CHAPTER – I
INRODUCTION

1.1 General Introduction

Chemistry and natural product chemistry in particular, is a very broad subject that bears a profound relationship with all phase of drug discovery, design and development. Natural product chemistry is the study of primary and secondary metabolites synthesized by plants or animals. Natural products have been a fertile area of chemical investigation for many years, driving the development of both analytical chemistry and of new synthetic reactions and methodologies.[1,2]

Nowadays several drugs are synthesized in the lab, which are copies or just modifications of naturally occurring compounds obtain from plants. These drugs are used for treatment of different disease such as anti-fungal, anti-microbial, anti-infective agent, analgesic, anti-tumor, and anti-viral etc.[3] Plants have been proved to be an important natural source of compounds for many drugs and large numbers of compounds that have been isolated so far are currently under clinical trials. The use of plant and plant products as a medicine could be tracked back from the beginning of the human civilization. It is Ayurveda; a traditional practitioner where medicinal plants are used for treatment without knowing the actual bioactive constituent of plants.[4] Nowadays beside Ayurveda, allopathic, and homeopathic medicinal system are in used, through which plant products become an alternative source of medicine for living beings.

Research in chemistry of natural products has endless potential and especially important for the country like Nepal which has a rich biodiversity. Himalayan country Nepal, the presence of extreme range of altitude, climate, and soil within a small geographical area has created a striking vertical zonation and diversity in flora and fauna. Nepal extends along the Himalayan range between the latitudes of 36^{o} 22´ N and 30^{o} 27´ N to longitudes of 80^{o} 04´ E and 88^{o} 22´ E. Two-third of the area is occupied by hills and mountains, with the altitude ranges from 60 to 8848 m above the mean sea level. Plant diversity in Nepal can be illustrated from the fact that over 1000 of species of Himalayan plants have originally been discovered and described from Nepalese flora.[5] Among the 7000 species of medicinal plants recognized all

over the world, more than 900 type of precious medicinal plants are said to be found in Nepal.[6] Because of extraordinary biodiversity and presence of rare and more valuable crude drugs of vegetable origin Nepal has been regarded as natural showroom of bidiversity.[7] More than 700 species of medicinal plants grow wild in the country, majority of which are used in folk herbal remedies. However, in Nepal over fifteen thousand tons of medicinal herbs representing some 100 species are harvested from the wild for commercial and industrial purpose and large number of medicinal herbs collected from the forests and pastures, and traded for foreign country.[8] The uncontrolled commercial extraction has significantly eroded the country's medicinal plant resources and particular species have gradually become more difficult to find in a given locality once where they flourished.

1.2 Description of the Plants

Ageratum houstonianum
Family: Asteraceae
Common name: Blue goat weed
Biological activity: antifungal[9]
antidiabetic[10]

Calotropis gigantea
Family: Asclepiadaceae
Common name: Aank
Biological activity: antipyretic, sedative, analgesic, hepatoprotective, anti-diarrhoeal[11]

Ageratum houstonianum
Family: Apocynaceae
Common name: Baramase phool
Biological activity: anti-diabetic, antimalarial, antioxidant, anticancer etc.[12,13]

Thevetia peruviana
Family: Apocynaceae
Common name: Pahelo karbir
Biological activity: antifungal, antibacterial, antitermite etc.[14]

Thevetia peruviana (seed)
Family: Apocynaceae
Common name: Pahelo karbir
Biological activity: antifungal, antibacterial, antitermite etc.[14]

1.3 Pancreatic Cancer

Cancer is characterized by proliferation of abnormal cells which multiply out of control, destroying healthy tissue and endangering life. The uncheck growth of cancer cells spreads throughout the body, interfering with the ability of cells, organs, and other structure to perform their normal function. Cancer is presently responsible for 25% of death in developed country and 15% of all death worldwide. It can therefore,

be consider as one of the foremost health problem, with about 1.45 million new cancer cases being expected yearly.

Anticancer chemotherapy being a very active field of research, nowadays, a huge amount of information on this topic can be generated every year. Most of anticancer drugs work by interfering DNA synthesis that kill the tumor cells that are rapidly growing, stop mitosis or actual splitting of cells and halt the process of cancer growth. Some drugs causes cells to under- go apoptosis (so called “self programmed death”). However, chemotherapeutic drugs are sometimes feared because of their toxic side effects. Most of anticancer can induce genotoxic, carcinogenic, and tetratogenetic effect in non-tumor cells.[15,16] Because of side effect in non-tumor cells of chemotherapeutic agents despite their high efficacy in the killing of target malignant, there is need of alternative or complementary drugs that are effective on cancer cells and show minimum toxicity on normal cells.

Cancer can be classified in two ways: by the type of tissue from which they arise and by the location in the body from which they start to growth. The first ways is known as classification by histology and is define internationally. The second method of classification is not very useful to clinician but very common to general public to talk about cancer as being cancer in the breast or lung or skin etc. There are five major histological class of cancer[17] as Carcinoma, Sarcoma, Myeloma, Leukemia, and Lymphomas.

Pancreatic cancer is a malignant neoplasm originating from transformed cells arising in tissues forming the pancreas. The most common type of pancreatic cancer, accounting for 95% of these tumors, is adenocarcinoma (tumors exhibiting glandular architecture on light microscopy) arising within the exocrine component of the pancreas. A minority arises from islet cells, and is classified as neuroendocrine tumors. The signs and symptoms that eventually lead to the diagnosis depend on the location, the size, and the tissue type of the tumor, and may include abdominal pain, lower back pain, and jaundice (if the tumor compresses the bile duct).

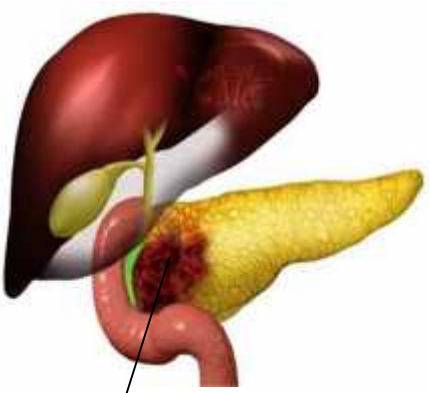


Fig: 1. Tumor on pancrease

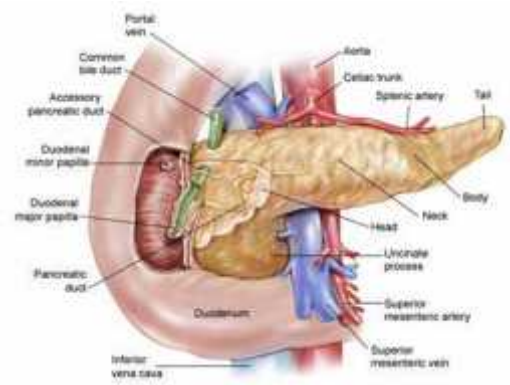


Fig: 2. T.s of pancreas

Pancreatic cancer is the fourth most common cause of cancer-related deaths in the United States[18] and the eighth worldwide.[19] Pancreatic cancer accounts for about 220,000 death each year in Asia pacific region, the risk factors as being smoking and diabetes for pancreatic cancer.[20] Human pancreatic cancer cells such as PANC-1 are known to exhibit marked tolerance to nutrition starvation that enables them to survive for prolonged period of time even under extremely deprive condition. Thus, elimination of this high tolerance to nutrition starvation as a novel approach in anticancer drug development.[20]

Plants secondary metabolites have an excellent source of new anticancer drugs. There are four major structural classifications of plant derived anticancer compounds viz; Vinca alkaloids, Epipophyllotoxin ligans, Taxane diterpenoids, and Campotothecin quinoline alkaloid derivatives. Vinca alkaloid belongs to important class of anticancer drug causes characteristic block during mitosis leading to apoptosis. Two Vinca alkaloids, namely, Vinblastine and Vincristine obtain from *Catharanthus roseus* commonly used in combination with others drugs for the treatment of cancers, such as lymphomas and leukemia.[21,22] Vinorelbine (VRBL) and Vindesine (VDS) are two semisynthetic analogous obtain from *C. roseus*, shows potential activity on leukemia's, lymphomas cancer, advance testicular cancer, breast cancer, lung cancer, and kaposi's sarcoma when treated in combination with other theraputic drugs.[23] Paclitaxol (taxol) is obtain from the bark of *Taxus brevifolia* and another species *Taxus baccata* (Lothsalla) have also been in use for cancer chemotheraphy.[24]

1.4 Justification of Study

Nepal occupies central part of great Himalayan range, its flora lie in transition zone between flora of west Himalayan and that of east Himalayan region. The Himalayan country Nepal is rich in many medicinal plants. These plants may have anticancer, antimicrobial, antifungal, antioxidant properties. Some medicinal plants may possess poisonous character. However, In Nepal quantitative analysis of the effort of extraction on natural plant population is still promising. Indiscriminate collection, not in accordance with any regulatory procedure or recognized management practices, has threatened the survival of some valuable species and reduces the quality of medicinal plants. Therefore, it is need to develop some effort in isolation of bioactive constituents of medicinal plant for the proper use. This research work is done to study the anticancer properties of some poisonous medicinal plants and also find out the biological activities of these plants, which may become a milestone in the field of anticancer drugs development.

1.5 Hypothesis and Objectives of the Study

Research in Chemistry, in last few decade, have shown interest in poisonous medicinal plants because they have been found as immense source of bioactive compounds especially in the field of cancer research. Therefore, the present research is focused mainly on the following objectives.

General objectives:

To study the bioactive constituents of some poisonous medicinal plants and to judge the medicinal value of isolated compounds in the field of anticancer drug development, along with their structure elucidation.

Specific objectives:

1. Qalitative phytochemical screening of four different poisonous medicinal plant species namely *C. gigantean, A. houstonianum, C. roseus,* and *T. peruviana* collected from central Nepal, Chitwan.
2. Brine Shrimp bioassay of crude methanol extract of respective plants.
3. Determination of anticancer and antimicrobial property of the methanol

extract of respective plants.

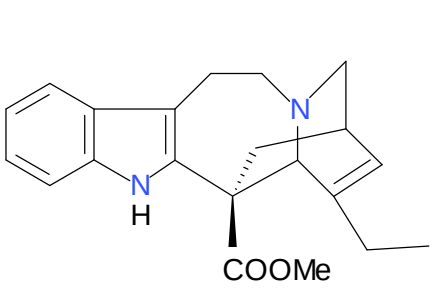
4. Isolation and identification of chemical constituents from the methanol fraction of aerial parts *C. gigantea* collected from central Nepal, Chitwan

CHAPTER-II

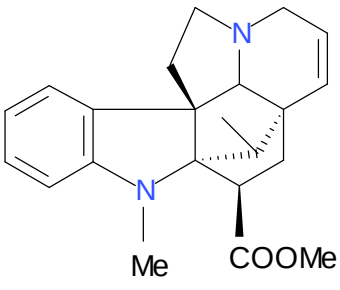
LITERATURE SURVEY

Medicinal plants being immense source of phytochemicals, the search of new pharmacologically active agents from natural resources such as plants and animals and microbs led to discovery of many clinically useful drugs.

Catharanthus roseus has long been cultivated for herbal medicine and as an ornamental plant. In ayurveda (traditional medicine) the extract of its roots and shoots those poisonous, was used against numerous diseases.[25] It has a variety of medicinal properties such as anti-bacterial,[26] antifungal,[27] antiviral,[28] and anticancer.[29] *Catharanthus roseus* has been found to be contained more than 120 Terpenoid Indole Alkaloids (TIAs), and many of the alkaloids exhibit strong pharmacological activities.[30] Vindoline, Vindolinine, Catharanthine, Vincristine, and Vincaleukoblastine are some indole alkaloids that have been isolated from aerial parts *C. roseus* by centrifugal partition chromatography in the pH-zone refining mode.[31] Previous studies have identified significant active compounds in *Catharanthus roseus* including Vincaleucoblastine or Vinblastine (the first isolated vinca alkaloid), Vincristine (anticancer), Ajmalicine (antihypertensive), and Serpentine (sedative).[30,32] Recently study of the crude aqueous *C. roseus* extract showed invitro cytotoxic effect on Jurkat cells and normal peripheral blood mononuclear cells (PBMc).[33]



