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PHYTOCHEMICAL INVESTIGATIONS AND ANATOMICAL STUDY OF TWO SPECIES OF *CALOTROPIS* FROM CHANDIGARH

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Keywords:

Calotropis, Asclepediaceae, Phytochemical Investigation, Anatomical study

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ABSTRACT: Medicinal plants have been used since prehistoric period for the cure of various diseases. Hence the herbal medicines occupy distinct position right from the primitive period to present day. *Calotropis procera*, a wild growing plant of family Asclepiadaceae, is well known for its medicinal properties. Different parts of this plant have been reported to exhibit anti-inflammatory, analgesic, and antioxidant properties. *Calotropis gigantea* found in dry waste places, therapeutically used as Anti-cancer, intermittent fever, paralysed part of body painful joints, swelling, heals wounds (leaves). The present study was undertaken to analyse the various active phytochemical constituents and to study detailed anatomical features of two species of *Calotropis* (family: Asclepediaceae) *i.e.*, *C.procera, C.gigantea*. A qualitative analysis was performed for detection of alkaloids, glycosides, terpenoids, steroids and flavanoids. HPLC (High Performance Liquid Chromatography) was used in identifying the qualitative and quantitative analysis of active principle index of two species of *Calotropis* present in the area of present study. Anatomical features of various plant parts were found useful for correct easy identification of commercial drug for ensuring purity.

INTRODUCTION: India being a tropical country is blessed with vast biodiversity of natural resources and ancient knowledge for its judicious utilization, which is in great demand all over the world. According to World Health Organization (WHO) the macroscopic and microscopic description of a medicinal plant is the first step towards establishing its identity and purity and should be carried out before any tests are undertaken. In recent years, ethno-botanical and traditional uses of natural compounds, especially those of plant origin, have received much attention as they are well known for their efficacy and are generally believed to be safe for human use [1].

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Plants are reported to have anticancer, antimicrobial, ant diabetic, anti inflammation, antioxidant properties [2]. New pharmacologically active agents have been searched from natural resources such as plants, animals and microbes leading to discovery of many clinically useful drugs [3]. Phytochemical evaluation is one of the tools for quality assessment, which includes preliminary phytochemical screening. HPLC is highly sensitive method for identification and quantification of any chemical in particular samples using UV and visible absorbance [4].

Calotropis procera, a wild growing plant of family Asclepiadaceae. The ethno-botanical uses of *Calotropis Procera* revealed the diversity of its medicinal uses and popular use of the plant for wide range of common ailments like fever, indigestion, cough, cold, asthma, vomiting etc [5]. The plant is known for its toxic properties like dermatitis and acts like a poison and produces

lethal effects [6]. *Calotropis gigantea* (Asclepiadaeceae) is found in dry waste places. *Calotropis gigantea* is frequently available in India and used for several medication purposes in traditional medicinal system [7]. The plant *Calotropis gigantea* is a plant with many curative principles and other economic values which include fungicidal, insecticidal, indicator of heavy metal etc [8].

MATERIAL AND METHODS:

Plant Collection and Identification: Fresh plant samples were collected from 2 different localities. The various useful parts like leaves and stem *etc.* separated and preserved for the study. Identification of various plants species done by comparing with authenticated herbarium specimens, later confirmed with the help of diagnostic keys and morphological description given in various floras.

1. **Anatomical study of part used:** The roots and stem of all two *Calotropis* species were fixed in F.A.A. (*i.e*. Formalin acetic acid-alcohol, 1:1:18) after trimming them to correct dimensions. Hand sections of fresh stem and leaf were cut using a sharp blade. Thin transverse sections were stained in safranin and then fast green, passed through alcohol grades for dehydration, and then mounted in D.P.X. Observations were taken from these sections using light microscope. These sections were also photomicrographed. Special identifying features of the plant part(s) were studied and identified.

