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SPECTROSCOPIC SIGNATURE, ANTIBACTERIAL AND ANTICANCER PROPERTIES OF *CALOTROPIS GIGANTEA* (LINN.) FLOWER

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Calotropis gigantea, Flower extract, UV-Vis, FTIR, Antibacterial, MTT assay

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ABSTRACT: *Calotropis gigantea* L. flowers were widely used as a natural herbal medication. The objective of the present study was to screen the phytoconstituents and to evaluate the antibacterial and anti-cancer properties of *Calotropis gigantea* flower extract. The UV-Vis and FTIR spectroscopic techniques were employed to screen the phytochemicals. The antibacterial property of the extract was evaluated through serial broth dilution technique. Cell viability was performed using MTT assay on HeLa cell lines. The presence of Phenolic compounds was confirmed by UV-Vis peak at 271nm. IR spectrum confirms the presence of alcohol/phenols, primary amines, aldehydes, alkanes, alkenes, sulfoxide and halogen compounds. The extract showed antibacterial activity against *Enterococcus faecalis* (75µg/ml) and *Klebsiella pneumonia* (2µg/ml). The aqueous ethanolic extract of *Calotropis gigantea* flower exhibited less activity on HeLa cancer cell viability even at higher concentrations (1000µg/ml). The application of spectroscopic techniques promises to be of a great value in screening phytoconstituents of the extract because of their simplicity and cost-effectiveness. The extract is capable of inhibiting the bacterial growth and may possibly be functional for fortifying of potential antimicrobial compounds. The study provides preliminary scientific evidence for further research in purifying the compounds from aqueous ethanolic extract and to screen their efficacy.

INTRODUCTION: For decades, the herbal system of medication has been practiced in rural countries as a primary health maintenance system [1, 2]. WHO reported, 80% of the rural countries depending on herbal medicine. Natural herbal medicine credited as well being in contrast to the synthetics that are regarded as unreliable to human and environment [3]. Thus, over a decade an herbal 'Renaissance' happening in the development of potential therapeutic applications. Research on medicinal plants has drawn the attention of many scientists for either its contents or efficacies.

Calotropis gigantea (Family: Asclepiadeace) is a common folk medicinal plant commonly known as "giant milk weed". The plant has been widely studied, for the presence of potential bioactive components like cardenolides [4, 5, 6] and steroids [14, 15]. The plant exhibited multiple therapeutic properties such as anti-inflammatory, anticonvulsant, anxiolytic, sedative, anti-diarrheal, hepatoprotective, trauma, larvicidal, antipyretic, anti-diabetic and anti-tumor activity [16, 17, 18, 19, 20, 21, 22, 23, 24]. In addition to this, the flowers are extensively used by ancient medical system for the treatment of diabetes mellitus, bronchial asthma,

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rheumatoid arthritis, and nervous disorders [16, 25]. Furthermore, *in-vivo* studies have demonstrated that, the flower exhibited analgesic, anti-diabetic and anti-tumor activity [26, 27, 28, 29].

The detection and identification of phytochemicals by spectroscopic techniques promises to be of a great value because of their simplicity and cost-effectiveness. In addition, spectroscopic techniques furnish a wealth of qualitative and quantitative information about a given sample [30]. The UV-Visible spectroscopy is an elementary technique, to key out the main phytochemicals [31, 32]. Fourier transforms infrared spectroscopy (FTIR)) is known for its unique "fingerprint" of any compound [29, 30, 31]. FTIR spectroscopy was used with success for the identification of various functional groups in the extract [31, 32, 33]. Phytochemicals are secondary metabolites produced by plants but these metabolites are not essential for plant growth at the same time they possess certain protective values and also medicinal properties [34].

To detect the phytochemicals in the plant, employment of advanced spectroscopic techniques was necessary. However, the study on the phytochemicals of *Calotropis gigantea* flower and their efficacy was rare in literature. Therefore, the main objective was aimed to screen the phytochemicals using spectroscopic techniques and to evaluate the antibacterial, anticancer properties of *Calotropis gigantea* flower extract.

