the treatment of diseases. At present, there are innumerable number of such bioactive compounds isolated form crude extracts and their chemical structure were elucidated (Bajpai *et al.*, 2001). Moreover, plants have always been a source of a wide array of secondary metabolites with potential pharmacological properties (Russell and Duthie, 2011). Polyphenolic (flavonoids) compounds that occur ubiquitously in

foods of plant origin, have many beneficial health effects due to their potential antioxidant, anti-inflammatory and cancer-preventive activities (Li *et al*., 2014). Therefore, the objective of this study was to provide step-by-step visual demonstration of fractionation and isolation of biologically active plant secondary metabolites using column-chromatographic techniques. The plant *Calotropis gigantea* belonging to *Asclepiadaceae* is a lactiferous shrub commonly foundin tropical and sub-tropical regions around the world (Sastry and Kavathekar, 1990). *Calotropis gigentica* which is normally identified as weed plant is a wasteland plant. It is natural habitat of Asian countries and is used by tribal people for many diseases such as a toothache, sprain, ear ache, anxiety, pain, diarrhea, and mental disorders. Its various actions such as cytotoxicity, wound healing, antipyretic, and anticandidal activity have been documented (Wang *et al.,* 2008; Sartha *et al.,* 2009; Chitme *et al.,* 2005; Kumar *et al.,* 2010). Latex from the plant has many medicinal uses, it is used to treat boils, scabies, bruises, burns, cuts, sores, boils, wounds, and to stop bleeding. It contains triterpenes, cysteine proteinase, and galactin and is used for cold and heart condition also (Lachman-White *et al.,* 1987; Duke, 1985).

This paper provides details in extraction, isolation and characterization of bioactive compound from plants extract with common phytochemical screening assay, chromatographic techniques, such as UV, FTIR, NMR and Fourier Transform Mass Spectrometry (FTMS) (Siddiqui *et al*., 2011; Dai *et al.,* 2010).

MATERIALS AND METHODS

Plant extraction

Prepared and submitted to Botanical Survey of India, TNAU Campus, Coimbatore for taxonomical identification (BSI/SRC/5/23/2016/Tech./772) and identification voucher received for the same.

Calotropis gigantea stem powders were subjected to Soxhlet extraction. Methanol was used as solvent for phytochemical extraction. The extraction was performed overnight to obtain the extract. Further the extract was concentrated using a rotary evaporator and were stored at 4°C for further use.

Determination and isolation of the biologically active compounds Preparative Thin Layer Chromatography (TLCP)

For Preparative thin layer chromatography (TLCP) separations, glass plates coated (1mm) with silica gel GF254 Merck, Darmstadt, Germany were employed.

Preparation of Thin Layer Chromatography Plates (PTLCP)

25g of Merck silica gel was suspended in 50mL of de-ionized water and shaken vigorously for 45 seconds in rubber-stopper Erlenmeyer flask (500mL). The thickened slurry was poured into the glass and pulled with a ruler in two sides at 1mm trailing edge to prepare similar plates. Then, the plates were allowed to air dry 30 minutes in the oven at 50^0 C (until they turn white). After preparation of the plates, a few drops of CGSME were applied (using a capillary tube) to the bottom of each of the pre-coated and pre-heated (50°C for 30 minutes) glass plates. After 5 minutes of drying, each of the plates was placed in the separate glass chamber with solvent system Hexane: acetone (9:1 v/v) as the mobile phase as shown in figure 2. The different solvent systems were tried as mobile phase, of which Hexane: acetone (9:1) gave clearly visible spots hence tried with that mobile phase. After the movement of solvent at the top of the plates, each plate was removed from the glass chamber and separately air-dried. After air-drying, the produced spots were located by their fluorescence under long and short wave UV light (254 and 366 n respectively).

Column chromatography (CC)

The extract residue (10 g) was applied onto a silica gel column (200 g, 100cm x 3.5 cm), to isolate the active compound from the CGSME using hexane as a solvent and the polarity was increased by hexane then chloroform and fractions (100 mL)

each were collected. The obtained fractions were concentrated and monitored by TLC using hexane, acetone and methanol (97:2:1%) as mobile phase.