2. **Phytochemical study:** Leaves were washed with a solution of 5% mercuric chloride for 5 minutes and then thoroughly washed with sterile distilled water in order to remove any dirt or filthy particles present on the surface and were shade dried as well as oven dried and then made into fine powder, The solvent extracts were evaporated to dryness in rotary evaporator in petroleum ether, distilled water, ethanol and chloroform. The dried residues thus obtained were stored in screw capped vials at -4°C. Phytochemical screening of plant extracts was carried out by using standard procedures. Specific qualitative tests were performed to identify bioactive compounds of pharmacological importance. In brief, the phytochemicals such as alkaloids, tannins, cardiac glycosides, saponins, terpinoids and steroids were qualitatively determined as following:

2.1 Test for alkaloids (Mayer's test): 2.0 ml of extract was measured in a test tube to which picric acid solution was added. The formation of orange coloration indicated the presence of alkaloids.

2.2 Test for cardiac glycosides (Keller-Killiani test): 5ml of plant extract was treated with 2ml of glacial acetic acid containing one drop of ferric chloride solution. A brown ring of the interface indicates a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer, a greenish ring may form just gradually throughout thin layer which shows the presence of Cardiac glycosides.

2.3 Test for Tannins: The substance (extracts) mixed with basic lead acetate solution. Formation of white precipitate indicates the presence of Tannins.

2.4 Test for Saponins: Froth test for saponins was used. 1g of the sample was weighed into a conical flask in which 10ml of sterile distilled water was added and boiled for 5 min. The mixture was filtered and 2.5ml of the filtrate was added to 10ml of sterile distilled water in a test tube. The test tube was stopped and shaken vigorously for about 30 second. It was then allowed to stand for half an hour. Honeycomb froth indicated the presence of saponins.

2.5 Test for Flavonoids: 5 ml of dilute ammonia solution were added to a portion of the aqueous filtrate of plant extract followed by addition of concentrated H_2SO_4. Formation of yellow color observed in each extract indicated the presence of flavonoids.

2.6 Test for Steroids: One gram of the test substance (plant extracts) was dissolved in a few drops of acetic acid. It was gently warmed and cooled under the tap water and a drop of

concentrated sulphuric acid was added along the sides of the test tube. Appearance of green colour indicates the presence of Steroids.

2.7 Test for Terpinoids (Salkowski test):

5ml of each plant part extract was mixed in 2 ml of chloroform, and concentrated H_2SO_4 (3ml) was carefully added to form a layer. Formation of reddish brown coloration at the interface shows the positive results for presence of terpenoids.

3. **High performance liquid chromatography Extraction of Plant samples:** The air- dried, powdered samples of *Calotropis procera* (Leaves) and *Calotropis gigantea* (Leaves) sample was subjected to pass through 20 mesh size sieve. The sieved material was refluxed with methanol (99%) at the temperature of 80-85°C for 2-3 hrs on a water bath. The material was filtered and marc was further refluxed with methanol three times. Following this all the extracts were pooled together, concentrated under vacuum using Rota-evaporator. Finally the material was air-dried after removal of methanol.

4. **Chromatographic conditions:**

The composition of extracts was analyzed using RP-HPLC system (Shimadzu Corporation, LC2010C HT, Kyoto, Japan) coupled with ultra violet - photo diode array detector (UV-PDA). Chromatographic separation was performed at 40°C on C18 phenomenex column (250 x 4.6 mm, 5µm). The mobile phase consisted of (A) 1:1 (v/v) acetonitrile: methanol and (B) 0.1mM ammonium acetate in water at a flow rate of 1ml/min and injection volume 20µl. The column effluent was detected at 280 nm with an UV detector [9].

RESULTS:

Anatomical study of plant: The comparative account of anatomical features of plant parts *i.e.* leaf and stem of two *Calotropis* species are given in **Table 1** and **Table 2** and hand sections are shown in **Figure 1-4**.