MATERIALS AND METHODS:

Plant material:

The fresh blossom flowers with no apparent physical, insect or microbial damages were collected (April, 2014) from Botanical Garden, Karnatak University, Dharwad. Karnataka, India. The flower petals were carefully taken out (without anther, stamen or sepals) and were kept in laboratory hot air oven (Tempo instruments Pvt. Ltd.) at 40^{0}C till dry. Samples were powdered and covered with aluminium foil (to avoid exposures to light) and stored at 4^{0}C until further analysis.

Reagents:

Ethanol (AG-99.7%) was purchased from Sisco Research Laboratory (SRL) and Potassium bromide (IR-Grade) from Himedia. Muller Hinton (MH) broth medium was used to determine the minimal inhibitory concentration (MIC) for each test microorganism (NCCLS, 2000). Dulbecco's Modified Eagles Medium (DMEM), Fetal Bovine Serum (FBS), Penicillin and Streptomycin, Dimethyl sulfoxide (DMSO) and the MTT reagent were purchased from Himedia Laboratories Pvt. Ltd. Mumbai. The HeLa cancer cells were procured from NCCS, Pune.

Preparation of plant extract:

A known weight of the powdered sample (2g) was set in a thimble of the Soxhlet extractor (Borosil). Distilled water and ethanol (99.7%) was employed as solvents for extraction. Round bottom flask containing 70% aqueous-ethanol (100ml) solvent was placed on heating mantel (60^{0}C). The operation was carried on for 16 hours. Extract was filtered through Whatman No. 1 filter paper and kept on a water bath (60^{0}C) to vaporize the solvent. The obtained aqueous ethanolic crude extract of *Calotropis gigantea* flower (AECF) was changed to a screw-top glass bottles (with Teflon caps) and covered with aluminium foil to avoid exposure to light. The extract was placed in the refrigerator (4^{0}C) until use.

Spectroscopic signature:

UV-Visible spectroscopy (UV-Vis): The AECF was centrifuged at 3000 rpm (Remi R-8C) for 10 min and filtered through Whatman No. 1filter paper. The sample was diluted (1:10) with distilled water. An aliquot of diluted AECF was scanned in the wavelength ranging from 200-800 nm (Hitachi, U-3310) and the characteristic peak was detected.

Fourier transforms infrared spectroscopy (FTIR): FTIR analysis was performed by following the method described by Yin et al., 2013[33]. Briefly, KBr pellet prepared using 1.0 mg of flower extract. The pellet was analysed in the absorption mode of FTIR and spectra was recorded from 4000 to 500 cm^{-1} at a data acquisition rate of 4 cm^{-1} using an FTIR Nicolet 6700 FT-IR (Thermo scientific) equipped with a deuterated triglycine sulphate (DTGS) as a detector. The spectral interpretation was analysed in accordance with Barbara(2004),Yin et al., (2013) [33, 35].

Antibacterial activity:

Selection of bacterial strains:

The test bacteria used were two gram–positive (*Staphylococcus aureus* and *Enterococcus faecalis*) and two gram–negative bacteria (*Escherichia coli* and *Klebsiella pneumoniae*).The test bacteria are known to cause severe infections in human, as they are found in multiple environmental habitats [36].

Minimum Inhibitory Concentration (MIC):

The Minimum Inhibitory Concentration (MIC) of the AECF was determined by using serial broth dilution technique [37]. 1 mg/ml of the sample solution of the extract was prepared using sterile distilled water. Each of the test tubes were filled with 1 ml of sterile MH broth media and graded doses of sample solution was added. The test tubes were the inoculated (1×10^6cells/ml) with the selected organisms (*Streptococcus aureus, Enterococcus faecalis, Escherichia coli* and *Klebsiella pneumoniae*) followed by incubation at 37^oC for 24 h to allow the growth of the bacteria. The test tubes which showed lowest concentration as well as clear content were selected. This lowest or minimum concentration was considered as MIC. Test tubes containing medium, medium and sample, medium and inoculum were used as control. Bacterial growth was observed only in test tubes (solution was turbid) containing medium and inoculum and the other were clear showing no growth. Streptomycin was used as standard.

