Results and Discussion
4. RESULTS AND DISCUSSION

4.1 Ethnobotany

Throughout human history, the use of herbal medicines has always been central to all healing systems. Prior to our relatively recent reliance on the isolated, purified, often times synthetic chemical entities dominant in modern medicine today, plants were the primary source of medicines for the majority of the world’s population. This is still true today. Plants also provide the source material for a large percentage of modern drugs.

From historic time, the documentation of traditional knowledge especially on the medicinal uses of plants has provided many important drugs of modern day (Cox and Balick, 1996; Flaster, 1996). Traditional medicine still remains the main resource for majority (80%) of people in developing countries for treating health problems, particularly because medicinal plants are accessible and cheap (Maridass and Britto, 2008). It has been repeatedly estimated by the World Health Organization (WHO) that approximately 80% of the world’s population continues to rely on traditional medical practices, including herbal medicines, as their primary form of health care.

Extensive survey of the literature showed that Dichrostachys Cinerea is distributed in Africa, Temperate and Tropical Asia (Plate 2). Curtis and Mannheimer (2005) observed that Dichrostachys is an aggressively invasive species, dominating large areas and forming impenetrable thickets. The survey of the literature pertaining to the ethnobotanical uses of Dichrostachys showed that the plant occupied a key position in the traditional health care system not only in African continent but in India also (Nadkarni, 1976; Thammamna and Narayane Rao, 1990; Vedavathys Mirudula and Sudhakar, 1997; Van Wyk and Gericke, 2000; Von Koenen, 2001; Khare, 2007; Gundidya, 2011). Further, in India leaves are the most exploited part especially for the genitourinary system disorders. According to Gurib-Fakim (2006), the search for new molecules has taken a slightly different route where the science of ethnobotany and ethnopharmacognosy are being used as guide to lead the chemist towards different sources and classes of compounds.
It is in this context that the flora of the tropics by virtue of its diversity has a significant role to play in being able to provide new leads.

4.2 Botanical Pharmacognosy

Meena et al. (2009) suggests that understanding the knowledge on medicinal plants used in traditional system in relation to their use as therapeutic agents, pharmacological properties, medicinal plants being imported, medicinal plant parts being exported, endangered medicinal plants and availability of medicinal plants in different bio-geographical zones of India so that the data and information could be utilized in drawing strategies for rational and more scientific use of medicinal plants in a way that can be extended for future scientific investigation in different aspects. In this respect a detailed study on the botanical aspects of the pharmacognosy of *Dichrostachys Cinerea* (L.) is carried out and discussed further.

4.2.1 Macroscopic features

*Dichrostachys Cinerea* (L.) Wight & Arn. is an armed small tree growing up to 6 metres in height. It is found in scrub-jungles and dry forests. The branchlets are stem – thorns; the leaves are alternate or in clusters. They are bipinnate with 5 -15 pairs of pinnae; leaflets are 12 – 16 pairs. Inflorescence is axillary spike oblong; flowers polygamous; bicoloured. Upper flowers bisexual, yellow, lower flowers sterile ‘♂’, pink. Pods linear, flat, coiled; seeds 4 – 6, ovoid.

4.2.2 Microscopic features.

4.2.2.1 Leaflet

The leaflet is flat on the adaxial side and slightly convex on the abaxial side (Plate 3). It is 1.2 wide and 250µm thick along median part and 100µm thick along the margin.

The midrib is situated in the median part of the leaflet; it does not project beyond the level of the lamina. The adaxial epidermal layer of the leaflet is thus comprising
spindle shaped darkly stained cells. The adaxial epidermis bears stomata (Plate 3). The abaxial epidermis is thick and the cells are hemispherical with papillate outer tangential walls. The mesophyll tissue consists of seven or eight layers vertically elongated cells of varying heights. Differentiation of palisade and spongy parenchyma is not distinct (Plate 3)

The Vascular bundle of the midrib is circular and prominent, measuring of 450 µm in diameter. It consists of two or three layers of heavily thick walled, lignified bundle sheath fibres enclosing vascular strand. The xylem elements of vascular strand include two vertical rows of angular thick walled cells and a small cluster of darkly stained phloem elements. The vascular elements are surrounded by thick walled cells (Plate 4).

Calcium oxalate prismatic crystals are seen associated with the bundle sheath fibres in the form of a circle (Plate 4). Crystals are also sparsely distributed in the mesophyll tissue.

4.2.2.2 Stem

Both young and old stem were studied. The young stem measuring about 1.4 mm thick is roughly circular, in sectional views (Plate 5).