TABLE 1: COMPARISON OF *CALOTROPIS PROCERA* AND *CALOTROPIS GIGANTEA* ANATOMICALLY

Character	*Calotropis procera*	*Calotropis gigantean*
Leaf	**Epidermis:** Epidermal cells are small, polyhedral. Presence of thick culticle. Trichomes are less developed.	**Epidermis:** Epidermal cells are small, polyhedral. Elongated cells are covered with cuticle. Trichomes are multicellular and more developed.
	Collenchyma: 3-5 layered collenchymatous cells present on both sides of upper and lower epidermis.	**Collenchyma: 5-6 layered** collenchymatous cells are present on both lower and upper epidermis.
	Parenchyma: 4-8 layered parenchymatous cells are present which are thin walled, isodiametric to circular with intercellular spaces present.	**Parenchyma:** Presence of large parenchymatous cells without chloroplasts. They are very large thin- walled cells.
	Vascular bundles: Stele crescent shaped composed of bi-collateral and open vascular bundles. A strip of cambium present between xylem and phloem tissue.	**Vascular bundles:** Stele crescent shaped composed of bi-collateral and open vascular bundles. A strip of cambium present between xylem and phloem tissue.
	Lamina: Mesophyll is seen to be differentiated into palisade and spongy tissue. Below upper epidermis there are 4 rows of palisade mesophyll tissue.	**Lamina:** Mesophyll is seen to be differentiated into palisade and spongy tissue. Below upper epidermis there are 3 rows of palisade mesophyll tissue.

TABLE 2: COMPARISON OF *CALOTROPIS PROCERA* AND *CALOTROPIS GIGANTEA* ANATOMICALLY

Character	*Calotropis procera*	*Calotropis gigantean*
Stem	**Epidermis:** It is single layered consisting of uniseriate cells with thick cuticle. Uni and multicellular hairs clothe epidermis almost completely. Cells are barrel to rectangular and are completely arranged.	**Epidermis:** It is single layered consisting of uniseriate cells with thick cuticle. Uni and multicellular hairs clothe epidermis almost completely. Cells are barrel to rectangular and are completely arranged.
	Cortex: Consist of 5-6 layers of collenchymatous cells which are present near	**Cortex:** Consist of **7**-9 layers of collenchymatous cells which are present near epidermis and 9-10 layers

	epidermis and 12-15 layers of parenchymatous cells which are present near endodermis. **Endodermis:** It forms a wavy ring around the vascular tissue. These cells are barrel – rectangular shaped and are compactly arranged. More prominent. **Pericycle:** It is in the form of small patches of sclerenchymatous fibres. More prominent. **Vascular tissue system:** Secondary growth is prominent. Primary phloem is completely obliterated. Patches of secondary phloem occur above and close to the cambium. Secondary xylem forms broad and extensive region.	of parenchymatous cells which are present near endodermis. **Endodermis:** It forms a wavy ring around the vascular tissue. These cells are barrel – rectangular shaped and are compactly arranged. Less prominent. **Pericycle:** It is in the form of small patches of sclerenchymatous fibres. Less Prominent. **Vascular tissue system:** Secondary growth is less prominent than *C.procera*. Primary phloem is completely obliterated. Patches of secondary phloem occur above and close to the cambium. Secondary xylem forms less extensive region than *C.procera.*

Calotropis procera Leaf Figures (1-2):