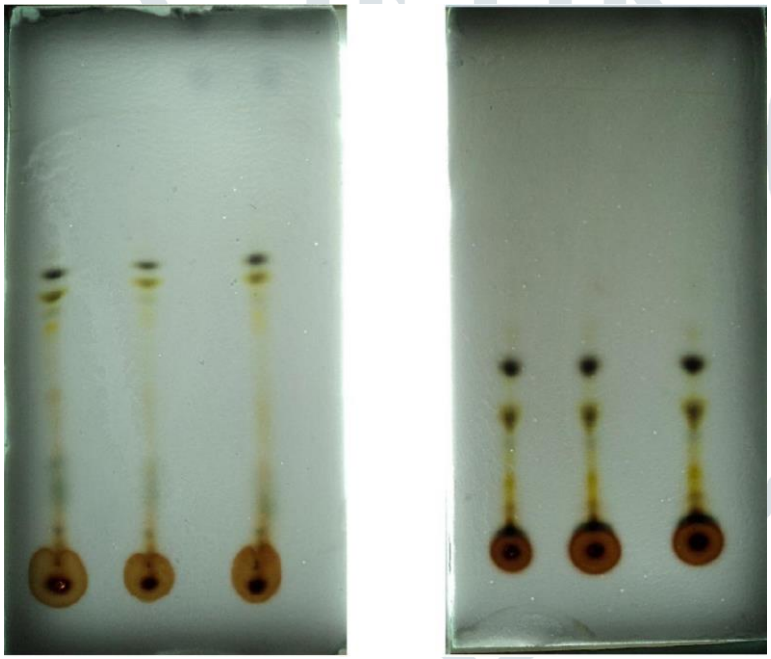
Spectral analysis

The spectral studies like UV, FT-IR, GC-MS, ^{1}H - NMR and ^{13}C – NMR analysis were carried out as per standard procedures. The spectral studies were carried out in SRM University, Chennai.

RESULT AND DISCUSSION

The qualitative phytochemical analysis CGSME showed the presence of biologically active phyto ingredients such as phenols, flavonoids, glycosides, sterols, saponins, tannins, and alkaloids. The medicinal value of plant lies in its bioactive phytochemical constituents that produce definite physiological actions on the human body and these phytochemicals are produced as secondary metabolites to defend the plant from the environment. Two compounds (n- Hexadecanoic acid and Phenol 2, 4 bis (1, 1- dimethyl ethyl)) were isolated from the methanolic extract of stem of *Calotropis gigantea*. These two compounds were identified through UV, FT-IR, GC-MS, ^{1}H - NMR and ^{13}C – NMR analysis.

Figure: Preparative Thin Layer Chromatography (PTLC) for CGSME using Hexane Solvent system



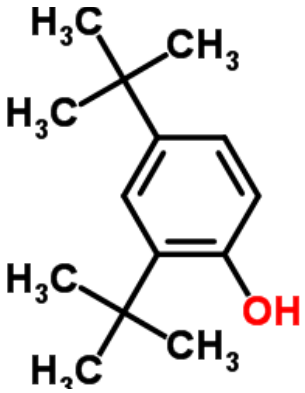
Hexane: Acetone (9:1 V/V) **Hexane: acetone (7:3)**

Phenol 2, 4 bis (1, 1- dimethyl ethyl)

The physical properties of Phenol 2, 4 bis (1, 1- dimethyl ethyl) - were Molar Refractivity (64.90 ± 0.3 cm^3), Molar Volume (221.2 ± 3.0 cm^3), Parachor (518.3 ± 4.0 cm^3), Index of Refraction (1.498 ± 0.02), Surface Tension (30.1 ± 3.0 dyne/cm), Density (0.932 ± 0.06 g/cm^3), Polarizability (25.73 ± 0.5 $10^{-24}cm^3$).

The Lipinski-type propertiesof Phenol 2, 4 bis (1, 1- dimethyl ethyl) are Molecular Weight (206.32), No. of Hydrogen Bond Donors (1), No. of Hydrogen Bond Acceptors (1), TPSA (20.23), No. of Rotatable Bonds (2).