It consists of thin, intact layer of epidermis and narrow cortex comprising five or six layers of thin walled elliptical parenchyma cells (Plate 5) Inner to the cortex, is the thick continuous cylinder of sclerenchyma which include mostly gelatinous fibres.

The vascular cylinder is thick and hollow enclosing wide pith. The outer part of the vascular cylinder consists of phloem where the cells are compressed. The xylem part includes narrow, thick walled lignified fibres and vessels mostly distributed in the outer zone of the xylem. The vessels are wide, circular and thin walled. The pith is angular in outline; the pith cells are circular, thin walled, less compact and tannin is located in many cells of the pith.
TS OF LEAFLET: Entire view

LEAFLET PEELING: Paracytic Stomata

LEAFLET: Epidermal hairs

LEAFLET : Vein islets

LEAFLET : Vein endings

AdS. Adaxial side; LM. Leaf Margin; MRB. Mid Rib Bundle;
PM. Palisade Mesophyll; SM. Spongy Mesophyll

PLATE 3
TS OF LAMINA WITH MIDRIB

TS OF LAMINA: Calcium oxalate crystals in bundle sheath fibres of the midrib bundle

AdS. Adaxial side; BS. Bundle Sheath; Cr. Crystals; Ph. Phloem;
VB, Vascular Bundle; X. Xylem

PLATE 4
4.2.2.3 Old stem

Old stem is about 2mm thick. It shows later stage of secondary growth (Plate 5). The epidemical layer is broken at several places forming wide shallow fissures. A thin layer of periderm is formed at the outer most part of the stem. The cortex is compressed in to dark thick cylinder of tannin filled cells. Inner to the cortex is a thick continuous cylinder of fibres where the cells are thick walled and lignified (Plate 5 and 6).

Secondary phloem is wide and continuous. It consists of randomly oriented sieve elements, dilated circular parenchyma cells possessing dark tannins. The phloem rays are slightly dilated. The secondary xylem cylinder consists of diffusely distributed wide circular fairy thick walled vessels (Plate 5 and 6) xylem figures are dense, thick walled and lignified. The vessels are up to 60µm wide. The pith is reduced in size and includes mostly tannin filled cells. The dimensions of cells of various tissue system is presented in Table 1.

Table: 1 Dimension of different types of cells in the stem of Dichrostachys cinerea.

<table>
<thead>
<tr>
<th>Types of cells</th>
<th>Stem (Length x Breadth values in µ)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Minimum</td>
</tr>
<tr>
<td>Phellem</td>
<td>109.3 x 32.8</td>
</tr>
<tr>
<td>Phellogen</td>
<td>87.4 x 32.8</td>
</tr>
<tr>
<td>Phelloderm</td>
<td>76.6 x 54.6</td>
</tr>
<tr>
<td>Sclerenchyma</td>
<td>32.8 x 21.9</td>
</tr>
<tr>
<td>Parenchyma</td>
<td>153 x 109.3</td>
</tr>
<tr>
<td>Trichome</td>
<td>125.7 x 29.7</td>
</tr>
</tbody>
</table>

Though analytical tests based on fluorescence in day light are not much used, as they are usually unreliable, owing to the weakness of the fluorescence effect, ultraviolet and florescent light are invariably used as part of quality control program by many
PLATE 5

YOUNG STEM

A PART OF YOUNG STEM

A PART OF THICK STEM

Co. Cortex; Ep. Epidermis, Fi: Fissure, Pi. Pith; Pe. Periderm; Ph. Phloem;
SC. Sclerenchyma of gelatinous fissures; SPh. Secondary Phloem; Ve. Vessels;
Xf. Xylem fibres; X. Xylem; XR. Xylem Rays.
PLATE 6

TS OF THICK STEM: Outer Zone of Sclerenchyma

TS OF THICK STEM: Secondary Phloem

TS OF THICK STEM: Secondary Xylem

Scl. Sclerenchyma; SPh. Secondary Phloem; SX. Secondary Xylem; Ta. Tannin Cells; Ve. Vessel; XF. Xylem Fibres; XR. Xylem Rays
commercial agencies. The quantitative fluorescence analysis produced by a compound in ultraviolet light is of much use (Evans, 2002). Table 2 illustrates the fluorescence properties of leaves and stem of the *Dichrostachys cinerea* under various conditions as required for a drug manufacturing firm.