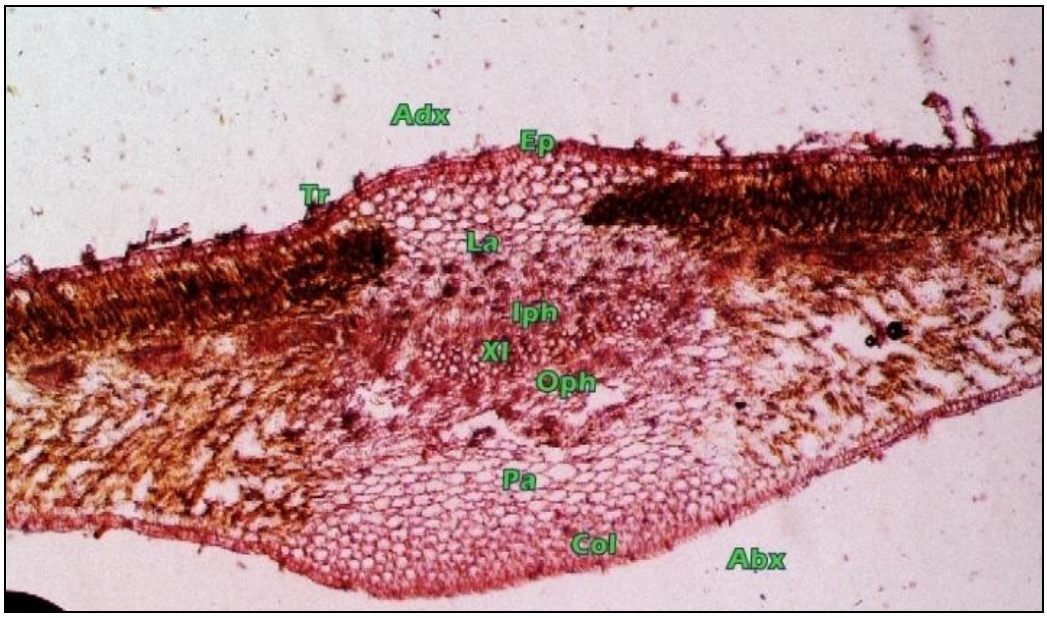

FIG 1: T.S OF *CALOTROPIS PROCERA* LEAF MIDRIB SHOWING Ep-Epidermis, Ads- Adaxial side, Abs- Abaxial side, Xl-Xylem, Iph-Inner Phloem, Col- Colenchyma, Pa- Parenchyma, Oph- Outer Phloem, Lc- Laticifers, Tr-Trichomes.

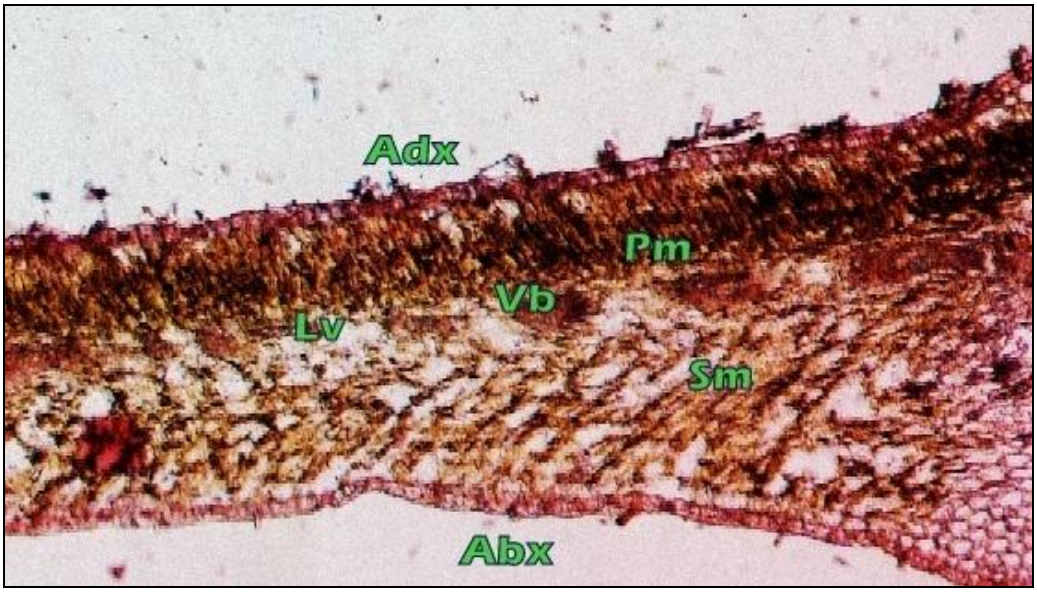

FIG 2: T.S OF *CALOTROPIS PROCERA* LEAF LAMINA SHOWING Ads- Adaxial side, Abs- Abaxial side, Pm-Palisade mesophyll, Sp- Spongy mesophyll, Lv- Lateral vein, Vb- Vascular bundles

