Research Article

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MORPHOLOGICAL, MICROSCOPICAL, PHYSICO-CHEMICAL AND ANTIMICROBIAL INVESTIGATIONS ON LEAVES OF *CALOTROPIS GIGANTEA* LINN.

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Received on: 25/02/14 Revised on: 19/03/14 Accepted on: 09/04/14

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DOI: 10.7897/2277-4343.05238

ABSTRACT
Calotropis gigantea Linn commonly known as Mudar or Yercum belongs to the family Asclepiadaceae. Leaves of this plant were investigated for its morphological, microscopical and phytochemical constituents to check the authenticity of the plant. Column fractionation was done. A study to evaluate *in-vitro* antibacterial activity of methanol extract of the leaves against gram negative bacteria such as *Salmonella typhi, Pseudomonas fluorescens, Pseudomonas aeruginosa* and *Escherichia coli* was carried out. The pathogens were tested by disc diffusion assay method and minimum inhibitory concentration was evaluated. An attempt has been made to compare the activity of extract with standard ciprofloxin. Maximum activity was seen in case of *Pseudomonas fluorescens* where the zone diameter was 32 mm (300 µg/ml). The antibacterial activity therefore shows clearly that the resistance to the pathogens may be minimized by these natural products of the plant origins.
Keywords: Antibacterial, Calotropis, leaves, microscopical, phytochemical.

INTRODUCTION

Herbal remedies are gaining their revival as many sufferers shifting from modern drugs and embracing complementary medicine. Worldwide most clinical useful prescription drugs are of plant origin[1]. India is considered as the storehouse of medicinal plants. Around 45 % of flowering plants are estimated to have medicinal importance. Also, India is the tenth among the plant rich countries of the world and fourth among the Asian countries[2]. Having time tested traditional system of medicine based on the natural products is the privilege of India. India is the largest producer of medicinal herbs and is appropriately called the botanical garden of the world. *Calotropis gigantea* commonly known as Mudar or Yercum belongs to the family Asclepiadaceae and is a shrub about 6 m high which is widely distributed in Eastern and southern parts of India[3]. Fibers of these plants are called madar or mader. The plant is known as aak in Ayurveda and used in cases of cutaneous diseases, intestinal worms, cough, ascites, asthma, bronchitis, dyspepsia, paralysis, swellings, intermittent fevers, anorexia, inflammations and tumors[4]. In large doses, Arka is known to act as a purgative and an emetic. In India, the genus is represented by two species. viz. *Calotropis gigantea* and *Calotropis procera*[5]. The first species being abundant while the second being restricted to forest areas. The experimental plant *Calotropis gigantea* Linn. produces white or violet colored flower in bunches, much branched, tall, erect, large and perennial with latex throughout. The powdered leaves of *Calotropis gigantea* L. has been investigated in a systematic way for pharmacognostical, preliminary phytochemical and pharmacological aspects, an attempt to rationalize its use as a drug of therapeutic importance and to help in the authenticity of this plant.

MATERIALS AND METHOD

Calotropis gigantea L leaves were collected from Chennai in Tamil Nadu, India, during September - October 2011. The plant was authenticated by Dr. P. Jayaraman, Director, Plant Anatomy Research Centre, Chennai, India. A voucher specimen (PARC/ 2011/746) was deposited in the same Institute. The macroscopical and microscopical studies were carried on preserved material (70 % alcohol). Free hand sections were taken stained and mounted in chloral hydrate and crystal violet. The photographs were taken by Olympus BX 50 camera attached to the microscope to determine anatomical characters. AOAC methods were applied to carryout proximate analysis of the samples for moisture, total ash, crude fiber, crude fats, proteins and carbohydrates[6]. The moisture and ash were determined using weight difference method. The plant material was shade-dried and uniformly powdered by passing through the sieve no.60 and then subjected to hot continuous extraction with chloroform followed by methanol and water extraction for a 24 hour cycle. The crude extract solution was concentrated by using rotary flash evaporator to produce a semisolid mass, and dried in lyophilizer

Tests for various phytochemicals of the extracts of water, methanol and chloroform were carried out using the procedures outlined by Pearson D[7]. Column chromatography was carried in a BOROSIL glass column of 100 cm x 3 cm (length and diameter). Silica gel - G (100 mesh) was used as the solid absorbent. Fifty grams of the methanol (2:1 v/v) extract concentrate was ground with a small amount of silica gel and loaded. The column was eluted with solvents of increasing polarity as the method outlined by Harborne JB[8].