**Table: 2 Fluorescent analysis of leaf of *Dichrostachys cinerea***

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Colour characteristics</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Leaves</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Visible light</td>
<td>Light</td>
<td>Visible light</td>
<td>UV light</td>
</tr>
<tr>
<td>Powder alone</td>
<td></td>
<td>Green</td>
<td>Green</td>
<td>Yellow</td>
<td>Yellow</td>
</tr>
<tr>
<td>Powder + water</td>
<td></td>
<td>Green</td>
<td>Yellowish green</td>
<td>Light brown</td>
<td>Light brown</td>
</tr>
<tr>
<td>Powder + 1N. NaOH Yellow in water</td>
<td></td>
<td>Yellowish green</td>
<td>Dark green</td>
<td>Yellowish brown</td>
<td>Light brown</td>
</tr>
<tr>
<td>Powder + 1N. HCl</td>
<td></td>
<td>Green</td>
<td>Green</td>
<td>Brown</td>
<td>Brown</td>
</tr>
<tr>
<td>Powder + 50% HCl</td>
<td></td>
<td>Green</td>
<td>Green</td>
<td>Brown</td>
<td>Dark brown</td>
</tr>
<tr>
<td>Powder + 50% H₂SO₄</td>
<td></td>
<td>Dark green</td>
<td>Brown</td>
<td>Brown</td>
<td>Black</td>
</tr>
</tbody>
</table>

**4.2.2.4 Root**

Both thin and thick roots were studied. The thin root is 2mm diameter. The root is highly fissured, the fissures being wide and irregular (Plate 7). The outer part of the root consists of wide homogenous periderm which is 300µm thick. The Periderm cells are thick walled, homogeneous and suberised. The periderm is followed by wide cortical zone. The cortical cells are large and compact. They are heavily loaded with starch grains.

Secondary phloem occur inner to the starchy cortex. It is narrow and includes small radial files of elements (Plate 7). The secondary xylem is undulate in outline due to the pressure of wide circular vessels located in the peripheral part of the xylem cylinder.
TS OF THIN ROOT: Entire view

Co. Cortex; Pe. Periderm; SPh. Secondary Phloem showing outer gelatinous fibres and inner sieve elements; XFi. Xylem Fibres; SX. Secondary Xylem; Ve. Vessels

PLATE 7
Narrow vessels occur in central part. In the outer part occur thick masses of gelatinous fissures. The vessels are the widest, vessel is 140µm in diameter.

4.2.2.5 Thick root

The thick root is more than 3mm in diameter. It has undergone secondary thickening by producing wider vascular cylinder and cortex. The periderm is 400µm thick and is highly fissured and broken (Plate 8). The cortex is nearly 550µm thick and consists of tangential rows of rectangular cells with dense accumulation of starch grains (Plate 8).

Secondary phloem is wider and consists of angular sieve elements with distinct companion cells and phloem parenchyma cells (Plate 9) outer to the intact phloem there is thick irregular masses of phloem fibres which include mucilaginous cells and are called gelatinous fibres or g-fibres (Plate 10). Calcium oxalate prismatic crystals are common in the outer phloem and they are associated with the g-fibres (Plate 10).

4.2.3 Powder microscopic observations

The powder of the root when examined under the Microscopic, exhibits the following inclusions.

4.2.3.1 Fibres.

Fibres are abundant in the powder (Plate 11). They are of two types some are wide, thin walled and short. They are called wide fibres, the lumen in wide and some inclusions are seen it the fibres (Plate 11). The wide fibres are 400µm long and 300µm.

4.2.3.2 Narrow fibres

The narrow fibres have thicker walls and narrow lumen (Plate 12). They are 650µm long and 15 µm thick. No inclusions are seen in the powder.
TS OF THICK ROOT: Entire view
Pe. Periderm; SPh. Secondary Phloem; SX. Secondary Xylem

PLATE 8
TS OF THICK ROOT: Outer portion of gelatinous fissures and starch grain

TS OF THICK ROOT: Inner intact sieve elements of secondary phloem and xylem

Co. Cortex; MFl. Mucilaginous or gelatinous Fissures; Pe. Periderm;
SG. Starch Grains; SPh. Secondary Phloem; XF. Xylem Fibres;
XR. Xylary Ray; XPa. Xylem Parenchyma

PLATE 9
TS OF THICK ROOT; Secondary Phloem and Gelatinous Fibres

TS OF THICK ROOT; Calcium oxalate crystals near the Fibres

Cr. Crystals; GFi. Gelatinous Fibres; SC. Sclerenchyma;
SPh. Secondary Phloem

PLATE 10
4.2.3.3 Vessel elements

Vessel elements of different shape and size are seen spread in the powder (Plate 11 and 13). They are short and wide (Plate 13), long and slightly wide and long, narrow and cylindrical. They have wide, circular single perforations at the ends. The perforation may be horizontal or oblique. Pits on the lateral walls are wide dense and angular in outline (Plate 13). The vessel elements are of 140 – 210 μm long and more than 100 μm wide. The dimensions of vascular elements from mature stem are given in table 3. Since the source is mature stem the data mainly represent secondary tissue.