Structure



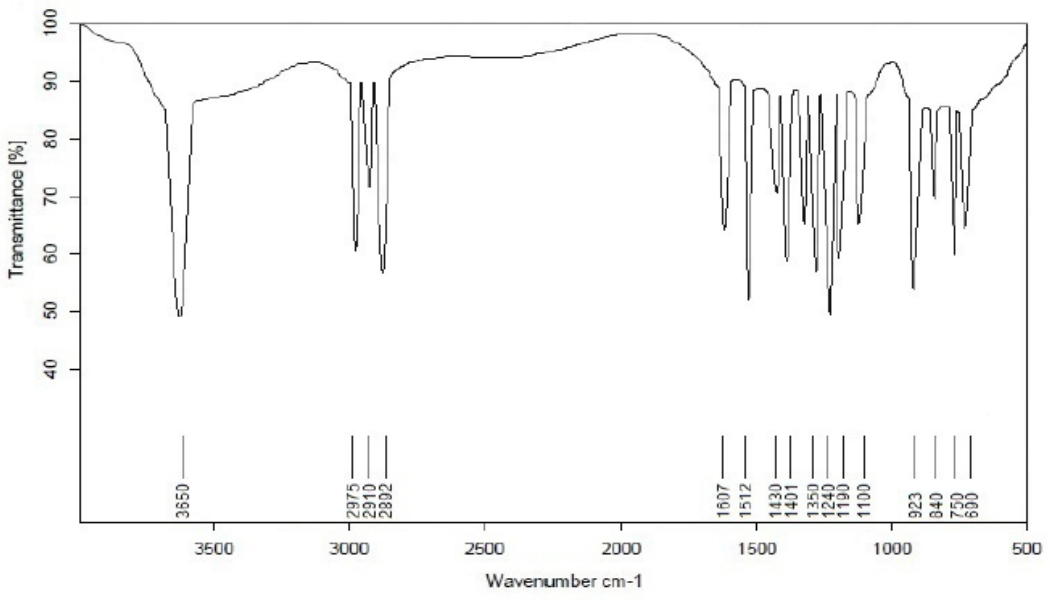
Molecular formula - $C_{14}H_{22}O$

Molecular weight - 206.329 g/mol

FTIR analysis

The spectrum as shown in fig lot of absorption bands indicates the presence of active functional groups in the compound. The FTIR spectrum of compound using the compound Phenol 2, 4 bis (1, 1- dimethyl ethyl) indicates a strong band at 3650 cm^{-1} indicates Intermolecular hydrogen bonded OH (Strong), 2975 cm^{-1} Vinyl terminal (medium), 2910 cm^{-1} Cycloalkanes (Medium), 2892 cm^{-1} Methoxy (Medium), 1607 cm^{-1}, 1512 cm^{-1} Primary amines (Medium to strong), 1430 cm^{-1} Acetylene (Medium), 1401 cm^{-1}, 1350 cm^{-1} Aliphatic aldehydes (Very strong), 1240 cm^{-1} Tertiary butyl (strong), 1990 cm^{-1} Aromatic methane (weak), 1100 cm^{-1} Aromatic esters (Very strong), 923 cm^{-1} Cycloalkanes (medium), 840 cm^{-1}, 750 cm^{-1} and 690 cm^{-1} - Aromatic methane (Strong).

Figure: FTIR Analysis of Phenol 2, 4 bis (1, 1- dimethyl ethyl)