Catharanthine



Vindolinine

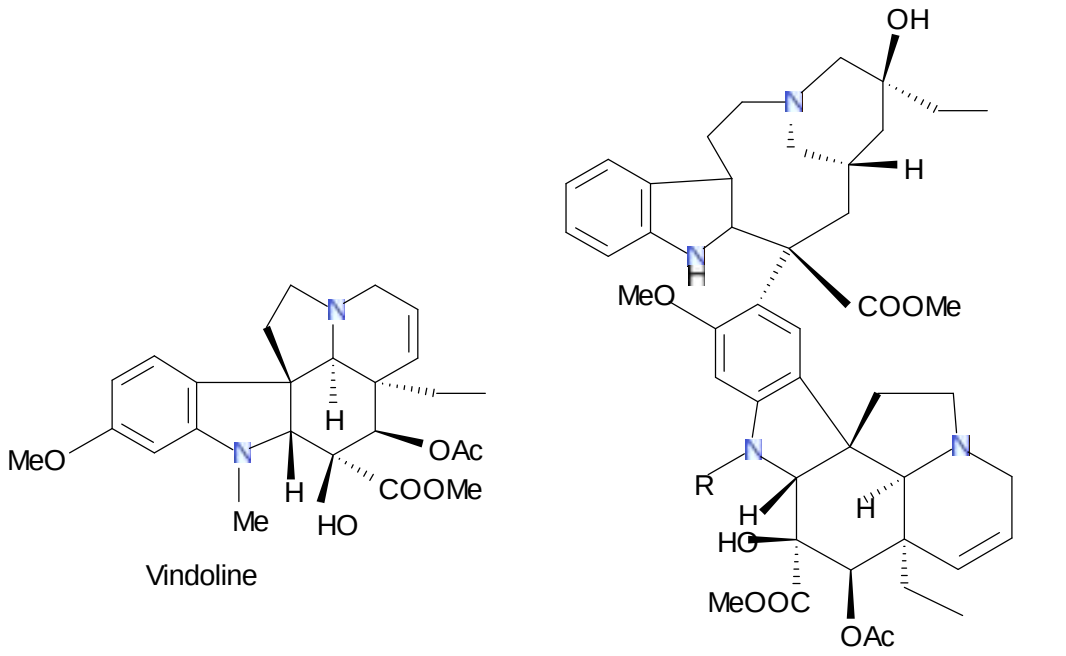
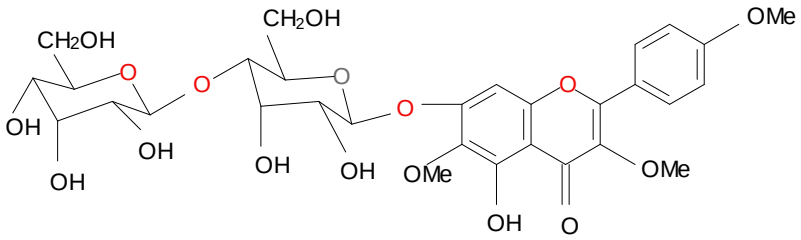
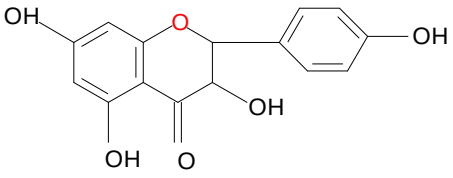


Fig: 3. Structures of interesting alkaloids found in the aerial parts of *Catharanthus roseus*.

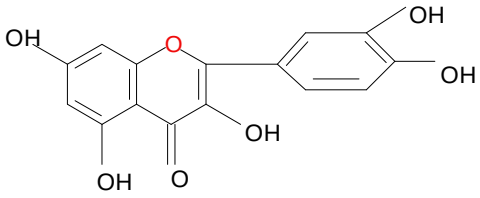
Thevetia peruviana is particularly known for its ability to produce cardiac glycoside such as nerrifolin and peruvoside, which have relatively high therapeutic index compared to that of digoxin.[34] It contains a milky sap which contain compound; thevetin, that is used as heart stimulant.[34] The fresh flowers of *Thevetia peruviana* have been found to contain quercetin, kaempferol and quercetin 7-0-galactoside.[35] Few reports are available regarding to tissue culture of this medicinally potent plant species i.e. somatic embryogenesis.[36] The flavonol glycosides isolated from the leaves of *T. peruviana* has inhibitory effect against HIV-1 reverse transcriptase and HIV-1 integrase.[37] The seed oil obtain from *T. peruviana* seed was used to make paint with antifungal, antibacterial, and antitermite properties.[14] Recent study on the, "Phytochemical evaluation and anti-spermatogenesis activity of *Thevetia peruviana* methanol extract on male albino rats'' showed the inhibition of spermatogenesis in rats, indicating the possibility of developing a herbal male contraceptive.[38]



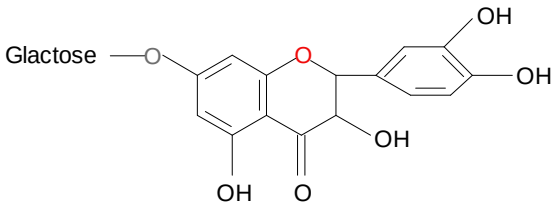
Flavone glycoside



Quercetin



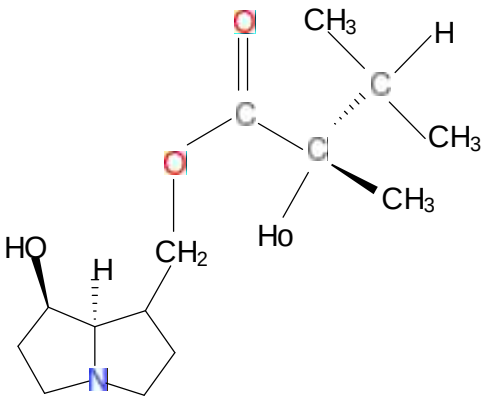
Kaempferol



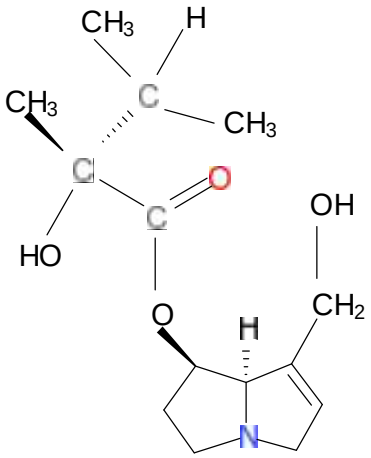
Quercetin-7-o-galactoside

Traditionally, *Ageratum houstonianum* was reported to be used as a poultice for tumors.[39] several phytoconstituents have been reported earlier from *Ageratum houstonianum*. Flavones like agehoustin A, B, C, D, eupalestin, agecorynin C[40,41] and Pyrrolizidine alkaloids like heliohoustine, lycopsamine, and retrohoustine[42] reported from the aerial parts of the plant. From the flowers flavones agehoustine E, F, G, lucidine dimethyl ether[43] reported. Triterpenes friedlin, friedelanol[44] and steroids β-sitosterol, stigmasterol[45] reported from the entire plant. Oxygen heterocycles ageratone, ageratum benzofuran[46,47,48] from the roots and Precocenes were reported from the leaf, stem, root, flowers and leaf essential oil of the plant.[49] The leaves and essential oil of the leaf reported for antifungal activity.[50] Besides already known lycopsamine, three new alkaloids were isolated from the aerial parts of the *Ageratum houstonianum* and their structure was eluciadted by spectrocopic means.[51] Again, study of hydro alcoholic extract of whole plants of *A. houstonianum* for its anti-

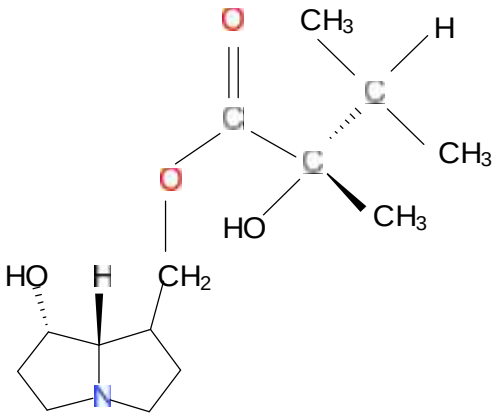
diabetic properties shown the good possibility of this plant as anti-diabetic properties.[10]



Retrohoustine

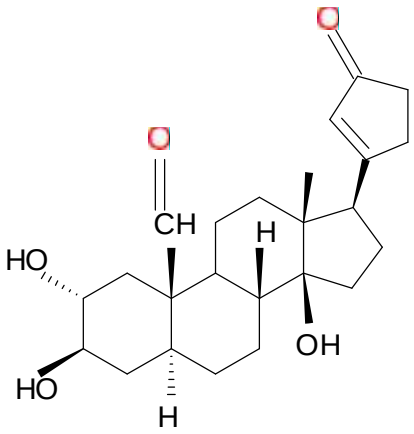


Isoretrohoustine

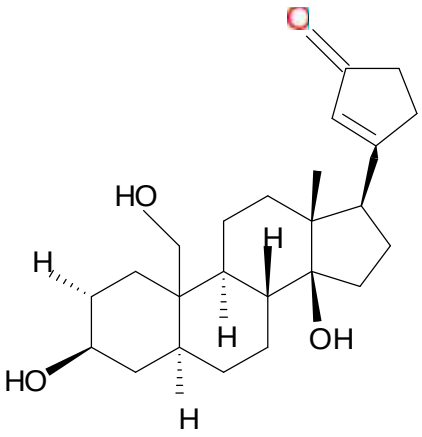


Heliohoustine

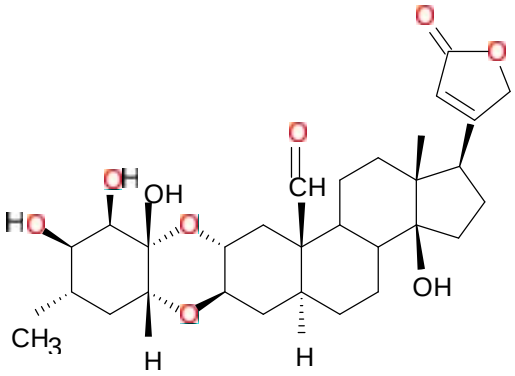
Chemical investigation *of Calotropis gigantea* reported the isolation of different types of phytochemicals such as cardenolides, flavonoids, glycosides, triterpenoids, steroids, anthocyanins etc. Along with different phytochemicals, considerable amount of macro and micro nutrients were documented in this plant. The leaves and latex of *C. gigantea* were found to have cardiac glycosides as Calotropagenin, Calotropin, Uscharin, and Calotoxin.[52,53] Three cardenolide glycosides as Coroglaucigenin, Frugoside and 4´-o-β-D-glucoryrenosylfrugoside, were documented from the root of *Calotropis gigantea* which are cytotoxic against various cell line of human origin.[54]



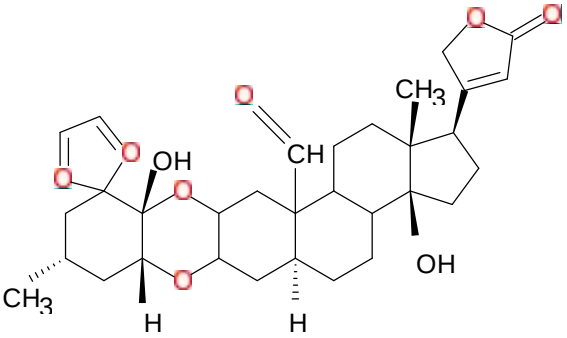
Calotropagenin



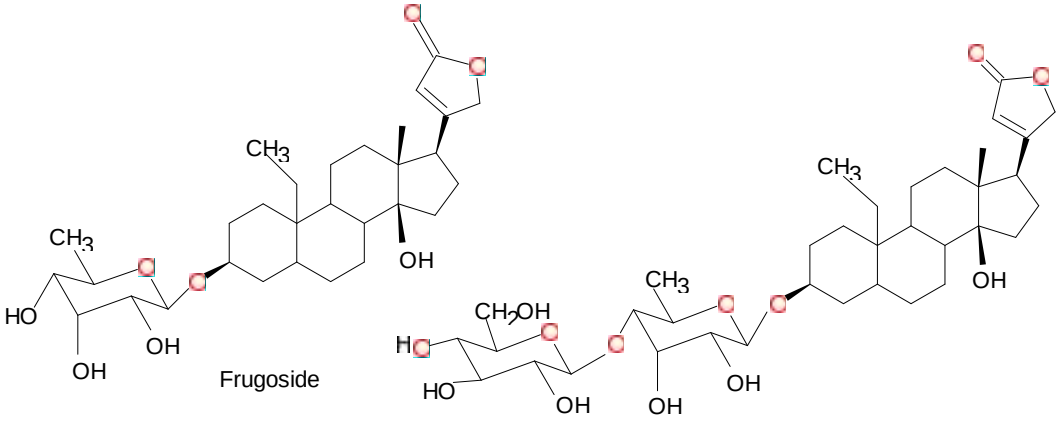
Coroglaucigennin



Calotropin



Uscharin

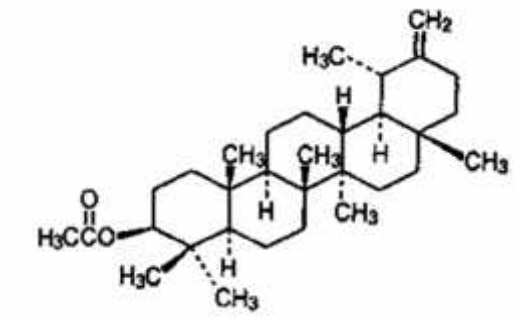


Frugoside

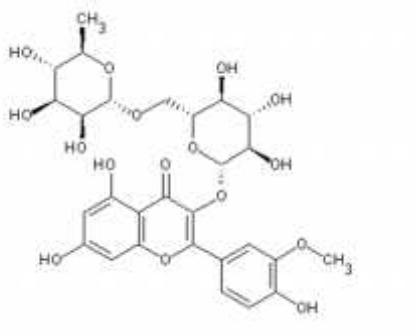
4-β-D-glucoside

Two new oxypregnane-oligoglycosides named Calotroposide A and Calotroposide B have been isolated from the root of *Calotropis gigantea*, and their chemical structure have been isolated by chemical and spectroscopic methods.[55] Giganticine, a novel insect anti-feedent non-protein amino acid has been isolated from methanol extract of

the root of *C. gigantean*.[56] Beside, the isolation and characterization of flavonols namely Isorhamnetin-3-O-rutinoside, Isorhamnetin-3-O-Glucopyranoside, Taraxasteryl acetate, a new flavonol trisaccharide was isolated from aerial part of *C. gigantea* and its structure was established as Isorhamnetin-3-O-(2-O-β-D-galactopyrenosyl-6-α-L-rhamnopy)-β-D-glucopyranoside by a combination of fast atom bombardment mass spectroscopy, ^{1}H-NMR and ^{13}C-NMR spectra and some chemical degradation.[57]