Anticancer activity:

MTT assay: The cytotoxicity of AECF on HeLa cancer cells was determined by the MTT assay described elsewhere [38, 39]. Cells (1×10^5/well) were plated in 100 µl of DMEM /well in 96-well plates. After incubation overnight, AECF was added in various concentrations (200, 400, 600, 800, 1000 µg/ml). After treatment with AECF for 24 h, 20 µl of 5 mg/ml MTT solution (pH 4.7) was added per well and incubated for another 4 h, the supernatant was removed, 100 µl DMSO was added per well and shaken for 15 min. The absorbance at 570 nm was measured with a micro plate reader (Bio-Rad, Richmond, CA), using wells without cells as blanks. The experiment was performed in triplicate. The effect of AECF on the viability of HeLa cancer cells was expressed as the % cell viability, using the following formula: % cell viability = A570 of treated cells / A570 of control cells × 100%.

RESULTS AND DISCUSSION:

Spectroscopic signature:

The UV-Vis spectrum of AECF was taken at 200 to 800 nm wavelength. The spectra showed the elevation at 271 nm with the absorption height of 0.769 ABS (**Fig. 1**). Many organic molecules absorb ultraviolet/visible radiation and this is normally because of the behaviour of a special compound. The characteristic wavelength for phenolic compounds may lay in the range 260-280 nm and the obtained peak may indicate the presence of phenolic compounds. The obtained spectrum was evident in identifying the specific bioactive class of molecule [31]. IR spectroscopy is the best approach to screen the different functional groups of the extract. The wavelength of light absorbed is attributed to the chemical bonding pattern as can be witnessed in the obtained spectrum (**Fig. 2**). IR spectrum confirmed the presence of alcohol/phenols, primary amine, aldehydes, alkanes, alkenes, sulfoxide and halogen compounds (**Table 1**).

The results are analysed in accordance with Barbara, 2004,Yin et al., 2013[33, 35]. The peak at 3423.06 cm^{-1}assigned as hydrogen bonded O-H stretching within alcohols/phenols. Adjacent peak 3396.57 cm^{-1}attributed as N-H stretching primary amine. The vibrations between 2960.43 cm^{-1}, 2920.78 cm^{-1} and 2848.50 cm^{-1} can be coupled into symmetric and asymmetric vibrational modes of aldehyde C-H stretching. The peak at 1633.73 cm^{-1} assigned as C-H bending alkenes compound. The characteristic methylene scissors lies in the region 1464.90 cm^{-1}corresponds to alkanes and O-H bending in alcohol at 1409.30 cm^{-1}.

A small vibration at 1256.44 cm^{-1} assigned as alkenes C-N stretching. The absorption band at 1070.30 cm^{-1} corresponds to sulfoxide with S=O stretching. The alkenes C=C bending assigned at 921.19 cm^{-1}.The alkane rocking bands 867.97cm^{-1}, 817.91$cm^{-1}$and 778.37 cm^{-1} assigned as methylene C-H out of plane bending. The vibration at 720.77cm^{-1}corresponds to alkanes C=C bending. The peak at 623.74cm^{-1}attributed as halogen compound with C-X stretching. The understanding

of the spectrum by the chemical bonds, which formed due to the vibrations in the molecules present in the extract. Geethu et al., (2014) [32] reported that, by understanding the infrared absorption spectrum, the chemical bonds in a compound can be determined. Judging from the band features in **Fig.2**, the AECF contains alcohol/phenols, primary amines, aldehydes, alkanes, alkenes, sulfoxide and halogen compounds. Ramamurthy and Kennan (2007)[40] reported that, FT-IR spectrum is able to predict the phytoconstituents in *calotropis gigantea*.

The obtained spectrum reveals some chemical constituents in the extract. Furthermore, similar type of studies demonstrated the possibility of spectroscopic techniques in identification of phytochemicals and also to screen the active phytoconstituents with therapeutic values [31, 32, 41]. It is most noteworthy that the application of the FTIR spectrum, has the particular advantages of being fast and effective, is easy to operate and allows for easier replication for an analysis of the extract; which is especially important in the field of traditional medicines [41]. Taken together, with information of spectral bands obtained from UV-Vis and FTIR, makes spectroscopy an effective tool for the detection and identification of compounds.