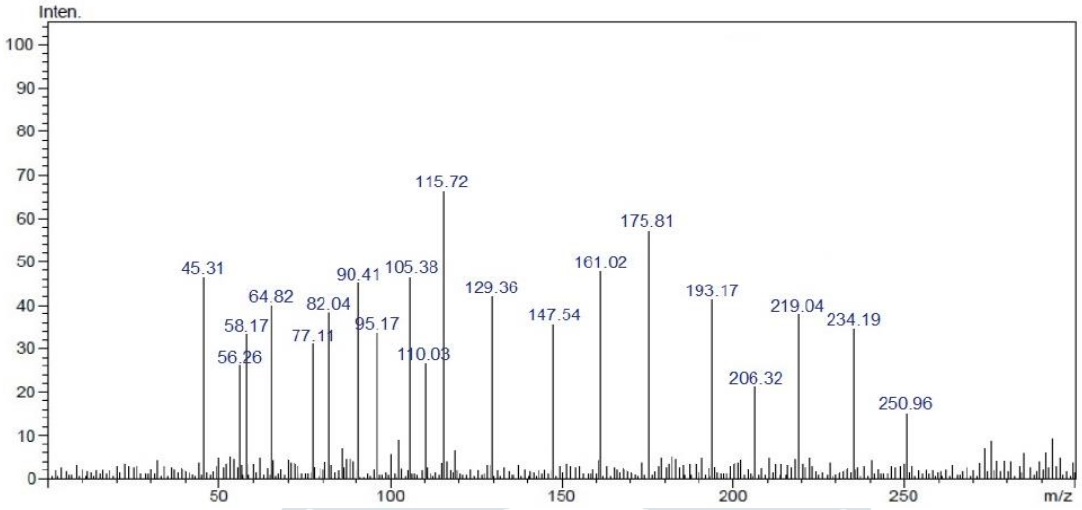


Mass analysis

EI-Ms m/z: The molecular ion peak is (M+) 206.32. The other fragments are 45.31, 56.26, 58.17, 64.82, 77.11, 22.04, 90.14, 95.17, 105.38, 110.30, 115.72, 129.36, 147.54, 161.02, 175.81, 198.17, 219.04, 234.19 and 250.96.

NMR analysis

Figure: NMRAnalysis of Phenol 2, 4 bis (1, 1- dimethyl ethyl)



The 1H and 13C NMR experiments of DI-I were carried out results obtained mentioned in figure. The 1H NMR spectrum showed Secondary (R_2CH_2) at δ 1.22, 1.36, Vinylic (C = C- H) at δ 4.91, Aromatic (Ar-H) at δ 6.65 and Phenolic (ArOH) at δ 7.16, 7.31.

The appearance of carbonyl group at δ 29.51 CH_3CO-, δ 31.45, 34.30, 34.55 RCH_2NH_2, δ 115.07 C = C (in alkenes), δ 124.62, 125.33 C in aromatic rings, δ 134.20, 146.85 C = C (in alkenes), and δ 152.60 C = O (in acids & Esters)

Figure: 1H NMRAnalysis of Phenol 2, 4 bis (1, 1- dimethyl ethyl)