Table: 3 Dimension of vascular elements in *Dichrostachys cinerea*

<table>
<thead>
<tr>
<th>Types of cells</th>
<th>Stem – maceration (values in μ)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Minimum</td>
</tr>
<tr>
<td>Vessel</td>
<td>655.8x153</td>
</tr>
<tr>
<td>Fibre</td>
<td>1836.2x39.3</td>
</tr>
<tr>
<td>Tracheid</td>
<td>262.3x43.8</td>
</tr>
</tbody>
</table>

4.2.4 Leaf constants

The quantitative microscopy of the leaf demonstrating Stomatal number, Stomatal index, Vein islet number and Palisade ratio are presented in table 4.

Table 4 Quantitative microscopy of the leaf

<table>
<thead>
<tr>
<th>Determination</th>
<th>Ranges</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomatal number</td>
<td>36-43</td>
<td>39.5</td>
</tr>
<tr>
<td>Stomatal index</td>
<td>23-27</td>
<td>25</td>
</tr>
<tr>
<td>Vein islet number</td>
<td>74-89</td>
<td>82.33</td>
</tr>
<tr>
<td>Palisade ratio</td>
<td></td>
<td>2</td>
</tr>
</tbody>
</table>
XYLEM ELEMENTS IN ROOT POWDER

VE, Vessel Elements; Fi. Fibres; WFi Wide Fibre.

PLATE 11
NARROW FIBRES

NARROW FIBRES: Enlarged

NFi. Narrow Fibres

PLATE 12
SHORT WIDE VESSEL ELEMENT

LARGE NARROW VESSEL ELEMENT

LONG NARROW CYLINDRICAL VESSEL ELEMENT

Pe. Perforations; Pi. Pits; Ta. Tail.

PLATE 13
Over the more than 170 years since Schleiden declared that the cell was the fundamental unit in plants, microscopy has been applied to plant materials and thousands of microscopic characterizations have been developed for the botanicals used in ayurvedic, Chinese, Egyptian, and Western herbal medicine.

WHO (1998) has cautioned adulterations in botanical products, as complete or partial substitution of one botanical for another, excessive amounts of impurities such as foreign matter (e.g., dirt, twigs, insect fragments), deteriorated or substandard material, contaminants (e.g., excessive microbial contamination), or any condition that would otherwise lessen the claimed or expected value of the product.

The value of botanical microscopy in the quality assurance of herbal ingredients is remarkable and well acceptable globally. Virtually all national and international regulatory authorities accept botanical microscopy as one of four primary methodologies for the identification of crude drug materials: namely, macroscopic appearance, organoleptic characters, microscopic characteristics, and presence or absence of chemical substances (Houghton, 1998).

In whole material or material that is coarsely chopped or sliced, a deviation of the arrangement of tissues from what is expected in the material under examination is an indication of the presence of an adulterant. However, once material is powdered, adulterants can only be detected if the microscopist is astute enough to find fragments of tissue that are not characteristic of the botanical being studied or that occur at far higher or lower frequencies than expected (Stahl, 1973). Such fragments might include leaf epidermis with diagnostic trichomes and stomatal complexes, floral parts, crystals, or a crystal sheath surrounding a vein or attached to fibres.

Powdered aerial plant parts are far easier to identify than roots and rhizomes because many of their diagnostic features can be found in surface views of fragments; in underground organs, the arrangement of tissues that is diagnostic is lost in powders. In powdered samples, fragments of epidermis, cork, fibers, and vessels typically remain
intact and recognizable; however, delicate tissues such as the cambium and sieve cells are completely disintegrated and trichomes are often shattered, making characterization difficult. Crystals, which are highly diagnostic, may or may not separate from the tissue in which they occur, depending on how they are arranged in the plant. Unlike whole material, which can be identified using an authoritative description, powder cannot be reliably identified as to species unless it is compared against a Botanical Reference Material.