***Calotropis procera* Stem Figures (3-4):**

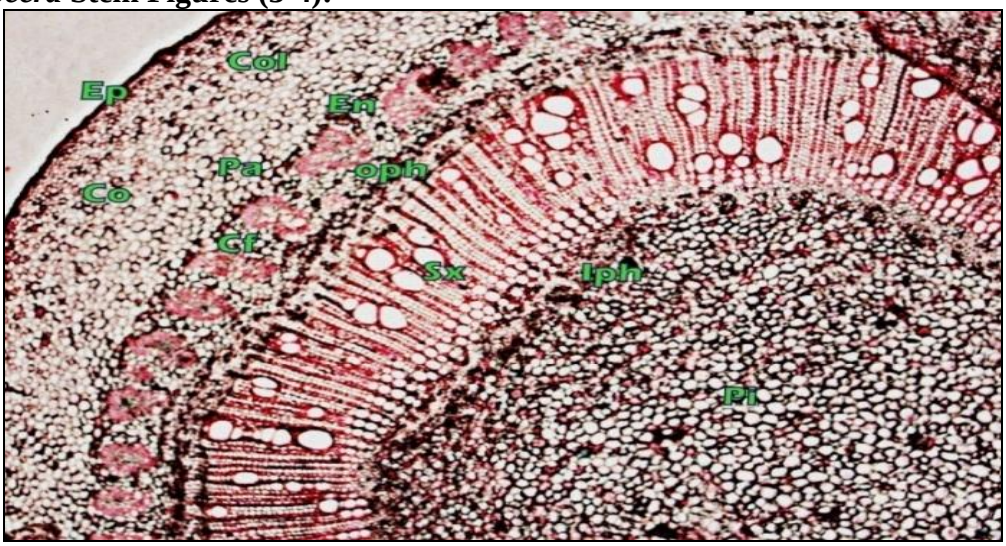

FIG 3: T.S OF *CALOTROPIS PROCERA* STEM SHOWING Ep- Epidermis, Co- Cortex, Col-Collenchyma, Pa-Parenchyma, Cf- Cortical fibres, En- Endodermis, Oph- Outer phloem, Sx- Secondary phloem, Iph- Inner phloem, Pi-Pith

FIG 4: T.S OF *CALOTROPIS PROCERA* STEM SHOWING VESSELS AND XYLEM FIBRE

***Calotropis gigantea* Leaf Figures (1-2):**

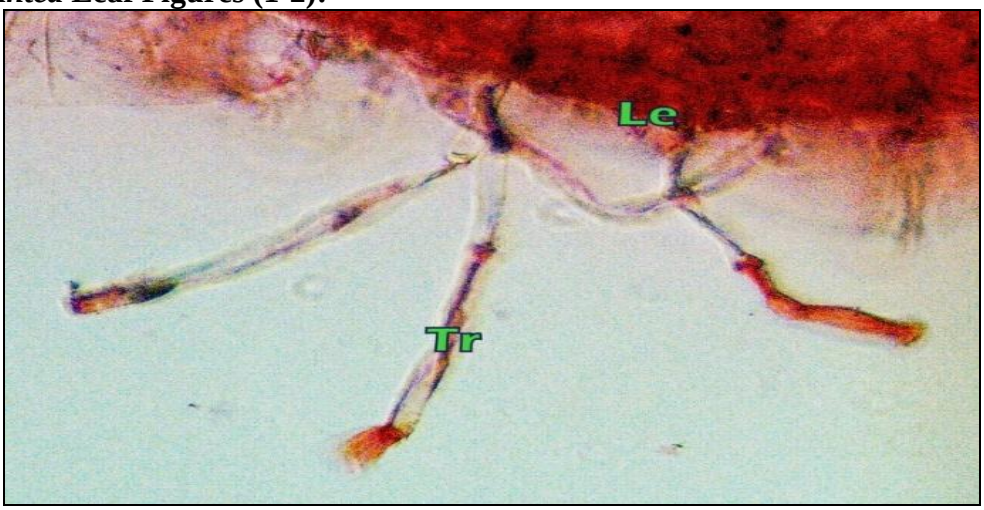

FIG 1:T.S OF *CALOTROPIS GIGANTEA* LEAF SHOWING LE-Lower Epidermis, Tr- Trichomes on lower Epidermis.