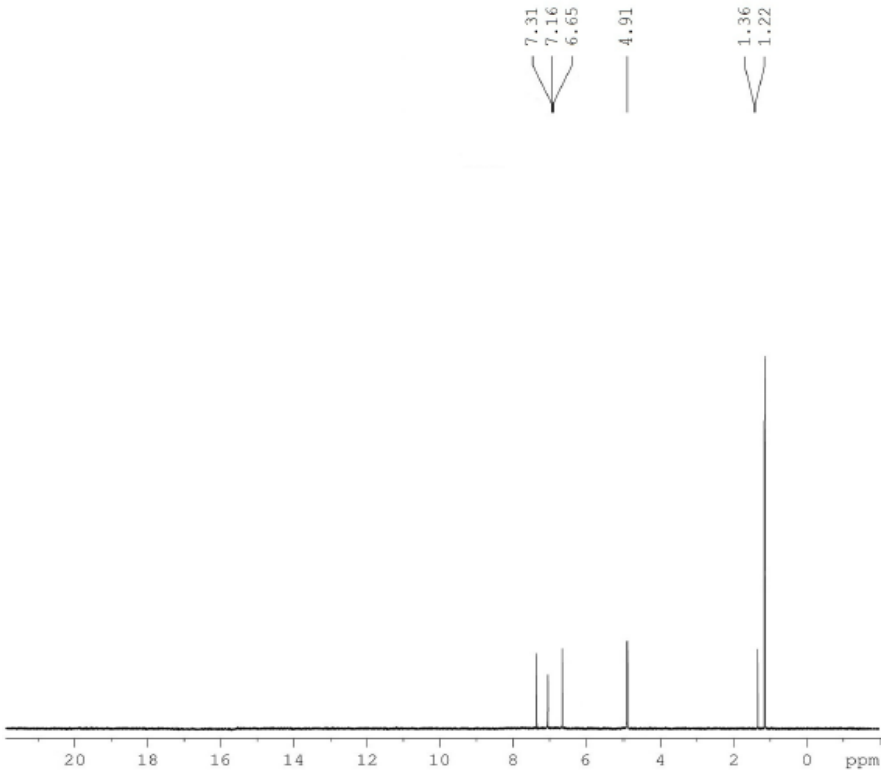
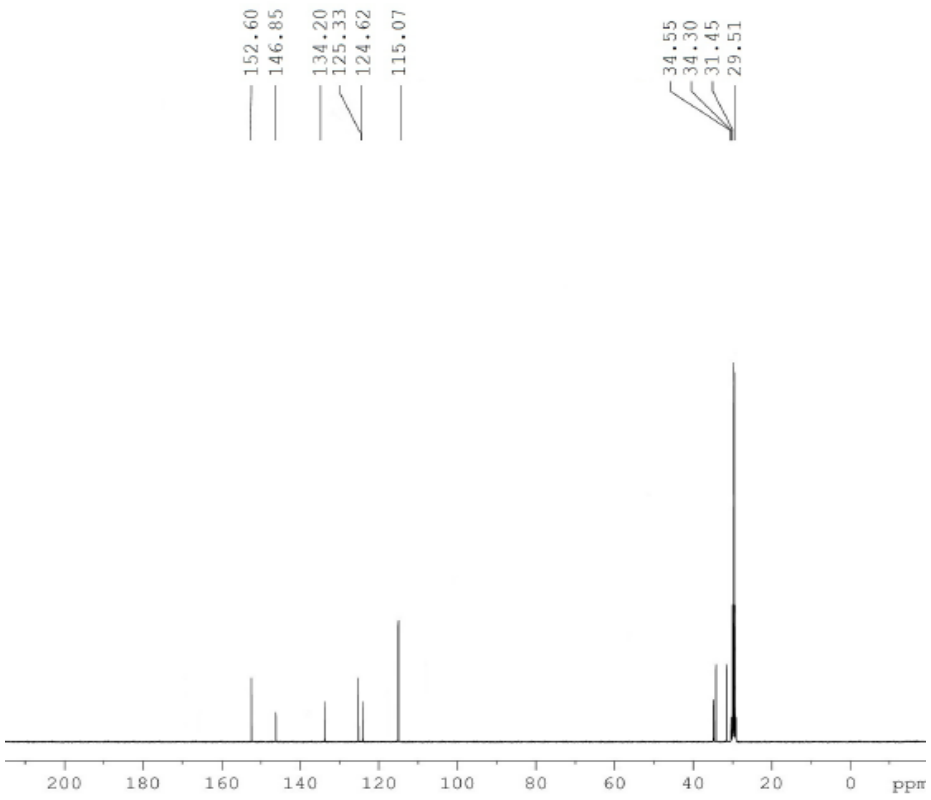


Figure: 1H NMRAnalysis of Phenol 2, 4 bis (1, 1- dimethyl ethyl)