A group of species that can be diagnosed microscopically using whole material but that is extremely difficult to identify when powdered is the commercial species of *Echinacea* (AHP, 2004; Langer 2001). *E. angustifolia* and *E. pallida* roots remain difficult to distinguish macroscopically, even in whole form.

A number of other quantitative values are utilized for the microscopic evaluation of botanicals (predominantly leaves). These include palisade ratios, vein islet and stomatal numbers, and stomatal index.

The palisade ratio of many plants remains constant regardless of geographical location of the plant. The palisade ratio, however, is not applicable to monocot leaves due to a lack of consistent differentiation within the mesophyll (Mukherjee, 2002). Various botanicals exhibit consistent values that can allow for the differentiation of closely related species (e.g., *Erythroxylum* spp). The vein or veinlet termination is the ultimate free termination of a vein or branch of a vein (Trease and Evans 1966).

The quantification of stomata is a specific assessment tool for leaves. Although the stomatal number varies greatly with the age of the leaf, the stomatal index remains highly consistent.

The preceding discussion provides an understanding of cell and tissue types essential for the anatomical characterization of plant material. However, it is the arrangement of tissues within plant organs that is most critical for plant identification. For practical purposes, tissue arrangement is predominantly observed by viewing transverse (cross) sections. Longitudinal sections are not informative regarding tissue arrangement,
although radial longitudinal sections can provide diagnostic information about the type of secondary wall thickenings of tracheary elements and the kinds of secretory tissue present, as well as help to distinguish between fibers and sclereids.

In the present study, thus *Dichrostachys cinerea* has been characterized as a reference material to a degree so as to ensure the identity, purity, and consistency of the material. The reference material is representative of the species tested. For botanicals, this means that the identity of the plant has been confirmed botanically, macroscopically, microscopically and chemically, by molecular means.

### 4.3 Phytochemical analysis

The direct approach in drug discovery from herbal medicines is to isolate active ingredient(s) from the respective herbs or plant source. Whether or not this approach is feasible mainly depends on (1) the concentration of the bioactive component(s) in the herb or plant, (2) the degree of difficulty in purification, and (3) the availability of the herb or plant; in particular whether the plant is an endangered species. An impressive number of chemicals have been isolated either from medicinal plants or synthesized on the basis of natural lead compounds. Considering the extremely high cost and long time of new drug development, as well as the high drug attrition rate, an imminent task for pharmaceutical companies is to explore new ways for drug Research and Development. Pan et al. (2013), consider that herbal medicine as a source of new compounds for drugs is going to become a global trend in the pharmaceutical industry. Therefore, the use of herbal/plant medicine has been the single most successful strategy for the development of novel therapeutic agents, and this trend will be continued in the future.

#### 4.3.1 Phytochemical analysis of *Dichrostachys Cinerea*

Phytochemicals, as the word implies, are the individual chemicals from which plants are made. Of the roughly 350,000 species of plants believed to exist, one-third of those have yet to be discovered. Of the quarter million that have been reported, only a fraction of them have been chemically investigated. Table 5 given below explains the result of the preliminary phytochemical analysis in *Dichrostachys Cinerea*. 

46
Table: 5 Phytochemical analysis of leaf and stem of *Dichrostachys cinerea*

<table>
<thead>
<tr>
<th>Phytochemical test</th>
<th>Cold extract</th>
<th>Ethanol extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Leaves</td>
<td>Stem bark</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Saponin</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Tannin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Steroids</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Tri terpenoids</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Flavinoids</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Glycosides</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Note: + = Presence - = Absence

4.3.2 Analytical, preparative separation and detection of an alkaloid in leaf.

Modern methods of isolation of natural products, in contrast to traditional methods, utilize principles of extraction that are based on the polarity (relative solubility in organic solvents), solubility in water, and various alterational solubilities based on salts and pH (relative acidity or alkalinity). Chromatography is one of the most useful means of separating mixtures of compounds, as a technique to both purify the components and identify them. In chromatography, the mixture is separated by differential distribution of the components between a stationary phase and a mobile phase.

4.3.3 Thin Layer Chromatography (TLC)

The plate 14 shows the thin layer chromatogram developed for analysis of the alkaloid. The orange brown spot appeared with an *Rf* value of 0.6 indicate the presence of an alkaloid in the leaf ethanol extract of *Dichrostachys Cinerea*. The mobile phase used for the TLC separation is Toluene : Chloroform : Ethanol : Water with a ratio of 28.5 : 25.5 : 45.5 : 1.