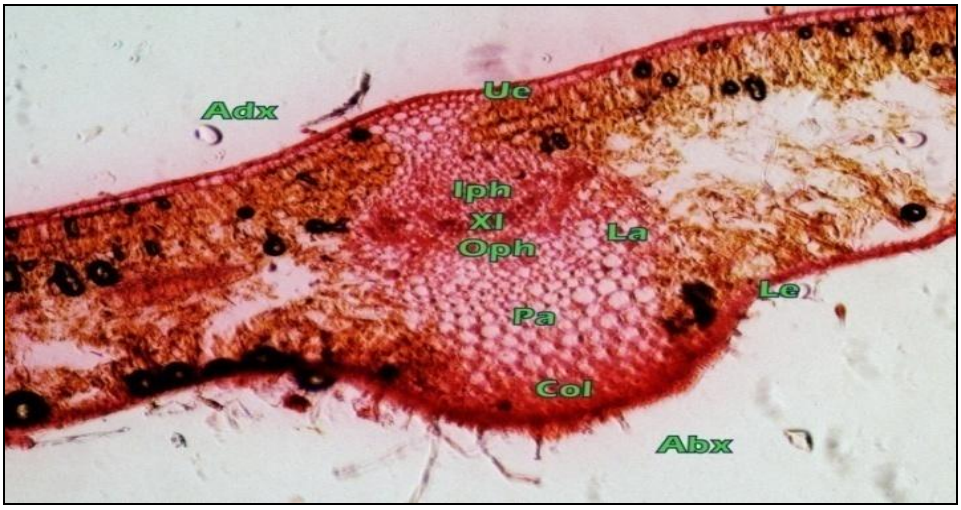

FIG 2: T.S OF *CALOTROPIS GIGANTEA* LEAF MIDRIB SHOWING Uep- Upper Epidermis, Lep- Lower Epidermis, Ads- Adaxial side, Abs- Abaxial side, Col-Collenchyma.

Calotropis gigantea Stem Figures (3-4):

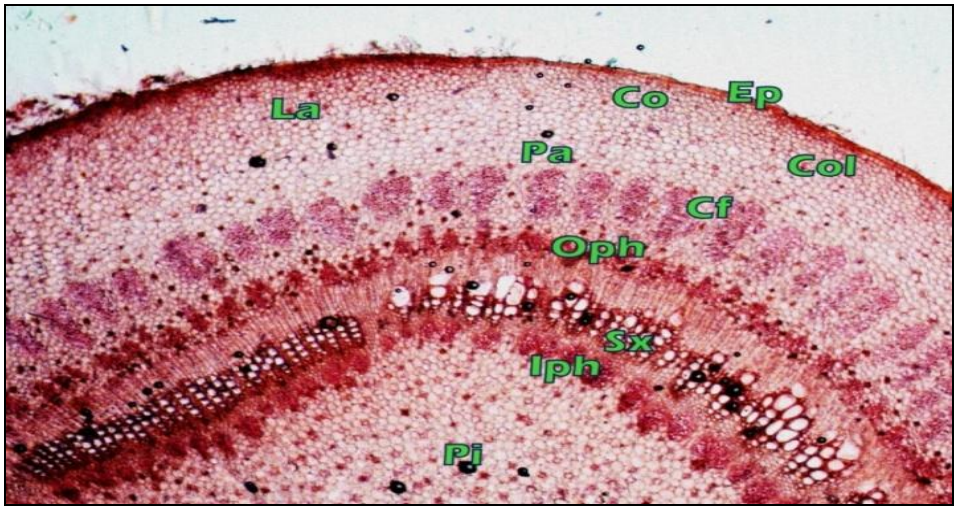

FIG 3: T.S OF *CALOTROPIS GIGANTEA* STEM SHOWING Ep- Epidermis, Co- Cortex, En- Endodermis, Pi- Pericycle, Cf- Cortical fibres, Col- Colenchyma, Pa- Parenchyma,Oph- Outer Phloem, Iph- Inner Phloem, Sx- Secondary xylem, La-Laticifers, Pi-Pith.