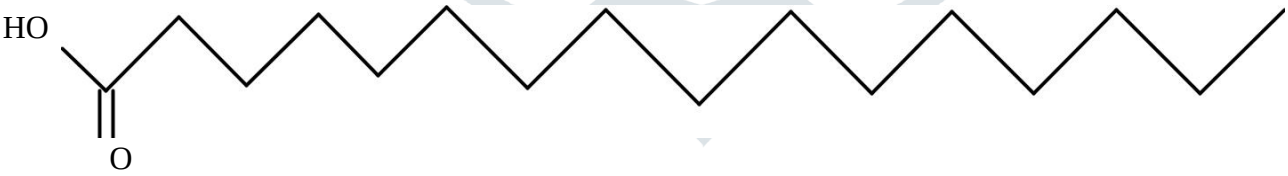


n- Hexadecanoic acid

The physical properties of n- Hexadecanoic acid are Molar Refractivity (77.73 ± 0.3 cm^3), Molar Volume (287.2 ± 3.0 cm^3), Parachor (690.5 ± 4.0 cm^3), Index of Refraction (1.453 ± 0.02), Surface Tension (33.3 ± 3.0 dyne/cm), Density (0.892 ± 0.06 g/cm^3) and polarizability (30.81 ± 0.5 $10^{-24}cm^3$)

The Lipinski-type properties of n- Hexadecanoic acid are Molecular Weight (256.42), No. of Hydrogen Bond Donors (1), No. of Hydrogen Bond Acceptors (2), TPSA (37.3), No. of Rotatable Bonds (14).

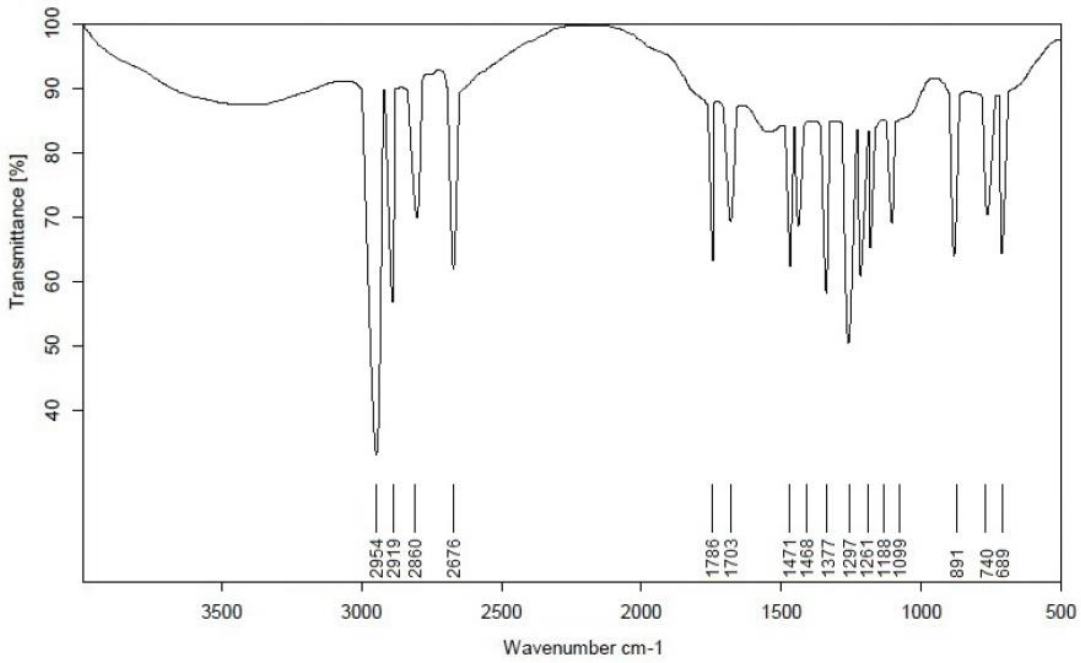
Structure



FTIR analysis

The spectrum as shown in fig lot of absorption bands indicates the presence of active functional groups in the compound. The FTIR spectrum of compound using the compound Hexadecanoic acid indicates 2954 cm^{-1} Acids (Medium), 2919 cm^{-1}, 2860 cm^{-1} Alkanes (Strong), 2676 cm^{-1}, 1786 cm^{-1}, 1703 cm^{-1}- Aromatic Methane (Weak), 1471 cm^{-1}Vinyl terminal (Medium), 1468 cm^{-1}, 1377 cm^{-1}- Propyl (Strong), 1297 cm^{-1} - Aromatic Esters (Very Strong), 1261 cm^{-1}, 1188 cm^{-1}, 1099 cm^{-1}- Aromatic Ketones (Strong), 891 cm^{-1}- Cycloalkanes (Medium), 740 cm^{-1} - Alkanes (Strong) and 689 cm^{-1}- Meta Disubstituted (Very strong).