Testing of antibacterial activity

The microorganism *Salmonella typhi* (ATCC 00215), *Pseudomonas fluorescens* (ATCC 06341), *Pseudomonas aeruginosa* (ATCC 02150) and *Escherichia coli* (ATCC 10263) were used as test organism. The testing of antibacterial activity of the plant extracts was carried out *in vitro* by Kirby-Bauer disc diffusion technique[9,10]. Culture of bacteria was made on Muller Hinton agar plates. Sterile paper discs 5 mm diameter (Himedia) were placed over the plate at an equidistant position. The discs were loaded with 10 µl of the drug at the concentration of 100 µg/ml, 150 µg/ml, 200 µg/ml, 250 µg/ml and 300 µg/ml. DMSO was used as solvent. Separate control disc was also included using the solvent. Ciprofloxin was used as standard for comparison. The plates were incubated at 37°C for 24 hours. The microbial growth was determined by measuring the diameter of zone of inhibition. Minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. The MIC is determined by agar dilution method[11]. The observations taken for each characterization in three replications was subjected to Analysis of Variance (ANOVA) test[12].

RESULTS

Morphological and anatomical characteristics of the experimental plant

Morphologically the plant is erect, tall, large, much branched and perennial shrub or small tree that grows on a height of 5.4 m, with milky latex throughout[13]. Bark is soft and corky, branches stout, leaves sub sessile, opposite, decussate, broadly ovate, oblong, elliptic or obovate, acute, thick, glaceous, green colored with fine cottony pubescent hair on young (Figure 1). Flowers are in umbellate cymes and tomentose on young. Seeds broadly ovate, acute, flattened, minutely tomentose, brown colored and silky.

The anatomical features reveal an upper and lower epidermis with abundant stomata. The presence of aseptate trichomes were with cystoliths and laticifers. The leaf is bifacial. The upper epidermis consists of a single layer of rectangular cells with a fairly thin cuticle. Numerous trichomes covered. Stomatal number was nearly the same on both the epidermis. Since stomata could not be clearly observed in the photographs, wavy walled, small 2-4 subsidiary cells. The mesophyll was clearly differentiated into palisade and spongy parenchyma. Two to three layers of thin walled long cylindrical cells (Figure 2 and 3), Spongy parenchyma, isodiametric parenchymatous cells with intercellular spaces, numerous cluster crystals scattered in the mesophyll were observed.

The upper and lower epidermis of the midrib is similar to that of lamina except that the cells are smaller (Figure 4). Trichomes are densely observed on the midrib. Under the upper epidermis a projecting prominent part, consisting of 2-3 layers of collenchymatic cells was observed. Palisade parenchyma is slightly interrupted and vasculature prominent.

Pharmacognosical characteristics

Conventional parameters such as fluorescence characteristics, ash values, exhaustive extractive values have been determined. The results are summarized in Table 1 and Table 2.

Before proceeding with the phytochemical tests the aqueous, chloroform and methanol extracts were prepared. The yield was recorded (Table 3)

Phytochemical properties

The qualitative phytochemical properties of plant sample are given in Table 4. Test for alkaloids, steroids, tannins, saponins, flavanoids, gum, proteins, glycosides and fats and oils were carried out for the samples.

Since the phytochemicals were abundant in methanolic extracts it was column fractionated and active principle was isolated for further antimicrobial studies.

Identification of the active principle(s) involved in the therapeutic value of the leaves of the experimental plant

The results presented in Table 5 depict the column fractionation of the methanolic extract for the Thin layer Chromatography (TLC) as the aqueous and chloroform extracts were not promising.

F5-F6 and F7-F8 were selected and used for further experiments to evaluate the therapeutic potential of the fraction. Compound identification was done by employing the UV and FT-IR spectroscopic techniques. The TLC analysis of the active fractions was later analyzed by UV light depicted in Table 6.

The results were supported by the FT-IR spectrum (Figure 5). The functional groups detected on comparison with standard compounds are given in Table 7. Based on their wavelength the functional groups were estimated.

The findings were collaborated by TLC, column chromatography and FT-IR studies of methanolic extract to conclude a cumulative activity and presence of compounds like alkaloids, flavanoids and saponins.

Antibacterial studies

All the microbes used in the present study were sensitive to the methanolic extract of the plant. Maximum activity was seen in case of *Pseudomonas fluorescens* where the zone diameter was 32 mm (300 µg/ml). The antibacterial activity for the bacteria *Salmonella typhi* and *Escherichia coli*, *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* is shown in Table 8.This *in-vitro* study demonstrated that folk medicine can be as effective as modern medicine to combat pathogenic microorganism.