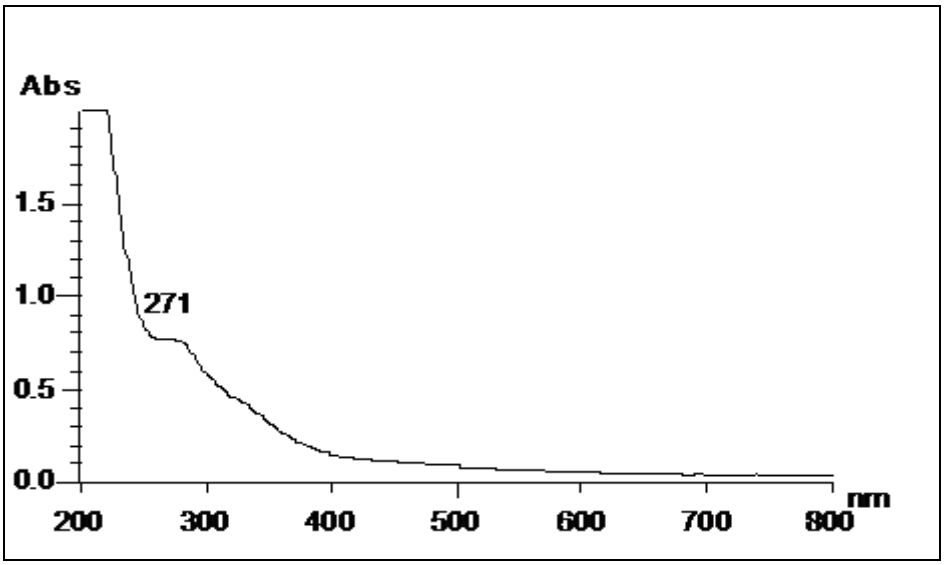

FIG. 1: UV-VIS SPECTRA OF AECF EXTRACT.