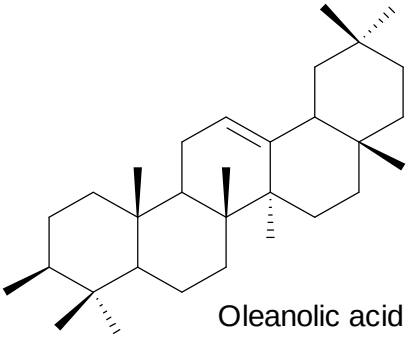


Taraxasterylacetate

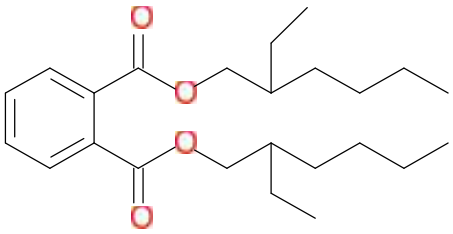


Isorhamnetin-3-0-rutinoside

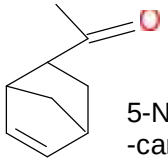
Again GC-MS analysis of the chloroform extract of aerial parts of *C. gigantea* revealed the presence of eight different types of terpenes namely 5-norborene-2-carboxylic acid, Phytol, Squalene, α-amyrin, β-amyrin, Oleanolic acid, Taraxasterol etc.[58] Various chemical constituents found in Calotopis gigantea have been found to be shown anticancer properties. Recently, di-(2-ethylhexyl) phthalate (DEHP), a triterpenoids isolated from flower showed anticancer activity against Ehrlich asites carcinoma cells (EAC) in Swiss albino mice.[59]



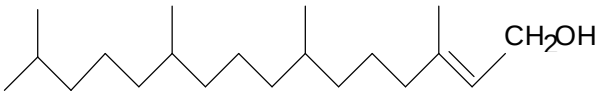
Oleanolic acid



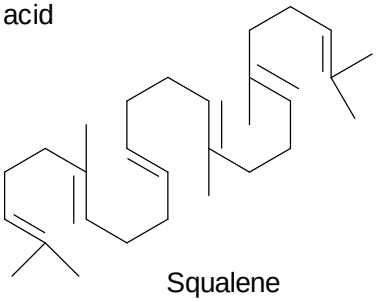
Di-(2-ethyalhexyl)phthalate



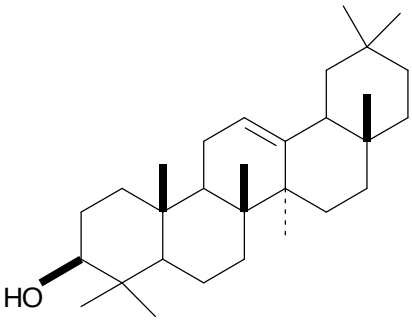
5-Noebornene-2
-carbocyclic acid



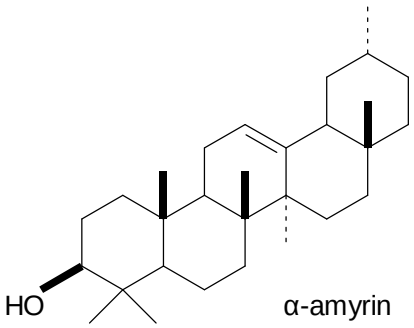
Phytol



Squalene

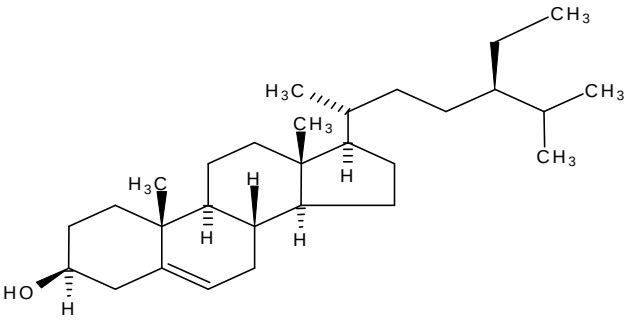


β-amyrin

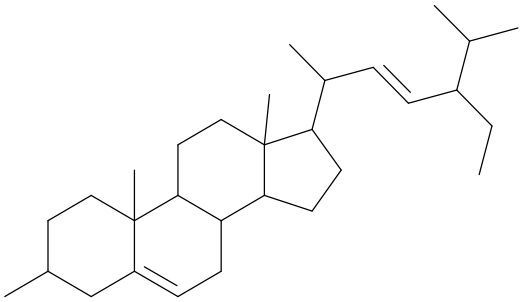


α-amyrin

Sterols occur as fats and oils in animals and plants. Sterol obtained from the plant sources is phytosterol and has 1,2-cyclopentanoperhydrophenanthrene unit. Stigmasterol and β-sitosterol are the two major sterol that have been isolated from root bark of *C. gigantea*.[60]



β- sitosterol



Stigmasterol

Beside, this various type of mineral such as phosphorous, potassium,[61] calcium, magnesium, zinc, copper, iron, manganese, boron, molybdenum, arsenic and etc. have been reported from this plant.[62,63] Among the various micronutrients, the amount of Iron (156.3 ppm) was the highest and molybdenum (0.1 ppm) was the lowest amount reported from the leaf sample of *C. gigantea*.[58]

CHAPTER-III

EXPERIMENTAL

3.1 Material

3.1.1 Equipments

1. Rotary evaporator
2. UV- Visible spectrophotometer (Shimadzu Spectrophotometer)
3. Incubator
4. UNITY plus-500 NMR spectrometer
5. Pre-coated TLC plates of E. Merck Company

3.1.2 Chemicals

1. Silica gel (mess no. 60-120)
2. DPPH
3. Dulbecco's modified Eagle medium (DMEM)
4. Nutrient Deprive medium (NDM)
4. Phosphate buffer (PBS)
5. WST-8 cell counting kit solution
6. DMSO
7. Methanol
8. Sea water

3.2 Methods

3.2.1 Plant Collection

Four different species of plants were collected from Central Nepal, Chitwan. The taxonomic identification of the plants was confirmed by Prof. Dr. R.P Chaudhary (CDB) TU, Prof. Dr. Mohan Siwakoti (CDB) TU, Mr. Bishanu Bhattrai, Assistant lecture, Birendra Multiple College, Chitwan and Mr. Mitra Pathak, Assistant officer,

National Herbarium Centre Godawari. The collected fresh plants materials were washed under tap water to remove other contamination such as dust, soils etc. The clean plants were air dried in shade for few days. The dried plants were ground into powder and stored in a labeled airtight bag.

3.2.2 Preparation of Plants Extract

100 gm of powder of each plant were extracted by maceration in methanol (200ml) for 48 hrs with frequent agitation. The mixture was filtered through muslin cloth followed by double filtration by filter paper. The same process was repeated for 4/5 times for complete extraction and filtrate was concentrated by Rotary Evaporator.

3.2.3 Phytochemical Screening

The method employed for phytochemical screening was based on the procedure put forward by Prof. I. Culie.[1] This method involves the selective and successive extraction of the plant material by using solvent of increasing polarity. By using different specific reagents, the presence of main groups of natural products was analyzed. The procedure is given in detail in Annex I.

3.2.4 Biological Screening

Biological screening involves the study of the effect of the crude plant extracts/fractions and isolated compounds at arbitrarily fixed dose levels in a species of organism and prediction of its effect over the entire dosage range. The present dissertation work involves the screening of methanol extract of five different plants for their brime-shrimp bioassay, anti microbial test and preferential cytotoxicity.

3.2.4.1 Brine Shrimp Bioassay

One of the objectives of this research work is to isolate and characterized the biologically active components. In this regard the crude methanol extract of different plant species were tested for their pharmacological potential by brine-shrimp bioassay. The procedure followed for the brine-shrimp toxicity assay was carried out according to Mayer *et al.* procedure.[64] This method is rapid inexpensive and simple technique to evaluate toxicity of crude extracts and isolated pure compounds.

A. Method

Brine Shrimp Bioassay process involves introducing the newly hatched brine shrimp naupalii (Atremia salina) to the crude plant extract/fractions or the isolated compounds. The method determines the LC_{50} values (µg/ml) for the isolated compounds and crude extracts by probit analysis[65]. Compounds of LC_{50} values less than 1000 ppm are considered as potentially pharmacologically active.

B. Required Materials

1. Eggs of Brine (*Atremia salina*)
2. Beaker of hatching
3. Test tubes (2x5cm)
4. Artificial sea water
5. Disposal pipette
6. Micro pipette

1. Sterilization of apparatus

All the apparatus were properly sterilized before using for bioassay.

2. Preparation of artificial sea water

Freshly prepared artificial sea water needed for entire bioassay was prepared by dissolving the following chemicals in distilled water.[66]

Table No. 1: Composition of artificial sea water

S.N.	Composition	Amount (g/l)
1	NaCl	23.50
2	Na_2SO_4	4.00
3	KCl	0.68
4	H_3BO_3	0.027
5	$MgCl_2.2H_2O$	10.68
6	$CaCl_2.2H_2O$	1.48
7	$NaHCO_3$	0.197
8	Na_4EDTA	0.0003

3. Hatching of shrimps

For the hatching of the shrimps, the brine shrimps eggs (about 50 mg) were sprinkled on the beaker filled with artificial sea water and illuminated with table lamp (60 watt) for 48 hours with temperature adjusted at 30^{o}C.

4. Preparation of samples

Twenty milligram of each sample to be tested was dissolved in 2 ml of DMSO. The solution thus prepared was used as stock solution. The test was conducted in test tubes and sterilized sea water (final volume 10 ml). Each of the extracts was tested at 1000, 100 and 10 µg extract per ml. The concentrations were obtained by transferring the corresponding volume from the stock solution to total nine test tubes for evaporation. After evaporation, 5 ml of sea water was added to each test tube with gentle shaking to ensure that the compounds diffused adequately in the aqueous solution. Three replicates were used for each treatment and control. The control was performed by adding the solvent used to dissolve the extracts in the assay.

5. Bioassay

After complete evaporation of solvent, ten matured shrimps in 5ml artificial sea water were transferred to all test tubes containing samples. Similarly, three controlled vials were taken and 10 matured nauplii were introduced in each vial. After 24 hours, the numbers of survivor were counted with the help of disposable pipettes.

6. Data analysis

LC_{50} value is the lethal concentration dose required to kill 50 % of the shrimps. It can be determined as follows.

If 'n' is the number of replicates (here three), 'x' is the log of constituents in mg/ml (log10, log100, log1000 for three dose level respectively) and 'y'is the probit for average survivor of all replicates.

We have,

$\alpha = 1/n [\sum y - \beta \sum x]$ …….. (1)

$$= \frac{\sum xy - \frac{\sum x \sum y}{n}}{\sum x^2 - \frac{(\sum x)^2}{n}} \quad \text{..... (2)}$$

Where,

From probit regression

$Y = \alpha + \beta X$ …….. (3)

$X = (Y - \alpha) / \beta$ …….. (4)

Where Y is constant having value 5 for calculating LC_{50}-value.

Thus,

LC_{50} = Anti log X …… (5)

In our present work, brine–shrimp bioassay of crude methanol extract of five different plants such as *Ageratum houstonianum, Calotropis gigantae, Catharanthus roseus, Thevetia peruviana* (seeds), and *Thevetia peruviana* (leaves) were carried out.

3.2.4.2 Antimicrobial Screening

Many compounds either of natural origin or from synthesis are examined in the search for new structure with activity against microorganism. The antimicrobial test evaluate the ability to kill or inhibit the growth of pathogenic microorganism of given plant extract/fraction/ or compounds. It provides the rationale for the selection of potentially bioactive compounds. In this study crude methanol extract of four different plants were taken of antimicrobial study.

First step in assessment of new antibacterial is screening step and in this step effectiveness of antimicrobial substance is evaluated by determination of zone of inhibition (ZOI), minimum inhibitory concentration (MIC), of the antimicrobial agent (WHO, 1991), and (or minimum bacterial concentration (MBC), for bacteria and minimum fungicidal concentration (MFC) for fungi.[67] MIC is the lowest concentration of antibacterial agent that will inhibit visible growth of an organism after overnight incubation. MBC is the amount of the concentration that will prevent growth after subculture of the organisms to antibacterial agent free medium.

1 Preparation of stock/working solution

25% of working solution was made by dissolving 125 mg of crude extract in 0.5 ml DMSO for each plant. It was diluted to 5% by dissolving 0.4 ml DMSO in 0.1 ml of 25% stock solution. 20% of working solution was made by dissolving 100 mg of crude extract on 0.5 ml DMSO in separate test tubes. After making stock/working solution the test tubes were capped, sealed and stored in refrigerator (2-8^{o} C) until use.[68]

2. Collection of Standard Cultures

The standard cultures of bacteria under study were collected from Chitwan Medical College, Bharatpur lab. After obtaining the culture, the test organisms were streaked on Nutrient Agar plates and incubated. From the isolated colony gram staining was performed. The organism thus obtained were tested for their purity and confirmed by their morphological, cultural and biochemical characteristics.[69] The organisms in the study include:

1) *Staphylococcus aureus*
2) Bacillus subtilis
3) Klebseilla pneumonia
4) Klebseilla oxytoca
5) Psedomonas aeruginosa
6) Citrobactes Sps
7) E. Coli
8) Proteus Vulgaris
9) Salmonella typhi
10) Salmonella parathyphi

3. Preparation of Standard Culture Inoculum

It was prepared from primary culture plates as described below

- The isolated colony was sub cultured on nutrient agar plates with the inoculating loops aseptically.
- It was transferred in a tube containing sterile nutrient broth and incubated for 4 hours at 37^{o}C.

