CHAPTER 4

EXPERIMENTAL SECTION

4.1 : General Experimental Remarks

i) IR spectra were recorded on SHIMADZU IR –Prestige 21 FT-IR spectrophotometer in the range of 200cm^{-1}-4000cm^{-1} using KBr pellets in neat condition. Absorption maxima were recorded in wave number (cm^{-1}).

ii) ^{1}H NMR and ^{13}C NMR (400MHz) spectra were recorded on a BRUCKER - ACF 300 spectrophotometer with Tetramethylsilane (TMS) (ð 0.00) as the internal standard in $CDCl_3$ (as solvent) at room temperature. All chemical shifts (ð) are quoted in ppm.

iii) GC-MS were recorded on PERKIN-ELMER Clarus GC/MS.

iv) Solvents like hexane, petroleum ether, ethyl acetate and methanol etc. (MERK, Qualigens) and commercially available reagents (MERK) were used as received without further purification unless otherwise specified and whenever needed, were purified by standard protocols prior to use. Anhydrous solvents were prepared by standard method and were freshly distilled before use.

v) Visualization of spots on TLC plates was achieved by development with iodine vapour.

All the chemicals used were of analytical grade except ethyl acetate, acetone, carbon tetrachloride and petroleum ether which were of laboratory grade. Silica gel (60-120 mesh) for column chromatography was obtained from S.D. Fine Chemicals Ltd. The solvents acetone,

methanol, benzene, petroleum ether, hexane, ethyl acetate and Silica gel for TLC were supplied by Qualigens Fine Chemicals, Mumbai, India. The solvents used for extraction and chromatographic separation were distilled at their respective boiling points before use.

4.2: Collection of the plant materials

The plant materials were collected from Kalinagar and Pachou of Jiribam, Manipur and Silchar, Assam, North-East India. Finally we selected two medicinally important plants species for study. One of the plant was taxonomically identified and authenticated by the Botanical survey of India, Howrah and the voucher specimen no. is CHN/I-I/(301) /2009/Tech. II/343. The plant selected for the present study was ***Croton caudatus*** **Geiseler.**

Another species has been identified at Botanical survey of India Shillong and locally at the Life Science department of Assam University through consultation with Dr. Manabendra Dutta chaudhury, Professor, Dept. of Life Science and hence the plant species selected for the study was ***Phyllantus acidus*** **(L.)Skeels**. Both the plant species were retaining in our laboratory for future references.

The root bark of *Croton caudatus* Geiseler and stem bark of *Phyllantus acidus* (L.)Skeels were collected, shade dried at room temperature and exhaustively extracted with distilled methanol by using soxlet apparatus and thus the crude extract was obtained.

4.3: Phytochemical tests

The crude extract of root bark of *Croton caudatus* Geiseler and stem bark of *Phyllantus acidus* (L.)Skeels thus obtained were subjected to preliminary phytochemicals screening following the methodologies

of ***Harbone (1998), Kokate (2001), Sofowora (1993) and Ngbege (2008).***

General standard screening procedure:

1. **Test for Acidic compounds**: To the extract, sodium bicarbonate solution was added. The production of effervescence indicates the presence of acids.
2. **Test for Alkaloids:** For detecting alkaloids in phytocemical screening, two types of reagents were used i.e., alkaloidal precipitants and spray or dip reagents.
   i) Dragendroff's- reagent: Five ml of the extract was added to 2ml of HCl. To this acidic medium, 1ml of Dragendroff's reagent was added. An orange or red precipitate produced immediately indicates the presence of alkaloids.
   ii) A small amount of the residue obtained after evaporating was dissolved in 5ml of 1% HCI, filtered and tested with Mayer's reagent, Dragandroff's reagent, Wagner's reagent and Hager's reagent separately. The observation exerted cream ppt, orange brown ppt, reddish brown ppt, and yellow ppt respectively to prove the presence of alkaloids.
3. **Screening of steroids and Terpenoids**
   i) Leibermann-Burchard reaction**:** After spraying the TLC plates with LB-reagent and heating at 100^0C for 5 minutes, terpenes gave yellow to violet colour while sterols gave pink colour when observed under UV light.
   ii) Salkawaski reaction**:** When the chloroform solution of the compound was treated with 2-3 drops of conc.H_2SO_4, brown

red colour appeared in the chloroform layer, the compound was a steroid.

iii) Teschugajew reaction: Chloroform solution of the compound was mixed with acetyl chloride in excess and a little of zinc chloride, and boiled. A colour with characteristic fluorescence was developed, a green colour for terpene (i.e. triterpenes) and yellow to pink colour for steroids.

iv) Winogradow reaction: Solution of the compound in chloroform was mixed with 90% trichloroacetic acid (in HCl) and mixture was boiled, a yellow colour appeared for triterpenes and pinkish yellow to blue for sterols.