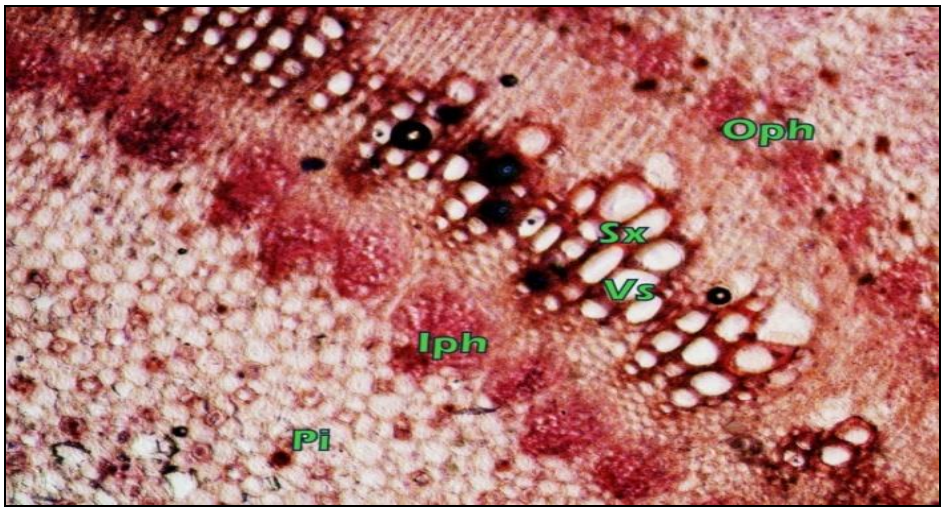

FIG 4: T.S OF *CALOTROPIS GIGANTEA* STEM SHOWING Iph- Inner Phloem, Oph- Outer phloem, Sx- Secondary Xylem, Vs- Vessels, Pi- Pith

Phytochemical studies: Different phytochemical constituents analysed from two species of *Calotropis* are shown in the table given below:

TABLE 3: PHYTOCHEMICAL STUDIES ON *CALOTROPIS PROCERA* LEAF EXTRACT

Phytochemicals	Petroleum ether	Distilled water	Ethanol extract	Chloroform extract
Alkaloids	_	_	+	_
Glycosides	+	+	+	+
Saponins	_	+	+	_
Tannins	+	+	+	+
Flavonoids	_	+	+	_
Steroids	+	_	+	_
Terpenoids	+	+	+	+

Note (+): Positive, (-): Negative

TABLE 4: PHYTOCHEMICAL STUDIES ON *CALOTROPIS GIGANTEA* LEAF EXTRACT

Phytochemicals	Petroleum ether	Distilled water	Ethanol extract	Chloroform extract
Alkaloids	_	+	+	+
Glycosides	_	+	+	+
Saponins	_	+	+	+
Tannins	_	+	+	_
Flavonoids	_	+	+	+
Steroids	_	+	+	+
Terpenoids	_	+	+	+

Note (+): Positive, (-): Negative

Active Principle analysis by HPLC:

HPLC profile of both species:

In methanol extract of *Calotropis procera,* two peaks were present at retention time 14.889 and 17.163 which were found to be of Calotoxin and Calactin respectively with peak area of 4692290 and 3315264, while in *Calotropis gigantea* four peaks were present at retention time 3.054, 10.26, 14.58 and 26.233 which were found to be of Catechol, Calotropagenin, Calotoxin and Chyrsin (Phenylpropanoid) respectively with peak area of 69856478, 6368090, 4439946 and 8661843.

Hence, the results reveal that Calotoxin was present in both the species i.e. *Calotropis procera* and *Calotropis gigantea*. Results were in accordance with results of Kanojiya and Madhusudanan, 2011. While on the other hand *Calotropis gigantea* consists of some additional compounds such as Catechol, Calotropagenin and Chyrsin (Phenylpropanoid) respectively.