4. Preparation of Media

Nutrient Agar

It was added in distilled water in the ratio of 28 g/litre in appropriate size of conical

flask and boiled with continuous shaking and autoclaved at 121^{o}C for 30 minutes. Sterilized media was allowed to cool about 50^{o}C. They were distributed in the sterile Petri-plates of size of 90 mm diameter in the ratio 25 ml per plate aseptically and labeled properly. Plates were left as such for solidification.

5. Qualitative Screening and Determination of Anti-microbial Activity

The antibacterial activities of crude extracts of medicinal plants were screened against the test organisms by agar well diffusion method as given by Dingle et al. Sterile Muller-Hinton Agar (MHA) plates of approximately 4 mm thickness were prepared. Before using the plates, they were dried under hot air over at appropriate temperature to remove excess of moisture from the surface of the media. The fresh inoculum comparable with turbidity standard was prepared. Then, a sterile cotton swab was taken out and was dipped into the prepared inoculum. The excess of inoculum was removed by pressing and rotating against the upper inside side wall of the tube above the liquid level and then swabbed carefully all over the plates. The plate was rotated through an angle of 60^{o}C after each swabbing. Finally the swab was passed round the edges of the agar surface. The inoculated plates were left to dry for few minutes at room temperature with the lid closed.

Then with the help of sterile cork borer no.5, wells were made in the inoculated media plates and labeled properly. So, the diameter of a well was 6 mm. Then 50 ml of the test compound was introduced into respective well with the help of micropipette. In one well pure DMSO was filed as control. The plates were then left for half an hour with the lid closed so that the extract diffused into media. The plate was incubated overnight at 37^{o}C.

After proper incubation (18-24 hours) the plates was observed for the zone of inhibition around, well which is suggested by clean zone without growth was noted. The triplicate assay was performed in the case of presence of zone of inhibition. The ZOI were measured using scale and mean was recorded.

3.2.4.3 Preferential Cytotoxicity

PANC-1 cancer cell preferential cytotoxicity assay was done on standard protocol[70,

[71]. PANC-1 cancer cells were seeded in 96- well plates ($1x10^4$/ cell well) and incubate on fresh Dulbecco's modified Eagle's medium (at Japan) at 37°C under 5% co_2/95% air for 24 h. the cells were then washed with PBS, and the medium was changed to both DMEM and nutrient- deprived medium (absence of glucose, amino acids, and serum) separately. Then it was followed by immediate addition of serial dilution of the test samples. After 24 hours of incubation, the cells were again washed with PBS, then 100 µlit of DMEM with 10% WST-8 cell counting kit solution was added to the wells, and plates was incubated for a further 2 hours. Then, the absorbance of the wells at 450 nm was calculated by following equation:

Cell viability (%) = $[Abs_{(test\ sample) -} Abs_{(blank)} / Abs_{(control) -} Abs_{(blank)}] \times 100$

The experiment for cytotoxicity was carried out at Institute of Natural Medicine, University of toyama, Sugitani, Japan.

3.2.5 Extraction

The aerial parts of *Calotropis gigantea* so called aanka were collected and washed under tap water to remove other contamination such as dust, soils etc. Then, it was shade dried for few days. The plant materials were ground into powder for extraction. About 300 gm of dried powder of *Calotropis gigantea* was extracted by maceration in methanol (500ml) for 48 hours with frequent agitation. The mixture was filtered through clean muslin cloth followed by double filtration by filter paper. The same process was repeated for 4/5 times for complete extraction and the filtrate was concentrated by Rotary evaporator.

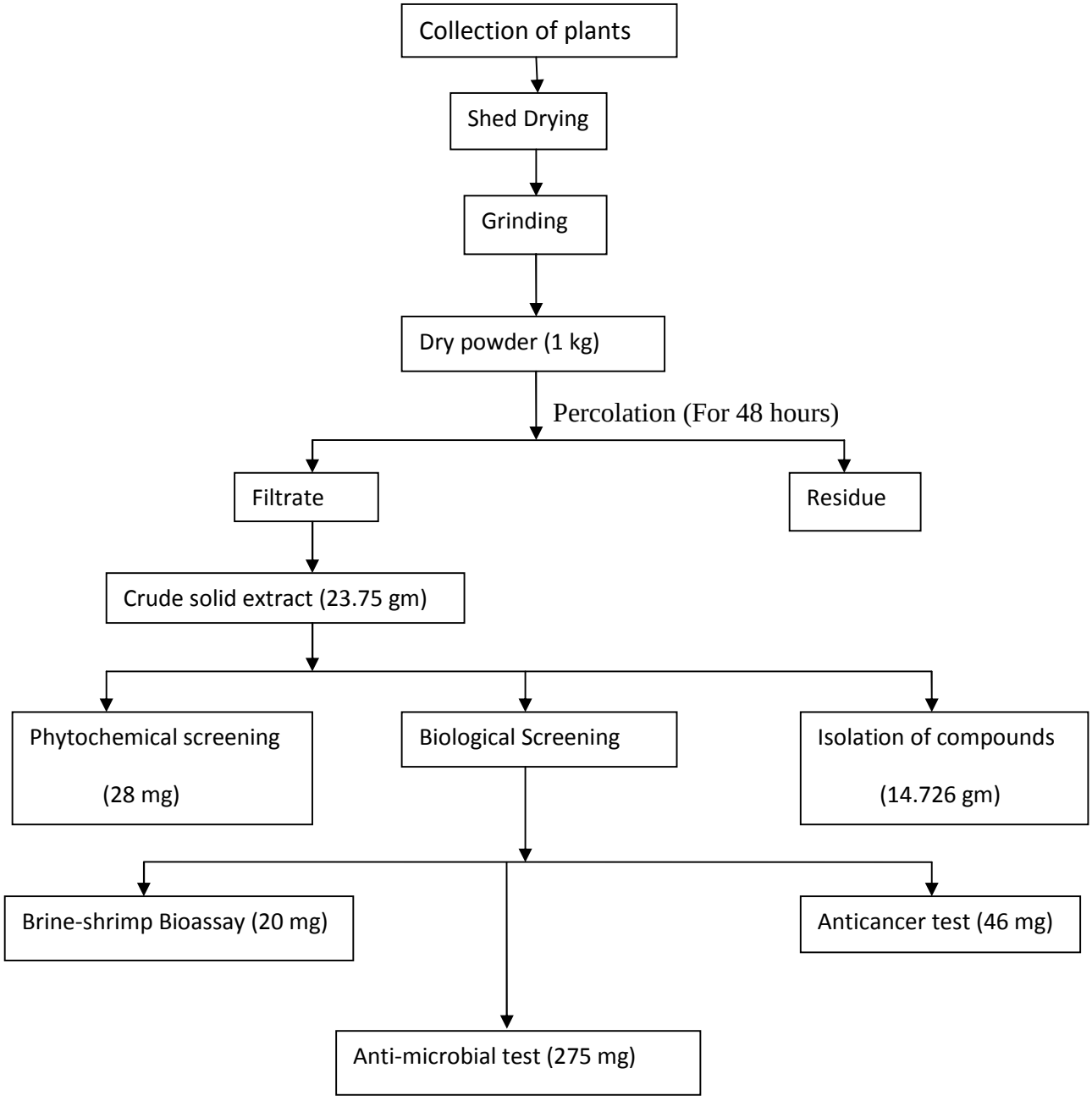


Fig: 4. Flow chart of extraction from aerial parts of *C. gigantea*

3.2.5.1 Separation of compounds from methanol fraction of *Calotropis gigantea* by column chromatography

The methanol fraction (15 g) of aerial part of *C.gigantea* was adsorbed on an equal amount of silica gel and loaded on to a silica gel (15 g, E-Merck, 60-120 mesh) packed column having internal diameter 2 cm with the adsorbent height 45 cm. The column was eluted with gradients of hexane, ethyl acetate and methanol to obtain no. of fractions.

Table No. 2: Elution and TLC Profile of Different Fractions

S. N.	Eluent	Fraction no.	Volume of Eluent (ml)	Solvent system for TLC	TLC report
1	100% Hexane	1-10	1000	1% EtOAc/Hexane	No spots
2	1% EtOAc/ Hexane	11-16	700	10% EtOAc/Hexane	2 spots(t)
3	10% EtOAc/Hexane	17-21	750	15% EtOAc/Hexane	1 spots (t)
4	15% EtOAc/Hexane	22-30	1300	20% EtOAc/Hexane	3 spots (t)
5	20% EtOAc/Hexane	31-33	1600	25% EtOAc/Hexane	2 spots
6	30% EtOAc/Hexane	34-38	600	25% EtOAc/Hexane	3 spots
7	30% EtOAc/Hexane	39-52	1950	40% EtOAc/Hexane	3spots(t)
8	40% EtOAc/Hexane	53-61	1200	50% EtOAc/Hexane	5 spots
9	40% EtOAc/Hexane	62-69	1050	50% EtOAc/Hexane	tailing
10	60% EtOAc/Hexane	70-77	1050	70% EtOAc/Hexane	tailing
11	70% EtOAc/Hexane	78-88	1500	80% EtOAc/Hexane	tailing
12	90% EtOAc/Hexane	89-93	600	100% EtOAc/Hex	1 spot(t)
13	100% EtOAc/Hexane	94-103	2000	1% MeOH/EtOAc	3 spots (t)
14	10% MeOH/EtOAc	104-107	450	100% EtOAc	tailing
15	10% MeOH/EtOAc	108-113	750	10% MeOH/$CHCl_3$	3 tailing
16	20% MeOH/EtOAc	114-116	600	30%MeOH/$CHCl_3$	3 spots (t)
17	30% MeOH/EtOAc	117-122	750	40% MeOH/$CHCl_3$	tailing
18	50% MeOH/EtOAc	123-130	1050	60% MeOH/$CHCl_3$	tailing
19	80% MeOH/EtOAc	131-137	1050	90% MeOH/$CHCl_3$	tailing
20	100% MeOH	138-150	1950	100% MeOH	tailing

t = tailing

3.2.6 Isolation and Characterization of Compounds

Depending upon the nature of compounds, column chromatography and fractional crystallization techniques were employed, for isolation and characterization of compounds.

COMPOUND CG_{21}

Methanol fraction 17-23 on elution with 10% EtOAc in Hexane showed same R_f value on TLC, they were combined and concentrated under reduced pressure on rotary evaporator. The purity was checked on TLC, one major spot with tailing was observed. The content was left undisturbed overnight. A white needle shaped crystals were appeared. The crystals were washed with methanol and recrystalized with 1:1 ethylacetate: hexane. The compound was found to be single spotted on TLC with R_f value 0.45 (20% ethylacetate in hexane). It was soluble in ethylacetate, chloroform, slightly soluble in hexane and insoluble in methanol. The melting point of compound was found to be 128^{o}C and yield was 74mg.

Compound CG_{22}

Cubic shaped crystals were appeared on the remaining crude methanol extract of *C. gigantea*. The crystals were washed with hexane, chloroform, ethyalacetate and in methanol according to increasing polarity. The compound found to be insoluble in hexane, chloroform, ethylacetate. It was completely soluble in water. It was recrystalized by distilled water and purity was checked on TLC. The compound was found to be single spotted on TLC with R_f value 0.66 (80% methanol in etylacetate). The melting point of compound was found to be 282^{o}C and yield of compound was 152 mg.

CHAPTER-IV

RESULTS AND DISCUSSIONS

4.1 Phytochemical Screening

Phytochemical screening of crude methanol extract and aqueous extract of five different plants was done on the basis of procedure developed by Prof. I. Culie.[1] The results obtained are given below.

Table No. 3: Phytochemical Screening for Methanol Extract OF Five different plants

S.N	Group of Compounds	Results				
		A.houstonianum **(Flower)**	***C.gigantea*** **(Aerial part)**	***A. roreus*** **(Aerial part)**	***T. peruviana*** **(seed kernal)**	***T. peruviana*** **(leaves)**
1.	Polyphenols	-	+	+	+	+
2.	Alkaloids	-	-	+	+	+
3.	Glycosides	-	+	+	+	+
4.	Quinones	+	+	+	+	+
5.	Anthocyanosides	+	+	+	-	-
6.	Anthracenosides	+	-	-	+	-
7.	Flavon glycoside	+	+	+	+	+
8.	Coumarin Glycosides	+	+	+	+	+
9.	Reducing Compounds	-	-	-	-	+

+ = Present - = Absent

Table No. 4: Phytochemical Screening for Highly Polar Solvent (Water) Extract of Five Different plants.