4. Test for Amino acids: One ml of the extract was treated with few drops of Ninhydrin reagent. Appearance of purple colour shows the presence of amino acids.

5. Test for Anthraquinones:

i) Five ml of the extract solution was hydrolyzed with diluted Conc. H_2SO_4 extracted with benzene. 1ml of dilute ammonia was added to it. Rose pink coloration suggested the positive response for anthraquinones.

ii) **Borntrager's Test:** About 0.5g of the extract was taken into a dry test tube and 5ml of chloroform was added and shaken for 5 minutes. The extract was filtered and the filtrate was shaken with equal volume of 10% ammonia solution. A pink violet or red colour in the ammonical layer was observed for the presence of anthraquinone.

6. Test for Carbohydrates (Molisch's test): Small quantities of alcoholic and extract were dissolved in 5 ml of distilled water and filter. To this solution, 2-3 drops of alpha-naphthol was added. To this about 1 ml of conc.H_2SO_4 was added along the sides of inclined test tube so as to form two layers and observed for the formation of violet colored ring at the interface for the presence of carbohydrates.

7. Test for Flavonoids

(i) (Shinoda or Pew Test): A small quantity of plant extract was dissolved in 5ml of ethanol and treated with few drops of conc. HCl and 0.5g of magnesium turning. The formation of an emulsion indicates the presence of flavonoids.

(ii) One ml of the extract, a few drops of dilute sodium hydroxide was added. An intense yellow colour was produced in the plant extract, which become colourless on addition of a few drops of dilute acid indicates the presence of flavonoids.

8. Detection of Glycosides: The extract was hydrolyzed with HCl for few hours on a water bath. To it, 1ml of pyridine was added and a few drops of sodiumnitroprusside solutions were added and then it was made alkaline with sodium hydroxide solution. Appearance of pink to red colour shows the presence of glycosides.

9. Test for Phenols: The extract was treated with little water and 2ml of Ferric chloride solution. The formation of green or blue colour indicates the presence of phenols.

10. Test for Pholabatannins: Few drops of 1% aqueous HCl was added to plant extract in a boiling tube and then allowed to stand. The

development of red precipitate indicates the presence of pholabatannins.

11. Test for Resins: Five milliliter of distilled water was added to the extract. The formation of turbidity indicates the presence of resins.

12. Test for reducing sugar (Fehling's Test): Few drops of Fehling's A and B in equal volume were added in dilute extracts and heated for 30 min. The formation of brick red colored precipitate indicates the presence of reducing sugar.

13. Test for Saponin: The extract was diluted with 20 ml of distilled water and it was agitated in a graduated cylinder for 15 minutes. The formation of 1cm layer of foam showed the presence of saponins.

14. Test for Tannins: Five ml of the extract and a few drops of 1% lead acetate were added. A yellow precipitate was formed, which indicates the presence of tannins.

15. Test for triterpenoids: Ten mg of the extract was dissolved in 1ml of chloroform; 1ml of acetic anhydride was added following the addition of 2ml Conc.H_2SO_4. Formation of reddish violet colour indicates the presence of triterpenoids.

4.4: Qualitative phytochemical screening of the two selected medicinally important plants (Chemical group test)

The root bark of *Croton caudatus* Geiseler and stem bark of the *Phyllantus acidus* (L.)Skeels were clearly washed with water, shade dried (air dried) and powdered. The powdered plant materials (300g) were defatted with petroleum ether. After wasing with petroleum ether the residue were extracted exhaustively with 200ml distilled methanol

by using soxhlet apparatus. The extract was filtered through cotton followed by vacuum suction. The solvent was then removed by distilling at 20-30^{0}C to give a methanol extract and the extract was used for chemical group analysis.