Table 1: Physico chemical studies

S. No.	Parameters	Values (% w/w)
1	Total ash	9.0
2	Water soluble ash	1.2
3	Acid insoluble Ash	1.1
4	Sulphated ash	2.0
5	Alchol soluble extractive	8.0
6	Water soluble extractive	6.3
7	Loss on drying	8.5

Figure 1: Twig of the experimental plant

Table 2: Fluorescence analysis of powdered leaves

S. No.	Treatment	Day light	UV Light	
			254 nm	365 nm
1.	Powder as such	Dark green	Light greenish	Light greenish
2.	Water	Green	Light greenish	Dark greenish
3.	5% NaOH	Light green	Light greenish	Greenish
4.	1N NaOH	Yellowish green	Dark greenish	Light greenish
5.	1N HCl	Green	Dark greenish	Light greenish
6.	50 % H2SO4	Light Yellow	Pale greenish	Light greenish
7.	50 % HNO3	Pale Brown	Yellow greenish	Light greenish
8.	Picric Acid	Greenish	Greenish	Light greenish
9.	Acetic acid	Whitish red	Light greenish	Brown
10.	Ferric chloride	Light reddish	Dark greenish	Brown
11.	HNO3 + NH3	Brown	Dark greenish	Light greenish

Table 3: Extraction of *Calotropis* (leaves) in different solvents

Weight of the powdered leaves	Solvent used 500 (mL)	Yield	Color of the extracts
50 g	Aqueous	35.11 g	Greenish
	Methanol	42.16 g	Greenish brown
	Chloroform	21.22 g	Brown

Table 4: Qualitative phytochemical analysis of powdered leaves

Phyto constituents	Chloroform extract	Methanolic extract	Aqueous extract
Alkaloids	+	+	+
Saponins	-	+	+
Glycosides	_	_	_
Carbohydrates	+	+	_
Tannins	+	+	+
Flavanoids	+	+	-
Steroids	-	+	+
Proteins	_	_	_
Terpenoids	_	_	_
Fats	_	_	_
Gums and Mucilages	+	+	+

(+) - present (-) – absent

Table 5: Column chromatography of methanolic extracts

S. No.	No. of fractions	Solvent/eluents	Color of the fractions
1	F1-F2	Chloroform (100)	Green
2	F3-F4	Chloroform: Ethyl acetate (75:25)	Light green
3	**F5-F6**	Chloroform: Ethyl acetate (50:50)	Dark green
4	**F7-F8**	Chloroform: Ethyl acetate (25:75)	Light green
5	F9-F10	Ethyl acetate (100)	Reddish green
6	F11-F12	Ethyl acetate: Water (75:25)	Light green
7	F13-F14	Ethyl acetate: Water (50:50)	Light green
8	F15-F16	Ethyl acetate: Water (25:75)	Green
9	F17-F18	Ethanol (100)	No color
10	F19-F20	Ethanol: Water (75:25)	Light yellow
11	F21-F22	Ethanol: Water (50:50)	No color
12	F23-F24	Ethanol: Water (25:75)	Light yellow
13	F25-F26	Water	No colour

Table 6: TLC of isolated fractions and UV analysis for active fraction

Fractions	Solvent System	No. of Spots	R_f Value	Color of the spot
F5-F6	Chloroform: Acetic acid: Water (90: 45:6)	One	0.52	Pink
F7-F8	Chloroform: Acetic acid: Water (90: 45:6)	One	0.49	Red

Table 7: Interpretation of FT-IR data of methanolic extract of leaves

S. No.	Stretching or Bending	Wave length (cm^{-1})	Functional group
1.	Stretching	3392.77	O-H group
2.	Stretching	721.81	C-Cl (Alkyl Halide)
3.	Stretching	1170.92	C-N (Amines)
4.	Stretching	1378.12	C-O (Carboxylic Acids and Esters)
5.	Stretching	1462.36	Aliphatic Nitro Compounds
6.	Stretching	1712.47	Esters
7.	Stretching	1734.94	Carboxylic acid
8.	Stretching	2851.27	Acid anhydride
9.	Stretching	2920.35	CH2 (Alkane)

Table 8: Antibacterial activity of the methanolic extract

Bacteria	Zone of inhibition (in mm)					Ciprofloxin (50 µg/ml)
	100 µg/ml	150 µg/ml	200 µg/ml	250 µg/ml	300 µg/ml	
Salmonella typhi	8 ± 0.12	11 ± 0.01	12 ± 0.11	13 ± 0.01	18 ± 0.12	38 ± 0.11
Pseudomonas fluorescens	10 ± 0.11	17 ± 0.01	21 ± 0.01	28 ± 0.01	32 ± 0.41	46 ± 0.00
Pseudomonas aeruginosa	11 ± 0.02	15 ± 0.22	17 ± 0.01	22 ± 0.01	25 ± 0.01	34 ± 0.01
Escherichia coli	7 ± 0.13	13 ± 0.23	18 ± 0.11	20 ± 0.01	22 ± 0.11	33 ± 0.01