S.N	Group of Compounds	Results				
		A.houstonianum **(Flower)**	***C.gigantea*** **(Aerial part)**	***B. roreus*** **(Aerial part)**	***T. peruviana*** **(seed kernal)**	***T. peruviana*** **(leaves)**
1.	Alkaloids	+	+	+	+	+
2.	Saponins	+	-	+	+	+
3.	Tannins	+	+	+	+	+

+ = Present - = Absent

4.2 Biological Screening

This dissertation work involves biological screening of methanol extract of five different plants in three sets namely, brine shrimp bioassay, antimicrobial bioassay and determination of preferential cytotoxicity, to evaluate the pharmacological potency of the plant extract and isolated pure compounds.

4.2.1 Brine Shrimp Bioassay

For the study of biological activity of plant material, the crude methanol extract of five different plants were considered. This method consists exposing the newly hatched brine shrimp nauplii to the plant extracts. The biological activities were evaluated on the basis of their toxicity towards this nauplii[72]. In this method, the LC_{50}-values (μg/ml) for different extracts were determined and those having LC_{50} values less than 10^3 are considered to be pharmacologically active. Results obtained during this study are shown in table no.5.

Table No.5: Calculation of toxicity of different fraction of the plant from 24 hours counts.

S.	S. N	Z	x	Y	xy	x^2	$\sum x$	$\sum y$	$\sum xy$	$\sum x^2$	β	α	X	LC_{50}
1	O_1	10 100 10^3	1 2 3	6.0 3.4 3.7	6.0 6.8 11.1	1 4 9	6	13.1	23.90	14	-1.15	6.66	1.44	27.54
2	O_2	10 100 10^3	1 2 3	5.4 4.4 2.0	5.4 8.8 6.0	1 4 9	6	11.8	20.20	14	-1.70	7.33	1.37	23.44
3	O_3	10 100 10^3	1 2 3	6.4 4.6 3.7	6.4 9.2 11.1	1 4 9	6	14.7	26.7	14	-1.35	7.60	1.92	83.17
4	O_4	10 100 10^3	1 2 3	8.4 7.4 6.4	8.4 14.8 19.2	1 4 9	6	22.2	42.40	14	-1.00	9.40	4.40	25118
5	O_5	10 100 10^3	1 2 3	9.4 7.0 6.4	9.4 14.0 19.2	1 4 9	6	22.40	42.60	14	-1.10	9.66	4.23	16982.4

O_1*: Ageratum houstonianum* O_2: *Calotropis gigantea* O_3: *Catharanthus roseus*

O_4: *Thevetia peruviana* (seed kernels) O_5: *Thevetia peruviana* (leaves)

Z = Concentration

LC_{50} = Antilog

We have,

$\alpha = 1/n [\sum y - \beta \sum x]$ ……. (1)

$$= \frac{\sum xy - \frac{\sum x \sum y}{n}}{\sum x^2 - \frac{(\sum x)^2}{n}} \quad \text{..... (2)}$$

Where,

From probit regression

$Y = \alpha + \beta X$ …….. (3)

$X = (Y - \alpha) / \beta$ …….. (4)

Where Y is constant having value 5 for calculating LC_{50}-value.

Thus,

LC_{50} = Anti log X …… (5)

4.2.2 Antimicrobial Screening

In the present work, antimicrobial inhibition studies of crude methanol extract of four different plants were studied. The results obtained from the study are tabulated below.

Table No.6: Showing ZOI by different microorganisms for methanol extract of four different plant species

Well diameter: 6mm Concentration of loaded extract = 5%, 20% and 25%

Plant name	**Concentration (%)**	**Diameter of Zone of inhibition (ZOI)**				
		control	***E. c***	***K. o***	***P. a***	***S. a***
Ageratum houstonianum	5	6	-	11	12	11
	20	6	-	11.5	12.5	11.5
	25	6	-	11.5	13.5	14.5
Calotropis gigantean	5	6	8	11	8	10.5
	20	6	11	11.5	12.5	11
	25	6	12	15	16	12
Catharanthus roseus	5	6	-	8.5	-	11.5
	20	6	-	10	-	16
	25	6	-	11.5	-	18.5
Thevetia peruviana	5	6	8.5	-	-	8.5
	20	6	11	-	-	10
	25	6	12.5	-	-	13.5

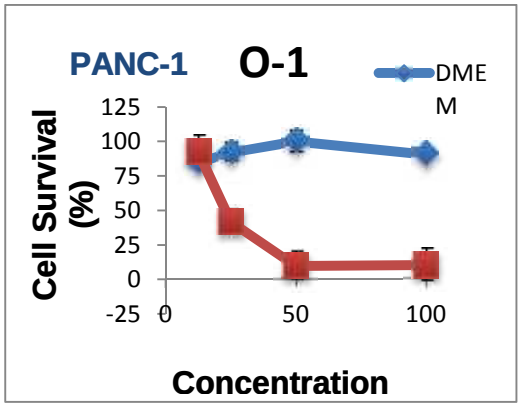
Escherichia coli (E. c); Klebsiella oxytoca (K. o); Pseudomonas aeruginosa (P. a); Staphylococcus aureus (S.a)

Methanol extract showed a variable activity against test organism. Different types of gram positive and gram negative organisms were examined four different plants namely *Ageratum houstonianum* (flowers), *Calotropis gigantean* (aerial parts), *Catharanthus roseus* (aerial parts), and *Thevetia peruviana* (leaves).

Depending on the measured values of the complete inhibition diameter in mm, the anti-microbial activity can be classified into the following types such as >12 mm zone of inhibition = high sensitivity, 9-12 mm zone of inhibition = moderate sensitivity, 6-9 mm zone of inhibition = less sensitivity, and <6 mm zone of inhibition = resistant[73]. From the above experiment, it has been observed that methanol extract of *Cathranthus roseus* at 125 mg/0.5 mL DMSO showed ZOI value 18.5 mm and showed greater sensitivity for *Staphylococcus aureus.* On the other hand, methanol extract of *Calotropis gigantea* (aerial parts) showed good values of zone of inhibition for all the tested organism at all concentration under taken including ZOI value 14.5 for *Staphylococcus aures* at concentration 125 mg/0.5 ml DMSO. All the four different extract showed ZOI values at all concentration for *Staphylococcus aureus.*

4.2.3 Preferential Cytotoxicity

Preferential cytotoxicity of aerial parts of *C. gigantea* on methanol extract was performed by using PANC-1 cancer cell lines. The test was conducted in two medium namely; Nutrient-deprived medium (NDM) and in addition of salts, amino acids, serum so called Dulbecco Modified Eagle medium (DMEM) but without addition of sugars and other nutrients[71]. The absorbance of sample wells at 450 nm was measured and % of cells survival verses concentration was drawn as follows.



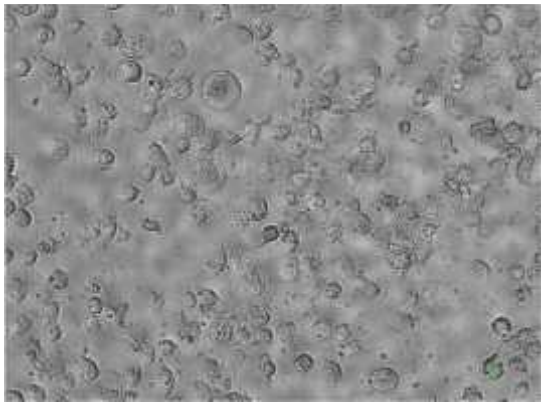
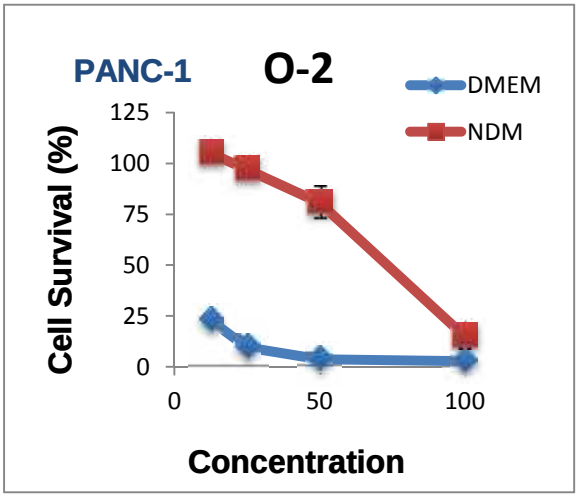


Fig: 5. Preferential cytotoxic activity of *Ageratum houstonianum*



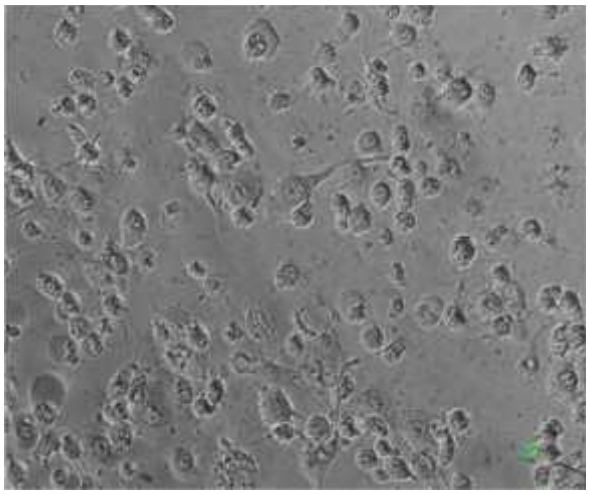


Fig: 6. Preferential cytotoxic activity of *Calotropis gigantea*

O-2/100 «g/mL

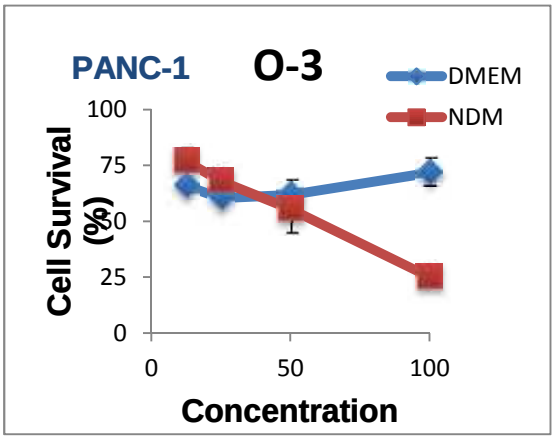


Fig: 7. Preferential cytotoxic activity of *Cathatharanthus roseus*

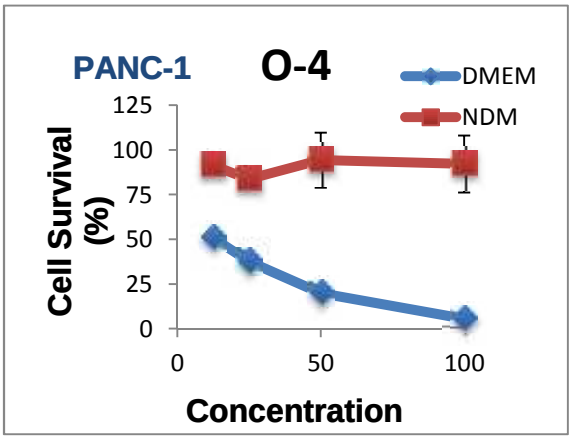


Fig: 8. Preferential cytotoxic activity of *Thevetia peruviana* (seed kernals)

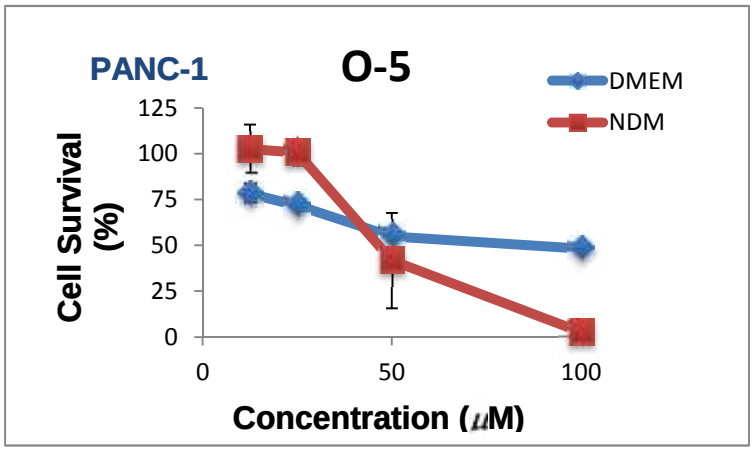


Fig: 9. Preferential cytotoxic activity of (*Thevetia peruviana* leaves)

Among the five different plants, 100% preferential cytotoxic activity or 0% cells survival was observed at the concentration 100 µg/ml in the cells cultured in either NDM or DMEM on *Calotropis gigantea*. In case of *Ageratum houstonianum and Thevetia peruviana* (leaves) 100% preferential cytotoxicity were obtained at concentration 50 µg/ml and 100µg/ml respectively when cell cultured in NMD. *Thevetia peruviana* (seed kernels) also shows 100% preferential cytotoxicity at the concentration 100 µg/ml when cells cultured in DMEM. Other plants showed milder or no activity even at high concentration.

4.3 Analysis and Identification of Isolated Chemical Constituents

4.3.1 Compound CG_{21}

Compound CG_{21} a white needle shaped crystalline solid, with melting point 128^0C. The compound was found to be single spotted on TLC with R_f value 0.45 (20%EtoAc/hexane). It gave positive Libermann-Burchard test with violet, blue and finally green color indicating the compound to be sterol. The mass spectrum showed parent molecular ion [M^+] peck at m/z at 414 which corresponds to the molecular formula ($C_{29}H_{50}O$).