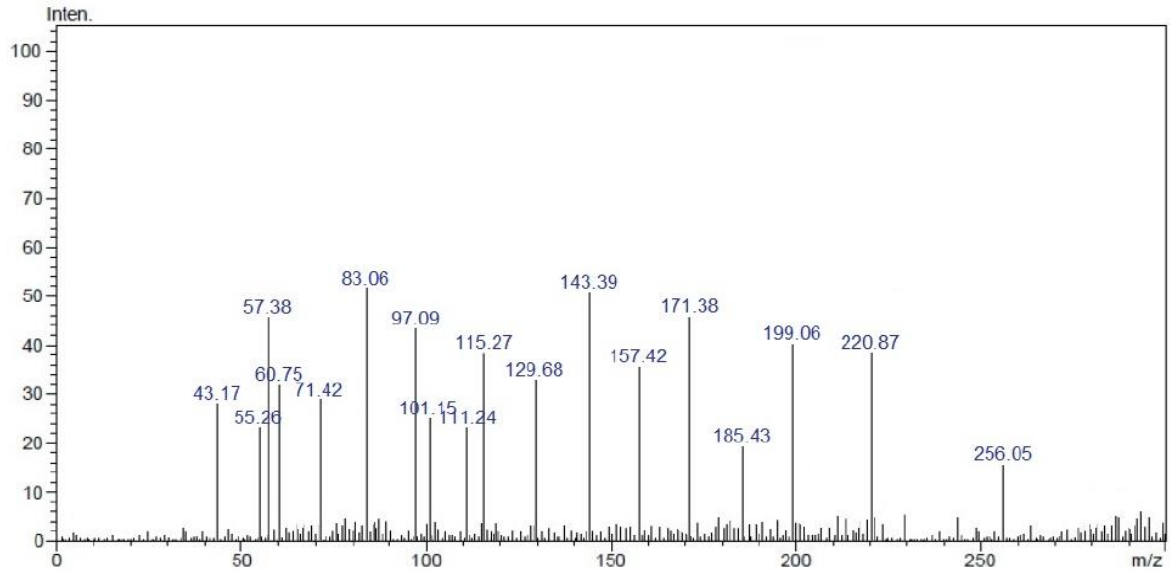
Figure: FTIR Analysis of n- Hexadecanoic acid



Mass analysis

EI-Ms m/z: The molecular ion peak is (M+) 256.42 The other fragments are 43.17, 55.26, 57.38, 60.75, 71.42, 83.03, 97.09, 101.15, 111.24, 115.27, 129.68, 143.39, 157.42, 171.38, 185.43, 199.06 and 220.87.

Figure: Mass Analysis of n- Hexadecanoic acid



NMR analysis

The 1H and 13C NMR experiments of DI-I were carried out results obtained mentioned in figure. The 1H NMR spectrum showed Primary (RCH3) at δ 0.88, Cyclopropane at δ 1.27, 1.28, 1.29, Secondary (R2CH2) at δ 1.32, 1.37, Amino (R-NH2) δ 1.40, Hydroxylic (R- OH) δ 1.64, Esters (CH-COOR), δ 2.35, δ 10.57 Aldehydic (CH-COOR).

The appearance of carbonyl group at δ 14.13 - RCH3, δ 22.77, 24.76, 29.17, 29.34, 29.52, 29.57, 29.70, 29.77, 29.86 - R3CH, δ 34.22 – RCH2 NH2 and δ 179.09 – C= O (in acids & esters).