PEAK TABLE: 1

Peak	RT	Area	Height
1.	14.889	4692290	332273
2.	17.163	3315264	22805

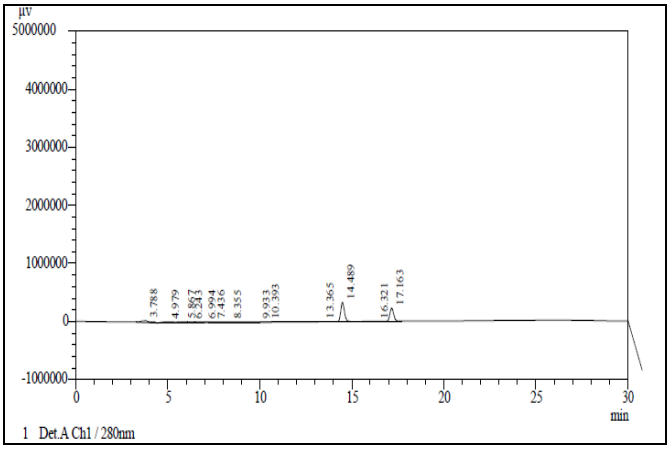

FIG 1: HPLC CHROMATOGRAM OF *CALOTROPIS PROCERA* METHANOL EXTRACT.

PEAK TABLE: 3

Peak	RT	Area	Height
1.	3.054	69856478	564987
2.	10.26	6368090	23645
3.	14.58	4439946	15489
4.	26.233	8661843	30254

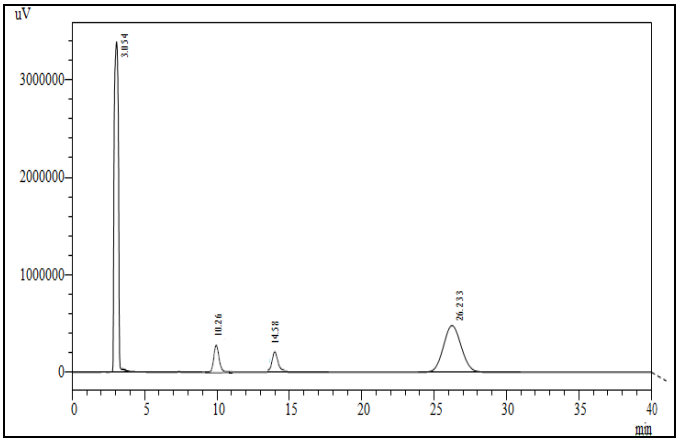

FIG 3: HPLC CHROMATOGRAM OF *CALOTROPIS GIGANTEA* METHANOL EXTRACT

DISCUSSION: Qualitative screening of phytochemical constituents of *Calotropis* species reveals the presence of alkaloids, glycosides, saponins, tannins, flavonoids, steroids and terpenoids in ethanol extract of both the species and distilled water extracts in *C. gigantea* leaf extract. *Calotropis procera* in distilled water revealed the presence of glycosides, saponins, tannins, flavonoids and terpenoids while alkaloids and steroids were absent. Other extracts had varied results. Pharmacological studies have demonstrated that *Calotropis* possess Calotoxin and Calotropegenin which exhibits a wide range of properties- anti-inflammatory and analgesic activity.

In present study, the observed alkaloids content is found maximum in *C. gigantea* by observing the peak area and additional alkaloids i.e. Catechol, Calotropagenin and Chyrsin. The anatomical features of stem and leaves of the species of *Calotropis* which are of diagnostic value have been studied. Focus of study has been towards obtaining the drug from leaves so these have been studied in detail to avoid and prevent adulteration of commercial drug by users. Certain diagnostic features of morphology and anatomy have been found to be useful in the correct identification of the species of *Calotropis* which are of medicinal value.

CONCLUSION: Approaches like screening, phytochemical profiling of these plants helped to get elite species among two species i.e. *C.gigantea*. The correct botanical identification of the herbal drugs of commerce shall help to check piracy of these drugs and hence make available true botanicals to the manufacturers of drugs and consumer.

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