The IR spectrum showed presence of –OH group (3425.58 cm^{-1}). The presence of bands at 2939.52 and 2870.05 cm^{-1} were due to asymmetric and symmetric –C-H stretching respectively. Absorption bands at 1643 cm^{-1} is assigned for C=C double bond stretching. Absorption bands at 1458.18 cm^{-1} and 1373.32 cm^{-1} were due to bending of gem-dimethyl groups. Absorption band at 1058 cm^{-1} showed the presence of –C-O group.

Two singlets at δ 1.00 and at δ 0.67 in ^{1}H-NMR are accounted for methyl group for C-19 and C-18 respectively. Peaks at δ 0.92 (d, J = 6.1Hz); 0.81 (d, J = 6.9 Hz); 0.83 (d, J = 6.9Hz) and 0.84 (t, J = 7.3Hz) were accounted for gem methyl groups at C-21, C-26, C-27, and C-29 respectively. A doublet at δ 5.35 (m) can be accounted for an olefinic proton at C-6. Multiplets at δ3.52 (ddd, J = 13.2, 16.0, 16.0 Hz) equivalent to single proton and was assigned for the proton of C-3 carbon. Thus the hydroxyl group at C-3 and double bond at C-5 assigned accordingly. Three multiplets equivalent to

two protons each appeared at δ 1.83, 2.00 and 2.27 were assigned for 3- CH_2 groups. The remaining protons have appeared as multiplets at δ 1.05-1.65.

From all the spectral analysis, the compound was identified as β-sitosterol, previously identified from large number of Nepalese plants species. The compound was further confirmed by Co-TLC.

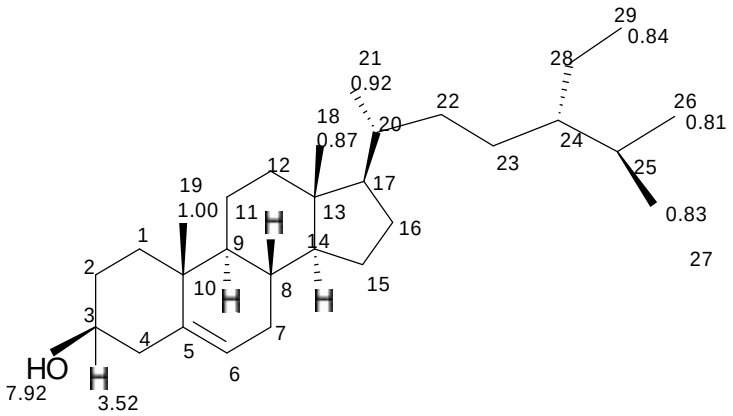


Fig: 10. ^{1}H NMR of CG_{21} (β- sitosterol)

4.3.2 Compound CG_{22}

Compound CG_{22} is cubic shaped crystals appeared on the remaining crude methanol extract of *C. gigantea.* It has melting point 282^0C, giving single spotted on TLC with R_f value 0.66 (80% methanol in ethylacetate). The yield of compound was 152mg. The structure of the compound was under progress.

CHAPTER- V
CONCLUSION AND RECOMMENDATION

5.1 Conclusion

Five different plants with potential poisonous and medicinal value were collected from the central parts of Nepal; Chitwan district. These plants were found to have high medicinal value with potential anticancer activity. Phytochemical screening report showed that, *Calotropis gigantean aerial part* contains polyphenol, alkaloids, quinines and flavonol glycosides, coumarin glycosides as rich components. Glycosides and Flavonol glycosides also reported from *T peruviana, A. houstonianum* flower, and *C.roseus* aerial part.

Brim-Shrimp bioassay result showed that *Calotropis gigantea* with LC_{50} value 23.54 µg/ml, *Ageratum houstonianum* with LC_{50} value 27.54 µg/ml and *Catharanthus roseus* with LC_{50} value 83.17 µg/ml were highly active. Form the antimicrobial test of crude methanol extract of four different plants, it was found that *Calotropis gigantea* showed specify ZOI value for all tested organism at all concentration including ZOI value 16.0mm for *Pseudomons aeruginosa* at concentration 125 mg/0.5 ml DMSO. *Catharanthus roseus* also showed high sensitivity at concentration 125mg/0.5 ml DMSO for *Staphylococcus aureus* with ZOI value 18.5 mm.

Anticancer test for PANC-1 cancer cell lines in either NDM or DMEM showed that *Calotropis gigantea* have high anticancer activity as it showed 100% preferential cytotoxicity or 0% cell survival in either Nutrient deprive medium or DMEM at the concentration 100 µg/ml.

Two different compounds: compound CG_{21} and compound CG_{22} were isolated from the crude methanol extract of aerial parts of *C.gigantea.* The structure of compound CG_{21} was elucidated from the spectral analysis as β- sitosterol and structure was verified by Co-TLC with authentic sample. The structure of compound another compound CG_{22} obtain from crude methanol extract of *C. gigantea* was however under progress.

5.2 Recommendations

Poisonous medicinal plants in Nepal might be immense source of bioactivity such as antimicrobial, anti-fungal, anti-bacterial, anticancer, antioxidant, and etc, but clear and throughout information is not available in the related field. Among the five different plants that, I have been included during my research work, some research work has been done for *C.roseus* in the field of anticancer drug development, although other plants such as *A. houstonianum*, *T.peruviana* have possibility in the related field. *T. peruviana* contains compound Thevetin in its milky sap, which used as heart stimulant. The methanol extract of this plant showed anti-spermatogenesis activity so that it is necessary for proper study of this plant as it is useful in country like Nepal.

Most of medicinal plants reported in Nepal remained as the major source of drugs but there are some traditional extraction systems in Nepal, where very little progressing of the products occurs in the area where they found. Some ethno botanical work and listing of the plants name with its description, phytochemical screening and antimicrobial activity only reported in different books of medicinal plants.

Regarding to Nepal, instead of extraction, thousand tones of medicinal herbs are collected from the forest and traded to foreign country. Therefore, researcher must be focused on the extraction, isolation and characterization of bioactive chemical compounds from the plants. It is important to find out biochemical importance of such poisonous medicinal plants and used them in the field of human welfare, their conservation and sustainable use for further generation.

ANNEX

Phytochemical Screening

General Procedure

Extraction and Fractionation

About 50 gm of air-dried plant material was extracted by methanol by cold percolation method. The methanol solvent was evaporated. This residue was fractionated successively between water and hexane, ethyl acetate and then methanol. Each fraction was concentrated to reduce its volume up to 50 ml.

1. Screening tests of the hexane extract

a) Test for Volatile Oils

Spot Test: 2 ml hexane extract was evaporated to get residue and it was mixed with 0.5 ml methanol. The solution was shaken vigorously and filtered. Few drops of filtrate were put on a filter paper by means of a capillary tube. A yellow spot on filter paper was persistent even after evaporation indicating the absence of volatile oil.

b) Tests for Basic Alkaloids

The hexane extract (10 ml) was concentrated to yield a residue and dissolved in 2 %(v/v) HCl (3 ml). This solution was equally divided into two test tubes.

i. Meyer's test: The first test solution was treated with three drops of Meyer's reagent. No white precipitate was obtained indicating the absence of basic alkaloids.
ii. Dragendorff's test: The second test solution was treated with three drops of Dragendorff's reagent. No white precipitate was obtained indicating the absence of basic alkaloid.

Saponification of the hexane extract

The remaining hexane extract was re-extracted by 5% KOH (W/V) (5 ml ´ 3 times) in a separating funnel to get aqueous alkaline solution and organic solution (the upper hexane layer). The upper hexane layer was equally divided into two test tubes for testing sterols, triterpenes, and carotenoids respectively. The lower aqueous alkaline solution was

acidified with conc. HCl until pH 3, and then it was again extracted by ether (5 ml ´ 3 times) to get etheric extract. Thus obtained combined upper etheric layer was used for the screening tests for fatty acids, coumarins, favone aglycones, emodins and quinones.

c) Test for Fatty Acids

The ether extract (2ml) was concentrated and one drop of solution was spotted on the filter paper. The persistence of yellow spot even after evaporation of solvent confirmed the presence of fatty acids.

d) Test for Coumarins

The ether extract (5 ml) was concentrated to yield a residue, which was dissolved in hot water (5 ml), cooled and the solution was divided into two test tubes. The first tube was taken as standard and the second tube was made alkaline by adding 10% NH_4OH (W/V) drop by drop until pH 8. When these two solutions (first and second) were observed in UV light, occurrence of an intense greenish yellow florescence was not observed in second solution (in comparison with standard) indicating the absence of coumarins.

e) Test for flavone Aglycones (Shinoda's reaction)

The ether extract (5 ml) was concentrated to yield a residue, which was dissolved in methanol (2 ml). To which a small piece of magnesium and 4-5 drops of conc. HCl were added. No pink or violet color was developed indicating the absence of flvone aglycones.

f) Test for Emodins (Borntiager's Test)

The ether extract (2 ml) was treated with 25% (v/v) ammonium hydroxide solution (1 ml) and was shaken vigorously. The test tube was allowed to stand for a few minutes to separate two layers. Neither the upper layer was decolorized nor the lower alkaline layer gained red color indicating the absence of emodins.

g) Test for Sterols and Triterpenes (Libermann-Burchard’s Test)

The organic portion was concentrated to yield a residue, which was dissolved in acetic anhydride (1 ml). To this solution, conc. sulphuric acid (2 ml) was added from the side of the test tube without disturbing the solution. A violet ring at the junction of two liquids was observed and the upper was green in color indicating the presence of sterols. The violet superior layer indicated the presence of triterpenes.

h) Test for Carotenoids

The hexane extract was concentrated and treated with conc. sulphuric acid (1 ml). An orange yellow color similar to the extract was not developed and not turned into red on long standing indicating the absence of carotenoids.

i) Test for Quinones

To the etheric extract (2 ml) freshly prepared ferrous sulphate solution (1 ml) and ammonium thiocyanate (few crystals) were added and treated with conc. H_2SO_4 drop by drop. The deep red color was not persistent indicating the absence of quinines.

2) Phytochemical screening for semi-polar (Ethyl Acetate) extract

a) Test for Volatile Oils

Spot Test: 2 ml ethyl acetate extract was evaporated to get residue and it was mixed with 0.5 ml methanol. The solution was shaken vigorously and filtered. Few drops of filtrate were put on a filter paper by means of a capillary tube. A yellow spot on filter paper was evaporated after evaporation indicating the presence of volatile oil.

b) Test for Polyphenols (Ferric Chloride Test)

ferric chloride solution (3 drops) was added. A greenish blue color was not developed indicating the absence of polyphenols.The Ethyl Acetate extract (1 ml) was mixed with water (1 ml). To this solution, 1% (w/v)

c) Tests for Alkaloid salts

The ethyl acetate extract (10 ml) was concentrated to yield a residue. To this residue, 2% (v/v) hydrochloric acid (4 ml) was added and shaken vigorously, then filtered. The filtrate was treated with 10% (v/v) ammonium hydroxide solution until pH 8. The solution was then extracted thrice with chloroform (10 ml). The upper alkaline layer was discarded. The lower combined chloroform layer was concentrated over a water bath. To this concentrated solution, 2% (v/v) HCl (5 ml) was added. Thus obtained solution was divided into two parts:

i. The first was treated with Meyer's reagent (3 drops). No white precipitate was obtained indicating the absence of the alkaloid salts.

ii. The second was treated with Dragendorff's reagent (3 drops). No white precipitate was obtained indicating the absence of the alkaloid salts.

d) Test for glycosides

The ethyl acetate extract (8 ml) was concentrated to half the original volume and divided into two test tubes.

i. The first test solution (2 ml) was treated with 25 % (v/v) ammonium hydroxide solution (2 ml) and shaken vigorously. A cherry-red color developed indicating the presence of glycosides.
ii. Molisch's Test: The second test solution was treated with Molisch's reagent (5 drops) and conc. H_2SO_4 was added drop wise from the side of the test tube without disturbing the solution. A violet ring at the junction of the two liquids was observed and on shaking, the solution was turned violet completely indicating the presence of glycosides.

e) Test for Quinones

To the ethyl acetate extract (2 ml), freshly prepared ferrous sulphate solution (1 ml) and ammonium thiocyanate (few crystals) were added and treated with conc. H_2SO_4 drop by drop. The deep red color was not persistent indicating the absence of quinones.