Vennapoosa *et al.* (2013), though observed the presence of alkaloid in *Dichrostachys Cinerea* leaf methanol extract, reported absence in ethyl acetate and petroleum ether.
Crude under UV light

Crude and Eluvent under visible light

TLC CHROMATOGRAM

PLATE 14
The present study makes use the ethanol extract for the isolation and purification of the alkaloid from Dichrostachys Cinerea leaf.

In TLC, ultraviolet (UV) absorption of the mobile phase solvents does not play a significant negative role in detection and quantification of the analytes, because the mobile phase is evaporated from the plate prior to the detection. The same system is used to confirm the presence of alkaloid in thin layer chromatogram (Plate 14) in the present study.

4.3.4 Preparative Column Chromatography

Column chromatography is most often used for the preparative scale separation of components from a crude plant extract. In the present study ethanol leaf extract is used to elute alkaloids by using the mixture of toluene, chloroform, acetone and ethanol with the ratio and flow rate mentioned in materials and methods. The purity and \( R_f \) value of the eluate is further confirmed with TLC and presented in Plate 14.

4.3.5 High Pressure Liquid Chromatography (HPLC)

The High Pressure Liquid Chromatography Chromatogram (Figure 1) shows the peak absorption with ultraviolet (UV) detection at 214nm.

Figure 1: HPLC Chromatogram of Pure Sample

The HPLC Chromatogram also demonstrate the presence of alkaloid as eluant from the ethanol extract of the Dichrostachys Cinerea in the present investigation.
4.4 Partial characterization of the alkaloid

The most important tools for structure elucidation of natural products are nuclear magnetic resonance (NMR) (Günther, 1995) and mass spectroscopic (MS) (de Hoffmann and Stroobant, 2002) techniques. In addition, infrared (IR) and ultraviolet-visible spectrophotometric (UV-Vis) methods are of importance. Also hyphenated techniques such as gas chromatography (GC)-MS, liquid chromatography (LC)-MS, and LC-NMR are handy. These techniques provide separation methods coupled with structural (spectroscopic) information. Although a very powerful analytical method, x-ray crystallography requires extreme sophistication (Stout and Jensen, 1989).

4.4.1 UV-Vis Spectroscopy

The ultraviolet visible spectroscopy plays an important role in detection of alkaloids in separations. The UV-Visible spectra (Figure 2) show absorption maxima at 214 nanometre in the UV range. The result is found to be in agreement with High Pressure Liquid Chromatography Chromatogram having the peak absorption detected at 214 nm by UV detection.

![Figure 2. UV Spectrum of Alkaloid (Eluate)](image)
4.4.3 LC-MS

LC-MS has grown into one of the most important and most widely used analytical techniques in natural products analysis with the introduction of Atmospheric Pressure Chemical Ionization and Electro Spray Ionization interfaces, which allow for the analyses of compounds regardless of their volatility. However, ESI and APCI tend to give only molecular weight information of the compounds. The analytical LC chromatogram of the column chromatography eluate (alkaloid) is given in Figure 3. The UV detection of the peak is maintained at 214 nm as in the case of HPLC.

The MS profile of the alkaloid depicted in Figure 4, accounts for many functional groups at different m/z ratio. The M+ peaks are obtained at 74, 102, 116, 216, 336, 474 and 502.

![Figure 3 LC Chromatogram of Alkaloid (Eluate)]
4.4.2 FT IR Spectroscopy

For IR measurements, it is common to report wavelengths in terms of wave numbers $\nu$ (cm$^{-1}$). All observable IR bands are due to the interaction of the electrical vector of the electromagnetic radiation with the electric dipole of non-symmetrical bonds. It turns out that IR spectroscopy can easily be used as a semi-empirical method for structural analysis because it was observed that there is a good correlation between the position of band maxima and organic functional groups or structural characteristics.

The group frequencies corresponding to the respective peaks found in the alkaloid are Peak 1- $R_2 = \text{CH}_2$, Peak 2,3 & 4 - C – O, Peak 5 - OH, Peak 6 and 7- Aromatic, Peak 8 C = C, Peak 9 – Esters and Amides, Peak 10 –C=C , Peak 11 – C=C, Peak 12, 13 and 14- C – H, Peak 15 – Alcohols and Phenols, Peak 16 and 17- N- H, Peak18- O – H (carboxylic acid). (Figure 5 and table 6)
Figure 5: FTIR Spectrum of Alkaloid (Eluate)

Table 6 FTIR Absorption Peak Vs Intensity
4.4.5 GC-MS

The combination of gas chromatography (GC) and mass spectrometry (MS) for the detection and identification of constituents of essential oils has become a powerful analytical tool in phytochemical analysis. The GC-MS combination allows for the separation of secondary metabolites and the acquisition of mass spectra of the separated components. Utilization of GC retention data along with MS fragmentation and comparison with spectral libraries allows for compound identification.