Figure: 1H NMR Analysis of n- Hexadecanoic acid

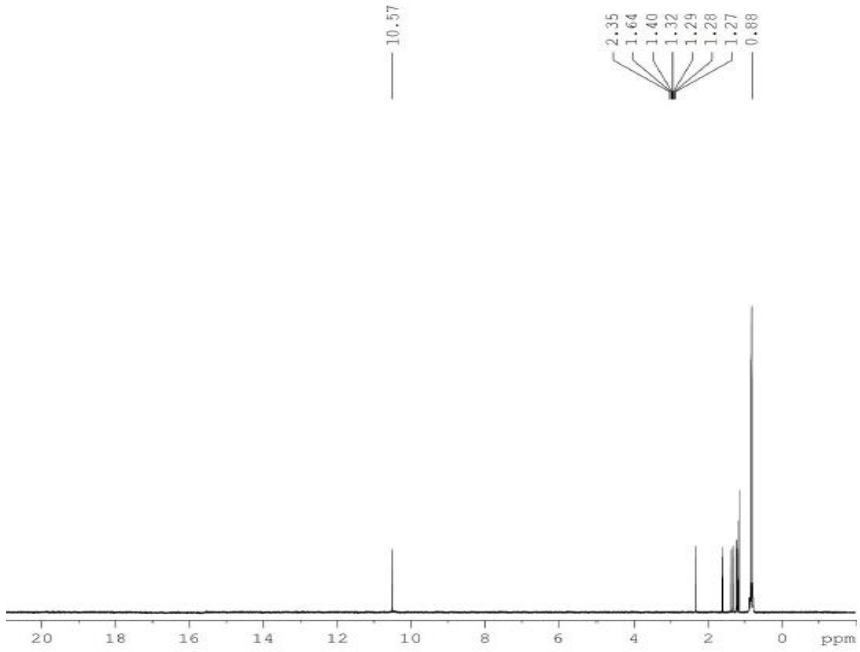
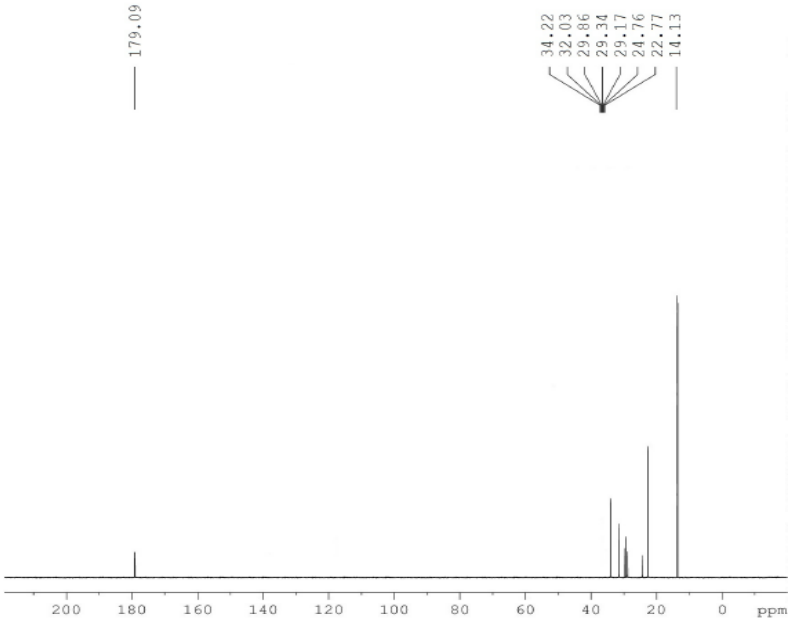


Figure: C13 NMR Analysis of n- Hexadecanoic acid



CONCLUSION

In this present study methods have been developed for separation of phytoconstituents using *Calotropis gigantea S*tem Methanolic Extract (CGSME). Thin layer chromatographic method showed remarkable separation of phytoconstituents which can be used for method development. Two compounds (n- Hexadecanoic acid and Phenol 2, 4 bis (1, 1- dimethyl ethyl)) were isolated from CGSME which has been characterized using IR, NMR and Mass spectroscopy. Column chromatography yielded good amount of Phenol 2, 4 bis (1, 1- dimethyl ethyl) and proved to simple and reproducible method for isolation. Since bioactive compounds occurring in plant materials consist of multi-component mixtures, their separation and determination still creates problems. Practically most of them have to be purified by the combination of several chromatographic techniques and various other purification methods to isolate bioactive compound(s).

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