Table 4.4.(a): Chemical group test for the extract of root bark of *Croton caudatus* Geiseler

Serial No.	Phytochemicals	Methanol extract
1	Alkaloids	+ve
2	Steroids	+ve
3	Saponins	+ve
4	Triterpenoids	+ve
5	Glycosides	-ve
6	Anthraquinone	-ve
7	Tannins	-ve
8	Flavonoids	+ve
9	Amino acids	-ve
10	Pholabatannins	-ve
11	Resins	+ve
12	Sterols	+ve
13	Carbohydrates	+ve
14	Reducing sugar	-ve

+ ve = Present, - ve = Absent

Table 4.4.(b): Chemical group test for the extract of the stem bark of *Phyllantus acidus* (L.)Skeels

Serial No.	Chemical constituents	Methanol extract
1	Alkaloids	-ve
2	Tanins	+ve
3	Saponins	+ve
4	Steroids	+ve
5	Terpenoids	+ve
6	Flavonoids	+ve
7	Phlobatinins	+ve
8	Cardic glycosides	+ve
9	Triterpenoids	+ve
10	Anthraquinones	-ve
11	Amino Acids	-ve
12	Carbohydrates	-ve
13	Reducing sugar	-ve
14	Resins	-ve

+ ve = Present, - ve = Absent

4.5: Results and Discussion

The qualitative analysis of the methanol extracts of the root bark of *Croton caudatus* Geiseler showed the presence of phytochemical constituents such as steroids, sterols saponins, triterpenoids, acids, flavonoids, resins and carbohydrates. At the same time phytochemcal constituents such as alkaloids, glycosides, anthraquinones, amino

acids, reducing sugar, pholabatannins, sterols, phenols terpenoids, and tannins were absent.

The qualitative analysis of the methanol extracts of the stem bark of *Phyllantus acidus* (L.)Skeels showed the presence of phytochemical constituents such as tannins, steroids, saponins, triterpenoids, terpenoids, phlobatinins, and Cardiac glycosides. At the same time phytochemcal constituents such as alkaloids, flavonoids, antraquinones, acids, saponons, cardiac glycosides, reducing sugar, phenols and resins were absent.

Different phytochemicals have been found in a variety of herbs and herbal extracts that contains saponins, terpenoids, flavonoids, tannins, steroids and alkaloids that can be of valuable therapeutic index. For example, the alkaloids contained in plants are used in medicine as anesthetic agents ***(Herourat et al., 1988).*** Saponins in plants have been reported to be responsible for the tonic and stimulating activities observed in Chinese and Japanese medical herbs ***(Alinnor, 2008***). It should be noted that steroidal compounds are of importance and interest in pharmacy due to sex hormones ***(Okwu, 2001).*** The diuretic and antibacterial activity of plant extracts containing flavonoids have been documented ***(Enwerem et al., 2001, 2003, Monache et al., 1996, Rao et al., 1996, Sofowora, 1993).***

Several workers have reported on the analgesic properties of alkaloids ***(Antherden, 1969, Harborne, 1973)*** as well as the anti-inflammatory and anti-bacterial properties of tannins ***(Duguid et al., 1989).***Tannins with its protein precipitating and vasoconstriction effect could be advantageous in preventing ulcer development ***(Aguwa and Nwankwo, 1988, Dahiru et al., 2006)***. The terpenoids have also been

shown to decrease blood sugar level in animal studies ***(Luo et al., 1999).*** Glycosides have hypoglycemic activities ***(Oliver, 1980).*** The steroids and saponins are responsible for central nervous system activities ***(Argal and Pathak, 2006).***

Phytochemical screening and qualitative analysis of root bark of *Croton caudatus* Geiseler and stem bark of *Phyllantus acidus* (L.) Skeels showed the presence of different phytochemical constituents **Table-4.4 (a), (b)** can also have various medicinal values such as central nervous system activity, tonic and stimulating activities, diuretic and antibacterial activities, in preventing ulcer development, anti-inflammatory and anti-bacterial activities etc.

4.6: Conclusion

In the present study, we have found that most of the biologically active phytochemicals were present in the methanol extracts of root bark of *Croton caudatus* Geiseler and stem bark of *Phyllantus acidus* (L.)Skeels. The medicinal properties of *Croton caudatus* Geiseler root bark and stem bark of *Phyllantus acidus* (L.)Skeels extracts may be due to the presence of above mentioned phytochemicals. Further studies are carried out to study the biological activity of the extracts to isolate the active compounds present in the extracts.

4.7 Bibliograpy

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