Figure 6: Total Ion Current Chromatogram of the Eluate (Pure) Sample

RT = Retention time; RI = retention index; TIC = total ion count; Area = % based on TIC; The gas chromatogram (Fig. ) shows the peak with a relative abundance of 100 for the peak with Retention time 38.42.
The MS spectrum of the Eluate (Pure) Sample with RT 38.42 (Figure 7) demonstrates m/z76, 113.2, 132.1, 149, 167, 279. . . The MS Spectrum shows only less fragmentation, insufficient for the full characterisation of the compound.

4.4.6 NMR Spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is becoming a powerful tool in the simultaneous analysis of many different compounds, and coupled LC-NMR has recently made some stunning advances in the analysis of natural products.

4.4.6.1 $^{13}$C NMR

The nuclear Overhauser effect is of great importance for $^{13}$C NMR. Typically, we run $^{13}$C NMR as $^1$H-decoupled spectra. This means that we saturate the proton frequencies. As a consequence, we do not observe couplings between $^{13}$C and $^1$H, so the carbon spectra are just single lines. Furthermore, due to the NOE, the intensity of the carbon signals is further increased. Due to the decoupling, we obtain only chemical shift information, and $^{13}$C-NMR spectra are much easier to analyze. In order to get proton information (i.e., how many protons are attached to a carbon), we need to turn off the decoupling that would result in very little signal intensity, limit the decoupling through
off-resonance decoupling, or use different mechanisms to determine the number of protons attached to a carbon (Vogler and Setzer, 2006).

The $^{13}$C NMR spectrum of the designated alkaloid is given in Figure 8 and Figure 9.

The spectra have been interpreted by comparing the reference spectra provided by Pretsch et al. (2009). The chemical shift ($\delta$, in ppm) ranges of carbon nuclei observed from the spectra are that of amines (10 – 60, 75 – 120) and other nitrogenous compounds (10 – 50, 55 – 110). Further, as a whole the spectra are indicative of aromatic compound.

4.4.6.2 $^1$H NMR

The $^1$H NMR spectra have been interpreted by comparing the reference spectra provided by Pretsch et al. (2009). The chemical shift ($\delta$, in ppm) ranges of hydrogen nuclei observed from the spectra are indicative of the presence of aromatic compounds in general and amines ($\delta$, in ppm, 0.5 – 4, 2.5 – 5, 2.3 – 3.1, 2.5 – 3.5, 3 – 3.7) and other nitrocompounds ($\delta$, in ppm 4.2 – 4.6) in particular. The $^1$H NMR spectra are presented in Figure 10, 11 and 12.

The complete $^{13}$C NMR as well as $^1$H NMR assignment for the alkaloid is not possible with the available data due to the fragmentation of the output result. However, the spectral data obtained in the present study through various techniques such as UV – Visible, MS, IR, $^1$H and $^{13}$C converges to the same conclusion that an alkaloid with high molecular weight is the eluant (pure) compound. Also the alkaloid could be assigned to aromatic heterocyclic group. The large size of the alkaloid itself suggests more sophisticated technologies such as x ray diffraction analysis for the complete elucidation of the structure.
Figure 8: $^{13}$C NMR Spectrum of Eluate (Pure) Sample
Figure 9: $^{13}$C NMR Spectrum of Eluate (Pure) Sample
Figure 10: \(^1\)H NMR Spectrum of Eluate (Pure) Sample
Figure 11: $^1$H NMR Spectrum of Eluate (Pure) Sample
Figure 12: $^1$H NMR Spectrum of Eluate (Pure) Sample
4.5 Bioactivity Studies

The driving force behind much phytochemical research is the discovery of new biologically active compounds for medicinal or agricultural uses. According to Setzer and Vogler (2006), biological assays, then, must be carried out in order to identify promising plant extracts, to guide the separation and isolation, and to evaluate lead compounds.