Saponification of the ethyl acetate extract

The half of the remaining ethyl acetate extract was re-extracted by 5% KOH (W/V) (5 ml ´ 3 times) in a separating funnel to get aqueous alkaline solution and organic solution (the upper ethyl acetate layer). The upper ethyl acetate layer was equally divided into two test tubes for testing sterols, triterpenes, and carotenoids respectively. The lower aqueous alkaline solution was acidified with conc. HCl until pH 3 and then it was again extracted by ether (5 ml ´ 3 times) to get etheric extract. Thus obtained combined upper etheric layer was used for fatty acids, coumarins, favone aglycones, and emodins.

f) Test for Fatty Acids

The ether extract (2 ml) was concentrated and one drop of solution was spotted on the filter paper. The persistence of yellow spot even after evaporation of solvent confirmed the presence of fatty acids.

g) Test for flavone Aglycones (Shinoda's reaction)

The ether extract (5 ml) was concentrated to yield a residue, which was dissolved in methanol (2 ml). To which a small piece of magnesium and 4-5 drops of conc. HCl were added. No pink or violet color was developed indicating the absence of flvone aglycones.

h) Test for Carotenoids

The ethyl acetate extract was concentrated and treated with conc. sulphuric acid (1 ml) An orange yellow color similar to the extract was not developed and not turned into red on long standing indicating the absence of carotenoids.

i) Test for Sterols and Triterpenes

The ethyl acetate fraction was concentrated to yield a residue, which was dissolved in acetic anhydride (1 ml). To this solution, conc. sulphuric acid (2 ml) was added from the side of the test tube without disturbing the solution. A violet ring at the junction of two liquids was observed and the upper was green in color indicating the presence of sterols. The violet superior layer indicated the presence of triterpenes.

j) Test for Coumarins

The ether extract (5 ml) was concentrated to yield a residue, which was dissolved in hot water (5 ml), cooled and the solution was divided into two test tubes. The first tube was taken as standard and the second tube was made alkaline by adding 10% NH_4OH (V/V) drop by drop until pH 8. When these two solutions (first and second) were observed in UV light, occurrence of an intense greenish yellow florescence was not observed in second solution (in comparison with standard) indicating the absence of coumarins.

k) Test for Emodins (Borntiager's Test)

The ether extract (2 ml) was treated with 25% (v/v) ammonium hydroxide solution (1 ml) and was shaken vigorously. The test tube was allowed to stand for a few minutes to separate two layers. Neither the upper layer was decolorized nor the lower alkali layer gained red color indicating the absence of emodins.

3) Phytochemical screening for polar (methanol) extract

a) Test for Polyphenols (Ferric Chloride Test)

The methanol extract (1 ml) was mixed with water (1 ml). To this solution 1 % (w/v) ferric chloride solution (3 drops) was added. A greenish blue color was not developed indicating the absence of polyphenols.

b) Test of Reducing Compounds (Fehling's test)

The methanol fraction (1 ml) was mixed with water (1 ml). To this solution, 1 ml Felhing's reagent (1:1 mixture of Fehling's reagent A and B) was added and then the mixture was warmed over a water bath for 30 minutes. The brick red precipitate was produced indicating the presence of reducing compounds.

c) Tests for Alkaloid salts

The methanol fraction (10 ml) was concentrated to yield a residue. To this residue, 2% (v/v) hydrochloric acid (4 ml) was added and shaken vigorously, then filtered. The filtrate was treated with 10% (v/v) ammonium hydroxide solution until pH 8. The solution was then extracted thrice with chloroform (10 ml). The upper alkaline layer was discarded. The lower combined chloroform layer was concentrated over a water bath. To this concentrated solution, 2% (v/v) HCl (5 ml) was added. This obtained solution was divided into two parts:

i. The first was treated with Maeyer's reagent (3 drops). White precipitate was obtained indicating the presence of the alkaloid salts.
ii. The second was treated with Dragendorff's reagent (3 drops), red brown precipitate was obtained indicating the presence of the alkaloid salts.

d) Test for glycosides

The methanol extract (8 ml) was concentrated to half the original volume and divided into two test tubes.

i. The first test solution (2 ml) was treated with 25 % (v/v) ammonium hydroxide solution (2 ml) and shaken vigorously. A cherry-red color developed indicating the presence of glycosides.
ii. Molisch’s Test: The second test solution was treated with Molisch’s reagent (5

drops) and conc. H_2SO_4 was added drop wise from the side of the test tube without disturbing the solution. A violet ring at the junction of the two liquids was observed and on shaking, the solution was turned violet completely indicating the presence of glycosides.

e) Test for Quinones

To the methanol extract (2 ml) freshly prepared ferrous sulphate solution (1 ml) and ammonium thiocyanate (few crystals) were added and treated with conc. H_2SO_4 drop by drop. The deep red color was persistent indicating the presence of quinones.

Hydrolysis of Methanol extract

The remaining methanol extract was hydrolyzed by refluxing with equal volume of 10% (v/v) HCl acid for 30 minutes. After cooling, the hydrolyzed extract was re-extracted thrice with solvent ether (10 ml). The lower acidic layer was used for the screening test for anthocyanosides. The upper combined etheric layer was dried over anhydrous sodium sulphate and filtered. The filtrate (etheric solution) was used for screening tests for anthracenosides, coumarin derivatives and flavonic glycosides.

f) Test for Anthocyanosides

The red acidic layer (4 ml) was treated with sodium carbonate until basic of the litmus paper. A godrej special grey color was developed gradually but not green or blue color indicating the absence of anthocyanosides.

g) Test for Anthracenosides

The ether extract solution (2 ml) was treated with 25% (v/v) ammonium hydroxide solution (1 ml) and was shaken vigorously. The test tube was allowed to stand for a few minutes to separate two layers. A greenish yellow color in the lower alkaline layer was observed but no red color indicationg the absence of anthracenosides.

h) Test of Coumarin derivatives

The ether extract solution (4 ml) was concentrated to yield a residue, which was dissolved in hot water (4 ml). After cooling, the solution was divided in two test tubes. The first test tube was used as control. To the second tube 10% (v/v) ammonium hydroxide solution was added drop by drop until pH 8 and was then absorbed under

ultra violet light. Yellow fluorescence in the control and not greenish yellow fluorescence in the senond test tube were observed indicating the absence of coumarin derivatives.

i) Test for Flavonic Glycosides

The methanol fraction solution (10 ml) was concentrated to yield a residue whice was dissolved in methanol. To this solution, a few pieces of magnesium and concentrated HCl (5 drops) were added. An orange yellow color was developed indicating the presence of flavonic glycosides.

4. Phytochemical screening for aqueous extract

a) Test for Polyoses

The aqueous extract solution (4 ml) was concentrated in porcelain basin to yield a residue. To this residue, conc. sulphuric acid (3 drops) was added and allowed to stand for 5 minutes. It was then treated with saturated methanolic solution of thymol (5 drops).Brown red color was observed indicating the presence of polyoses.

b) Tests for Saponins

The aqueous extract solution (2 ml) was concentrated and treated with hot water followed by shaking for 30 seconds. The formation of thick froth showed the presence of saponins.

c) Test for Basic Alkaloids

The aqueous fraction (10 ml) was concentrated to yield a residue. To this residue, 2% (v/v) hydrochloric acid (4 ml) was added and shaken vigorously, then filtered. The filtrate was treated with 10% (v/v) ammonium hydroxide solution until pH 8. The solution was then extracted thrice with chloroform (10 ml). The upper alkaline layer was discarded. The lower chloroform layer was concentrated over a water bath. To this concentrated solution, 2% (v/v) HCl (5 ml) was added. This obtained solution was divided into two parts:

i. The first was treated with Maeyer's reagent (3 drops). White precipitate was obtained indicating the presence of the alkaloid salts.
ii. The second was treated with Dragendorff's reagent (3 drops). White precipitate was obtained indicating the presence of the alkaloid salts.

PREPARATION OF REAGENTS

Meyer's Reagent

Mercuric chloride (0.679 g) was weighed in a 50 ml volumetric flask and dissolved in distilled water. To this solution, potassium iodide (2.5 gm) was added. The scarlet red precipitate was dissolved by shaking and then diluted with distilled water up to the mark of volumetric flask.

Dragendorff's Reagent

Bismuth nitrate (4 gm) was dissolved in 5N nitric acid (10 ml) to make solution A. Next, potassium iodide (13.5 gm) was dissolved in distilled water (20 ml) to make solution B. These solutions A and B were mixed together in a 50 ml volumetric flask.

Sodium Picrate Solution:

Picric acid (0.25 gm) was dissolved in distilled water (50 ml) to make aqueous picric acid solution. The solution was neutralized with sodium bicarbonate.

A strip of whatman no. 1 filter paper was dipped in the prepared solution. The paper was dried completely and protected from external contamination. Thus prepared sodium picrate paper was used for cyanogenic glycoside detection.

Molisch's Reagent:

α- Napthol (5 gm) was dissolved in methanol (50 ml).

Neutral Ferric Chloride Solution:

Ferric chloride (1 mg) was dissolved in distilled water (100 ml). To this aqueous ferric chloride solution, sodium carbonate was added little by little with stirring until the slight turbidity was persistent. The mixture was filtered and the colorless filtered was used as neutral ferric chloride solution.

1M HCl

Concentrated HCl (11.7 M) (8.5 ml) was diluted with distilled water up to the mark in 100ml volumetric flask to make 1 M HCl.

28% Ammonia

Liquor ammonia (28 ml) was diluted up to the mark in 100 ml volumetric flask to make 28% liquid ammonia.

References

1. Culie, I., Dingle, J., Red, W.W., *J. Sci. Food and Agri.,* **1953**, *4*, 149-153.

2. Perez, C.; Paul, M.; Bazerque, P. *Antibiotic Assay by Agar Well Diffusion Method*; Acta Bio medicine Experimental, 1990; Vol. 15, p 113-115.

3. Sapkota R., *Phytochemicals and Biological Studies on Floral parts of Nepalese origin Oroxylum Indicum*, M.Sc Dissertation submitted to Central Department of Chemistry, T.U., Kathmandu, Nepal, **2007,** pp 1-2.

4. Hamal S., *Phytochemicals and Biological Studies on Sarcococca hookeriana (Baillon),* M.Sc Dissertation Submitted to Central Department of Chemistry, T.U., Kathmandu, Nepal, **2006,** pp.1-3

5. Bajracharya, D. M., Joshi, R. M., Rajbhandari, K. R., Shakya, P. R., and Shrestha, T. B. *Endemism in Nepal Flora. In Proceeding of the National Conference on Science and Technology.* Kathmandu, Nepal; Royal Nepal Academy of Science and Technology, 2001; 73-76.

6. Manandhar N. P., "Plants and people of Nepal," Timber Press, U. S. A. 2000. P 50.

7. *Nepal in figures*, (1998) Central Bureau of Statistics, HMG Nepal, Thapathali, Kathmandu, Nepal.

8. Bhattrai N. K., Biodiversity- People Interface in Nepal, *Part two: Articles on Regional Aspects of Medicinal Plants use.*

9. Kurade N. P., Jaitak V., Kaul V, K., Sharma O, P., *Pharma Biol*, **2010**; *48(5),* 539-44.

10. http: / / www. Sciensage. Info / jasr

11. Mueen, A. A. K., Rana, A. C., Kixit, V, K., *Pharmacog. Mag*. **2005**, *1* 48-52.

12. htpp: // www. Comfsm. Fm/ dleeling/ botany/ 1999/ rosnak01. html. (Assumed date17 April 2013).

13. htpp: // www. drugdigest. org/ DD/ Printable Pages/ herbmonograph/ 0,11475,4108,00.html. (Assumed date 17 April 2013).

14. Kareu P.G., Kenji G.M., gachanja A.N. "Anti-termite and antimicrobial properties of paint made from Thevetia peruviana(pers.) Schum. Oil extract'' *Afri. J. Phar and Pharma* **2010**, *1:2* 087-089.

15. Chug K T, Wong T Y, Wei C I, Huang Y W and lin Y. Tannins and human health: A review. *J. food Sci and Nutri*, **1998**, *38(6)*, 421-464.

16. Philip P, A., Experiene with docetaxel in the treatment of gastric cancer. *Seminars in Oncology*., **2005**, *32(supp. 4)*: 24-38.

17. Mehndiratta, S., Meena, A.K., Koul, S. O. P., Lal Dhar, S. and K., *J. Pharm. Res.* **2011**, *4(1)*, 246-271.

18. Hariharan, D., Saied, A., Kocher, H. M., "Analysis of mortality rates for pancreatic cancer across the world". *HPB*, **2008**, *10 (1)*, 58–62.

19. http://www.cancerresearchuk.org/cancer-info/cancerstats/world/the-global-picture/cancer-overall-world#source2. (Assumed date 19 May 2013).

20. Ansary-Moghaddam A., Huxely R., Brazi F., Lawes, c., Ohkubo, T., Fang X., Jee, S. H., Woodward M., *Cancer Epi. Bio. Prev*. **2006**, *15(12)*, 2435 -40.

21. Gidding C. E. M., Kellie S. J., Kamps W. A., de Graaf S. S. N., Vincristine revisited. *Critical Reviews in Oncology/Hematology*, **1999**, *29(3)*: 105-119.

22. Van der heijden, R.; Jacobs, D. I.; Snoeijer, W.; Hallar, D.; Verpoorte ,R.; The *Catharanthus alkaloids*: Pharmacognosy and biotechnology. *Current Medicinal Chemistry* 2004, Vol. 11(5): p 607-628.

23. Cragg, G.M., Newman, D.J., *J. Ethnopharmacol.,* **2005,** *100,* 72-79.

24. Kingston, D.G.I., *Phytochemy.,* **2007**, *68(14):* 1844-1854.

25. http: // en. Wikipedia. Org/wiki/*Catharanthus roseus*. (Assumed date 17 April 2013).

26. Carew D. P., Patterson B.D.,. *Lloydia*, **1970**, *33*, 275-277.

27. Jaleel, C.A.; Monivannan, P.; Sankar, P.; Introduction of drought stress tolerance by Ketoconazole in *catharanthus roseus* is mediated by enhanced antioxidant potential and secondary metabolite accumulation. *Colloids Surfaces. B. Biointerfaces*: 2007, Vol. 60, p 201-206.

28. Fransworth N. R., Svoboda G. H., Blomster R. N *J. Pharmacol. Sci.* **1968**, *57*, 2174-2175.

29. Ram V. J., Kumarin S., *Drug New Perspect*, **2001**, *8*, 468-482.

30. Van der heijden, R.; Jacobs, D. I.; Snoeijer, W.; Hallard, D.; Verpoorte, R.; The *Catharanthus alkaloids*: Pharmacognosy and biotechnology. *Current Medicinal Chemistry,* 2004; Vol. 11(5): p 607-628.