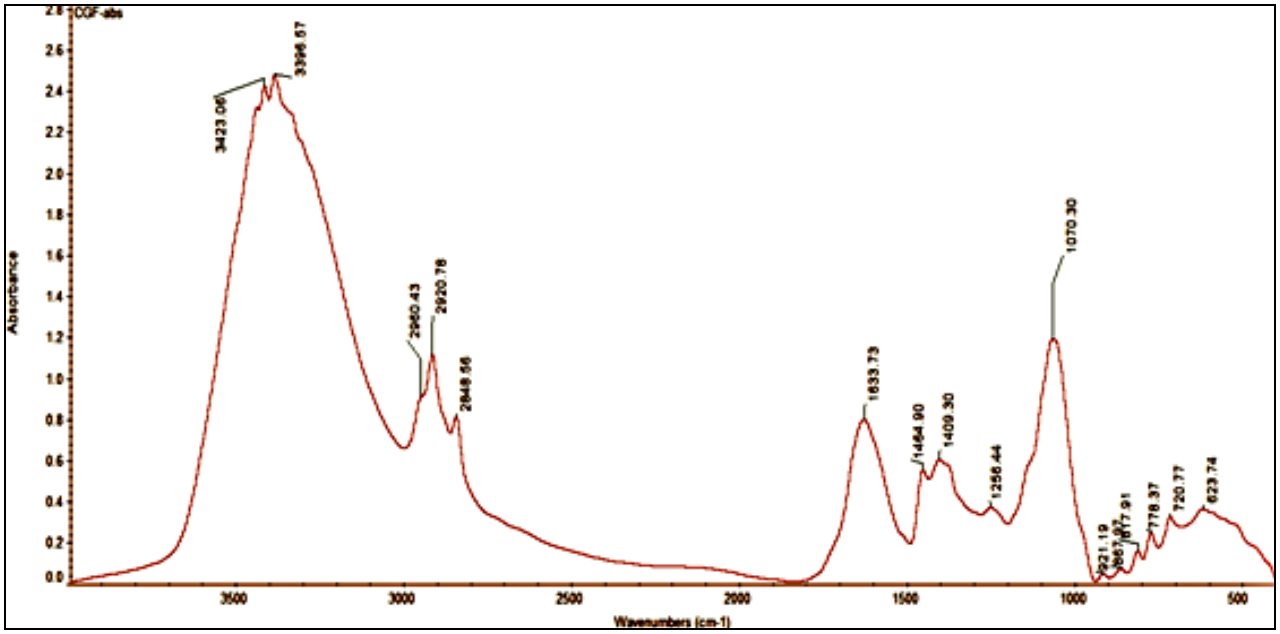

FIG.2: INFRARED SPECTRUM OF AECF EXTRACT

TABLE 1: CHARACTERISTIC INFRARED BANDS OF AECF EXTRACT.

SI.No	Wave numbers (cm^{-1})	Assignment	Functional group
1	3423.06	O-H stretch (Intermolecular bonded)	Alcohol/Phenols
2	3396.57	N-H Stretching	Primary amine
3	2960.43, 2920.78 & 2848.50	C-H stretching	Alkanes
		Methyl symmetric/Asymmetric stretch	Aldehydes
4	1633.73	C-H bending, Monosubstituted (overtone)	Alkenes
5	1464.90	C-H bending	Alkanes
		Methylene Scissors	
6	1409.30	O-H bending	Alcohol
7	1256.44	C-O stretching	Alkenes
8	1070.30	S=O stretching	Sulfoxide
9	921.19,	C=C bending, Monosubstituted	Alkenes
10	867.97 817.91 & 778.37	C-H out of plane bending, Methylene rocking	Alkanes
11	720.77	C=C bending	Alkanes
12	623.74	C-X stretching (X=F, Cl, Br, or I)	Halogen compounds

Minimum Inhibitory Concentration (MIC): Minimum inhibitory concentrations (MICs) are considered the 'gold standard' for determining the susceptibility of organisms to antimicrobials and are therefore used to judge the performance of all other methods of susceptibility testing [42]. The MIC assay is a quantitative method, attributed to the least nominal amount of test compound able to inhibit any visible microbial growth. The antibacterial activity as MIC value of AECF against tested bacteria was represented in **Table 2**. The MIC of AECF was 75µg/ml against *Enterococcus faecalis*, whereas 2µg/ml for *Klebsiella pneumoniae*. Then *Staphylococcus aureus* and *Escherichia coli* had highest value (500µg/ml), which was indicated by abundant bacterial growth in the tube. Habib and Karim, (2009) [27] reported the antibacterial activity of *Calotropis gigantea* flower. Bactericidal activity may be credited to plant bioactive compounds to make complex with bacterial cell wall and thus inhibiting the microbial growth [43]. Studies demonstrated that, crude plant extracts exhibit maximum antibacterial activities against gram positive bacteria than gram negative bacteria [33, 44, 45]. This may be due to structural variations observed in the bacterial cell membrane and cytoplasmic components between Gram-positiveand Gram-negative bacteria [46]. However, in the present study, the flower extract inhibitedboth Gram-positive and Gram-negative bacteria equally. Furthermore, the bactericidal activity doesn't depend on only phenolic compounds [47] but may possibly be strengthened by different functional groups. Taken together, our results suggest that AECF is capable of inhibiting the bacterial growth and may possibly be functional for fortifying of potential antimicrobial agents. Streptomycin used as standard and showed better MIC values against tested bacteria.

TABLE 2: THE MIC VALUES OF AECF EXTRACT

Bacteria	*Staphylococcus aureus*	*Enterococcus faecalis*	*Escherichia coli*	*Klebsiella pnumoniae*
Calotropis gigantea flower extract (µg/mL)	500	75	500	2
Streptomycin (Standard, µg/mL)	3.75	2.5	2.5	2.5

MTT assay:

For the investigation of anticancer property of AECF, MTT assay is considered as necessary and suitable tool. Enzyme-based methods using MTT rely on a reductive coloring reagent and dehydrogenase in a viable cell to determine cell viability with a colorimetric method. This method is far superior, because it is easy-to-use, safe, has a high reproducibility, and is widely applied in cell viability tests. Thus, this method is suited to screen the anticancer property of the extract. In the present study, we investigate the viability test to evaluate the live or death of HeLa cancerous cells. The observations of the MTT based assay performed on HeLa cell line are represented graphically in **Fig. 3**.