4.5.1 Antimicrobial assay

The emergence of pathogenic microbes with increased resistance to established antibiotics provides a major incentive for the discovery of new antimicrobial agents. Antimicrobial screening of plant extracts and phytochemicals, then, represents a starting point for antimicrobial drug discovery. The present study utilizes the disk diffusion technique to evaluate the antimicrobial potential of the alkaloid (Eluate) with respect to crude ethanol extract of *Dichrostachys Cinerea*. The result of the experiment is represented graphically (Figure. 13 - 18).

4.5.1.1 Antibacterial assay

The result shows that except *Salmonella* all other bacteria are susceptible to the eluate demonstrating the antibacterial activity of the crude extract supplemented by the alkaloid. However, the enhanced activity by the crude extract may be due to the synergistic action of the other components in the extract as suggested by Spelman and Duke (2006). Therefore, the alkaloid could be further worked upon in the scenario that established antimicrobial drugs have become less effective against many infectious agents.
Figure 13: Antimicrobial activity of Crude and Eluate against *Staphylococcus* bacteria
Figure 14: Antimicrobial activity of Crude and Eluate against *Pseudomonas* bacteria
Figure 15: Antimicrobial activity of Crude and Eluate against *Salmonella* bacteria
Figure 16: Antimicrobial activity of Crude and Eluate against *Streptococcus* bacteria
Figure 17: Antimicrobial activity of Crude and Eluate against *E. coli* bacteria
4.5.1.2 Antifungal assay

Antifungal assay was carried out with *Penicillium* and *Mucor* as test organisms. The results of the study are detailed in figure 18. Compared to ketoconazole, an effective fungicide control, both crude ethanol extract and the eluate (Alkaloid) showed remarkable inhibitory effect on both test organisms.

![Antifungal Activity](image)

**Figure 18: Antimicrobial activity of Crude and Eluate against *Penicillium* and *Mucor* Fungi**

4.5.2 Antioxident assay

4.5.2.1 DPPH scavenging activity

DPPH radical scavenging activity of *Dichrostachys Cinerea* ethanol extract and pure (Eluate) are presented in the figure 19. From the results, it is clear that Both crude (50.23%) and alkaloid sample (34.56%) are possessing significant inhibition percentage. This study confirms that the extract of *D. cinerea* contains more antioxidant phytochemicals. Further, the DPPH radical scavenging activity of the alkaloid when compared to the crude extract is promising.
4.5.2.2 Reducing Power Assay:

The potassium ferricyanide reduction method was used to evaluate the reducing power of the plant extract and the alkaloid, which is a widely used method in measuring antioxidant activity of phyto compounds.

In this assay, the antioxidants presented in the test solution can reduce the Fe3+/ferricyanide complex to the ferrous form by donating an electron. The colour of the test solution then changes from yellow to different shades of green and blue, which depends on the reducing power of each sample. In the present study, as shown in Figure 20, Crude extract exhibited the strongest reducing power (0.62 ± 0.001) and had no significant difference when compared with ascorbic acid (0.732 ± 0.046), while alkaloid sample showed less (0.302 ± 0.01) at a concentration of 1 mg /ml. Indicating that the
reducing power of both crude and pure sample is highly related to the amount of alkaloids compounds present in the extracts, which can serve as electron donor to terminate the radical chain reaction.

**Figure 20: Reducing power of Crude, Alkaloid (Eluate) and Ascorbic Acid**

4.6 Conclusion

The hugely diverse plant kingdom, consisting of some 250,000-300,000 species, continues to evolve and adapt to a multiplicity of environmental conditions and to protect from pathogens and predators. Whether by serendipity or design the human species appears, in contrast, to have stabilised its genetic code. Many characterised human endogenous receptors, important in physiological function, are activated by plant-derived chemicals; for example the opioid, and the more recently discovered cannabinoid, receptors. It is not unreasonable to hypothesise that many more structure-activity relationships, of physiological and pharmacological significance, involving plant molecules have yet to be characterised.
The present work conceived from the folklore medicine and necessitated by lack of organised studies throws light on the invasive growth of the population and evolution of secondary metabolism. The Pharmacognostic studies carried out in the present study is with an aim of filling the gap of a reference material. The phytochemical studies accounts for the periodic evaluation of the secondary metabolite profile of *Dichrostachys Cinerea*. The attempt to characterise the eluate (Alkaloid) limited to the identification of certain functional groups of the alkaloid. The future plan of the present investigation will need to focus on the pharmacognostic studies on morphologically similar plants such as *Acascia nilotica*, a possible adulterant. Structural elucidation studies starting from preparative HPLC purified Eluate (alkaloid) to end up with the structure of the alkaloid for pharmacological studies.