31. Renault J. H., Nuzillard J. M., Crouerour G. L., Thepenier P., Hanfor, M. Z., Oliver L. L., *J. Chromatography*, **1999**, *849* 421–431.

32. Sottomayor, M.; Ros Barcelo, A.; The *Vinca alkaloids:* From biosynthesis and .accumulation in plant cells, to uptake, activity and metabolism in animal cells. In A Rahman (ed.). *Studies in natural products chemistry (bioactive natural products).* The Netherlands: Elsevier Science Publisher, 2005; 813-857.

33. Ahmad N. H., Rahim R. A., Mat I., *Tro. Life Sci. Res*, **2010,** *21(2),* 101-113.

34. Mantu E. D., Sharma K. A., Cardenolide contents in different genotypes of *Thevetia peruviana* and *Nerium odorum*. Nucleus, **1980,** *23 (3)*: 213-225.

35. Thilagavathi R., Kavitha H. p., Venkatraman B. R., *E- J.Chem.* **2010**, ***7(4)***, 1584-1950.

36. Kumar A., *Plant cell tissue and Organ cult*. **1992**, *31(1):* 47-50.

37. Tewtrakul S., Nakamura N., Hattori M., Fugiwara T., Supavita T *Chem. Pharm. Bull*. **200**2, *50(5):* 630-5.

38. Gupta R., Kachhawa J. B., Gupta R. S., Sharma A. K., Sharma M. C., Dophal M. P., *Hum Fertil (Camb).* **2011 Mar**, *14(11):* 53-9.

39. Morton J. F., *J Crude drug Res*, **1975**; *13*: 97-121.

40. Quijano L., Calderon J. S., Gomez F., Rios T., *Phytochem*, **1982**, *21:* 2965-2967.

41. Quijano L., Calderon J. S., Gomez G. F., *Phytochem,* **1985,** *24(5):* 1085-1088

42. Wiedenfeld H., Andradecetto A., *Phytochem*, **2001**; *57 (8):*1269-1271.

43. Quijano L., Calderon J. S., Gomez F., Rios T., Further *Phytochem,* **1987**; *26(7):* 2075-2078.

44. Chandler R. F., Hooper S. N., *Phytochem,* **1979;** *18*: 711-724.

45. Hui W. H., Lee W. K.,. *Phytochem,* **1971**; *10*: 899-901.

46. Anthonsen T., Chantharasakul S., *Acta Chem,* **1970***; 24*: 721-725.

47. Siebertz R., Proksch P., Wray V., Witte L., *Phytochem*, **1988;** *27(12):* 3996-3997.

48. Brever M., Budzikiewicz H., Siebertz R., Proksch P., *Phytochem,* **1987;** *26(11):* 3055-3057.

49. Siebertz R., Proksch R., Witte L., *Phytochem*, **1990;** *29(7):* 2135-2138.

50. Kurade N. P., Jaitak V., Kaul V. K., Sharma O. P., *Pharm Biol*, **2010**; *48(5):*539-44.

51. WiedenFeld H., Cetto A. A., *Phytochem* **2001**, *57*, 1269-1271.

52. Tiwari *et al.,* : Vigynan Parishad Anusandhan Patrika, 1978; Vol. 21, p 177-178.

53. Chaudhary Rai H. N., *Bull. Bot. Surv. India,* **1961,** *3(2):* 171-173.

54. Kiuchi F., Fuako Y., Maruyoma T., Obata T., Tanaka M., Sassaki T., Mikage M., Haque M. E., Tsuda Y., *Chem. Pharm. Bull.* **1998**; *46(3):* 528-530.

55. Kitagawa I., Zhang R. S., Park J. D., Beak N. I., Takeda Y., Yoshikawa M., Shibuya H., *Chem. Pharm. Bull*., **1992,** *40*, 2007-2013.

56. Pari k., Rao P. J., Devakumar C., Rastogi J. N., *J. Nat. Prod*., **1998**, *61*, 102-104.

57. Sen S., Sahu N. P., Mahato S. P., Phytochemistry, **1992**, *31*, 2919-21.

58. Kumar P. S., Chezhian A., Raj, S., Sathiyapriya J., *Bangladesh J Pharmacol* **2012**, *7*: 1-5.

59. Habib M. R., Karim M. R., " Antimicrobial and Cytotoxic Activity of Di-(2-ethylhexyl) Phthalateand Anhydrosophoradiol-3-acetate Isolated from *Calotropis gigantean"* (Linn.) Flower Mycobiology, 2009; Vol. 37(1): p 31-36.

60. Habib M. R., Nikkon F., Rahman M., Haque M. E. , Karim M. R., *Pak. J .Biol.Sci.* **2007**, *10(22)*: 4174-4176.

61. Jackson M. L., "Soil chemical analysis". New Dheli, Prentice Hall of India, 1997, pp 10-144.

62. Baker D. E., Shur N. H., "Atomic and flame emission spectrometry". In: Methoda of soli analysis. U.S.A, American Society of Agronomy, 1982, pp 13-76.

63. Allen S. E., "Chemical analysis of ecological materials". 2nd ed. Blackwell science Publishers, Oxford, 1998.

64. Meyer B. N., Ferrigni N. R., Putman J. E., Jacobson L. B., Niahols D. E., McLaughlin J. I., *Planta Media.*, **1982**, 45, 31-34.

65. Finney D. J., " Probit Analysis", Third edition, Cambridge University Press, 1971, p 76-80.

66. Kafle A., M.Sc Dissertation Submitted to Central Department of Chemistry, T.U., Kathmandu, Nepal, **2009**, pp 24-27.

67. Ghimire B., *Phytochemical and Biological Studies on Lyonia oveliplia*, M.Sc Dissertation Submitted to Central Department of Chemistry, T.U., Kathmandu, Nepal, **2007**, pp 34-37.

68. Silverstein M. R., Webster X. F., “Spectrometric Identification of Organic Compounds”, sixth edition, John Wiley And Sons, Inc.
69. Cowan M. K., Taralo K. P., “Microbiology: a system approach”, Mc Graw- Hill., 2009.

70. Awale S., Feng L., Onozuka H., Esumi H., Tezuka, Y., Kadota S., *Bioorg. Med Chem*. **2008,** *16,* 181-189.

71. Awale S., Lu, J., Kalauni S. K., Kurashima Y., Tezuka Y., Kadota S., Esumi H., *Cancer Res.* **2006** Feb 1, *66(3)*, 1751-7. PMID: 16452235.

72. Monako P., Previlera L., *Journal of Natural Product*, **1984**, *47*, 1175.

73. Uma Devi P., Murugan S., Suja S., Selvi S., Chinnasamy P., Viayaanad E., *Pak. J. Nutrition*, **2007** *6(5)*,447-451.

Spectra

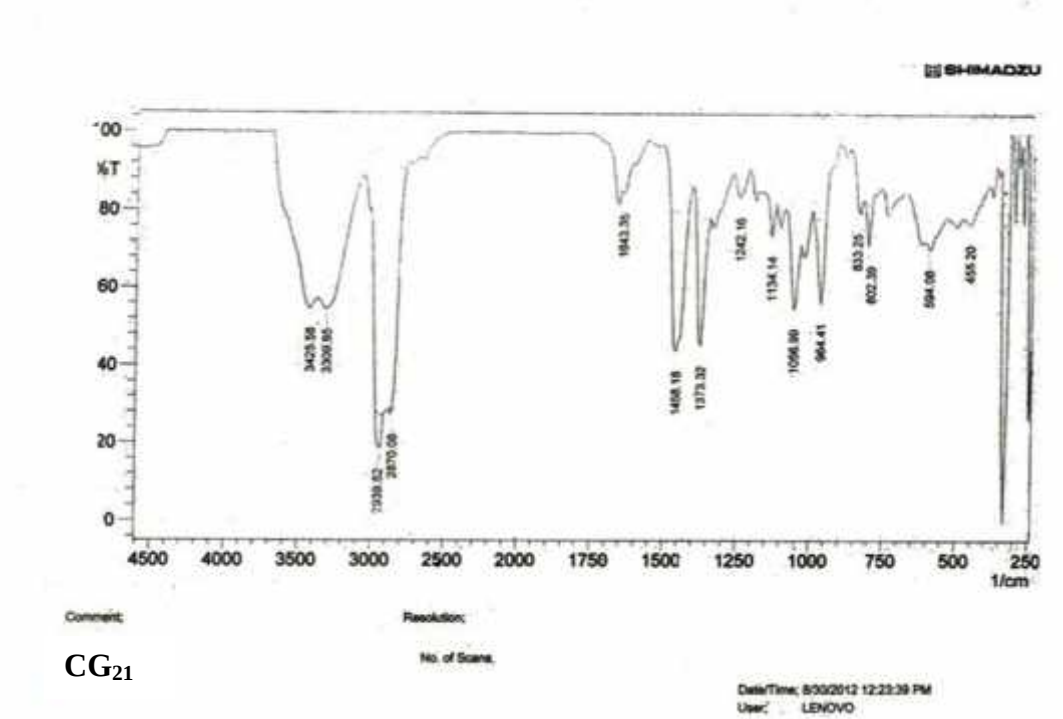
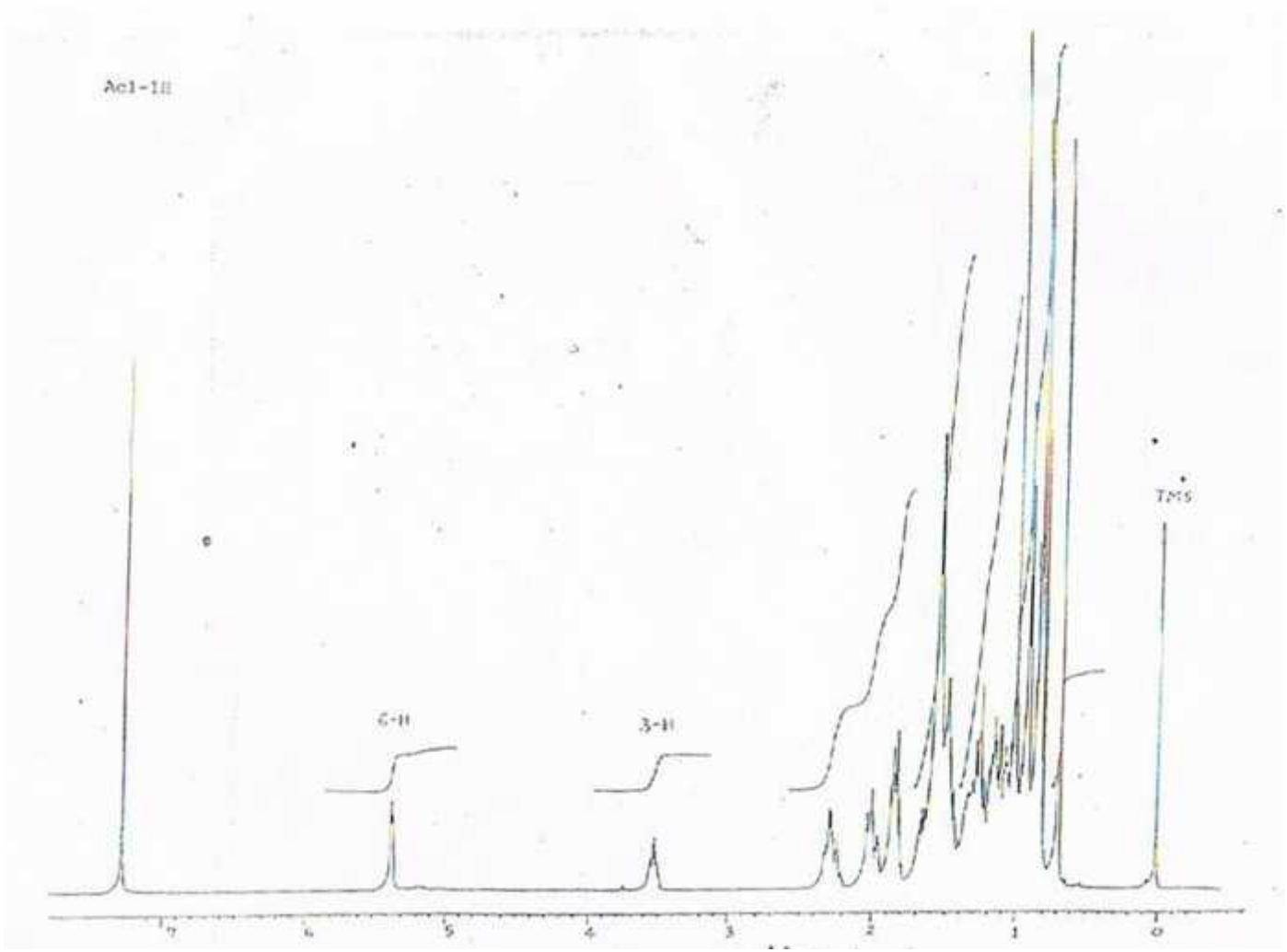


Fig: IR- spectra of β-sitosterol



^{1}HNMR of compound CG_{21} (β-sitosterol)

Photographs

C.roseus / Staphylococcus aureus

C.gigantea / klebeilla oxytoca

A. houstonianum /Escherichia coli