The bars in the graph represent the percent viability of cells caused after incubation with the extract. The extract showed less viability against HeLa cell line. The results indicated that even higher concentration of AECF has less effect on HeLa cancer cells. However, the extract was capable of inhibiting bacterial growth but showed less activity on HeLa cancerous cells. This may be attributed; the phytoconstituents present in the extract are not capable of inhibiting the HeLa cancerous cells and thus showed less activity. This may clearly elucidate that extract showing bactericidal activity could not be an anticancer agent. *In-vivo* studies reported that, the principle compounds of *Calotropis gigantea* flower showed potent anti-cancer activity [28] but reports on the *in-vitro* experiments are rare. Further, we have also compared the effect of 5-fluorouracil, a pyrimide analog anticancer compound on HeLa cancer cells. Results showed a decrease in cell viability. As 5-fluorouracil is a purified compound compared to aqueous ethanolic crude extract of *Calotropis gigantea* flower, a direct comparison of effective dosage between extract cannot be evaluated [48]. Taken together, in the present study AECF cannot exhibit remarkable anticancer property.

As aqueous ethanolic crude extracts of *Calotropis gigantea* has been used in the study, deciding dosage of effective compounds responsible for the bactericidal activity is difficult. However, identification of active compound possessing antibacterial effect will be of great therapeutic value. Though, the present study offers proof of preliminary evidence that a *Calotropis gigantea* flower, which is commonly available worldwide, showed antibacterial effect but not be a remarkable in anticancer properties. The study open avenues for further research in purifying the compounds from aqueous ethanolic extract and to screen their efficacy.

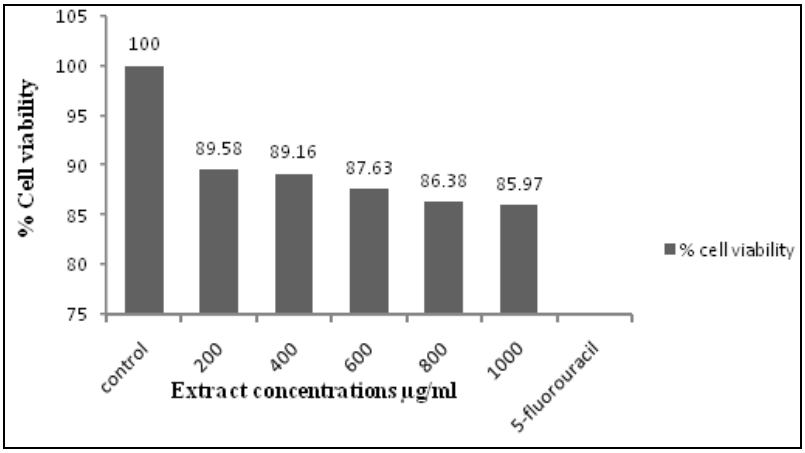

FIG.3: PERCENT CELL VIABILITY OF HELA CELLS AFTER TREATMENT WITH AQUEOUS ETHANOLIC EXTRACT OF *CALOTROPIS GIGANTEA* FLOWER. 5-FLUOROURACIL IS USED AS POSITIVE CONTROL. THE DATA REPRESENT THE MEAN OF TRIPLICATES

CONCLUSION: Our results indicate that, extract prepared from *Calotropis gigantea* flower encompass rich source of Phenolic compounds. With regards to functional group of compounds, the flower extract showed the presence of alcohol/phenols, primary amines, aldehydes, alkanes, alkenes, sulfoxide and halogen compounds. All the findings of the present study warrant further research wherein *Calotropis gigantea* flowers need to be a further experimental analysis, for the evaluation of a potential drug in the treatment of bacterial diseases, but this work proposes the beneficial aspects of medicinal plant.

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