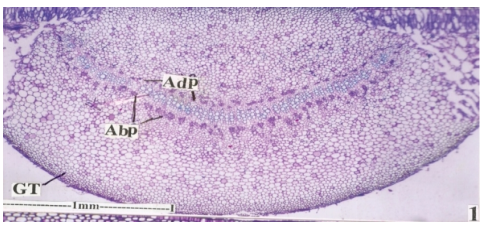

Figure 2: Transverse section of midrib of leaf

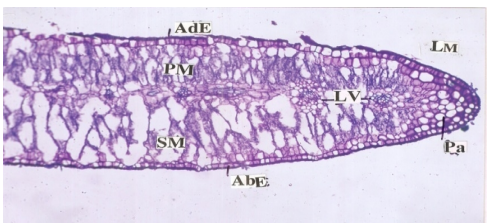

Figure 3: Transverse section of lamina

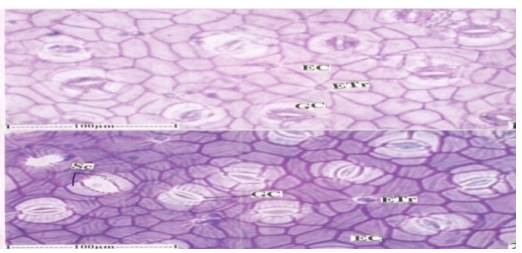

Figure 4: Epidermis in para dermal section (Upper and lower)

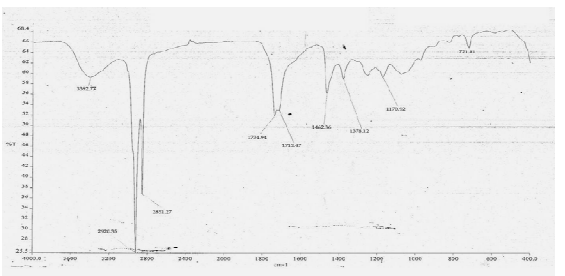

Figure 5: FT-IR spectrum of the active fraction

DISCUSSION

Allopath relies largely on drugs, which have substances arnalgamated in a fashion, where chemical counter balances the undesirable effects of the other, to give beneficial effects. The advantage of herbal extracts is that the active principles present are diluted and there is only less chance of taking dangerous overdoses. Around 80 % population in developing countries relies on community healers and use medicinal plants for protection from illness. Inspite of tremendous development in the field of modern medicine, plants still rank in modern as well as traditional medicine throughout the world. The present investigation including the morpho-anatomical characters, physico-chemical values and pharmacognostical studies[14] will serve as standard reference for identification and authenticity of the drug from its substitute and adulterants. This report would assist in the identification of the crude drug in future.

It was reported that the methanol and ethyl acetate extract of leaf and latex of *Calotropis gigantea* had amino acids, anthraquinones, flavanoids, phenolic compounds and showed antimicrobial activity against four clinically important bacterial species and six plant fungal pathogens. The results obtained from previous study inferred that the leaf and latex extracts of *Calotropis gigantea* effectively inhibited (concentration ranges from 1 mg/ml – 8 mg/ml) the growth of test organisms[15-18]. In comparison, the present study the methanolic leaf extracts were most effective against *Salmonella typhi* and *Escherichia coli,* gram negative bacteria. In 2010[19] it was reported that the latex of *Calotropis gigantea* is a rich source of useful components that has medicinal properties and one of the main applications in controlling the heart muscle. The crude latex extract had many proteins, which are highly basic in nature and exhibited strong dilatation activity. Blood vessel experiment of the latex from *Calotropis gigantea* was studied in the green frog (*Rana hexadactyla*). Thus, the use of medicinal plants, still play a vital role to cover the basis health needs in the developing countries like India[20,21]. Therefore, it is the

need of the time for the screening of medicinal plants to verify the use of natural medicine as an antibacterial. Thus looking to minimize the side effect of antibiotics[22].

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Cite this article as:
Poonam Sethi. Morphological, Microscopical, physico-chemical and antimicrobial investigations on leaves of Calotropis gigantea Linn. Int. J. Res. Ayurveda Pharm. 2014;5(2):193-197 http://dx.doi.org/10.7897/2277-4343.05238

Source of support: Nil, Conflict of interest: None Declared