SYSTEMATIC ANALYSIS AND EXTRACTION OF TRITERPENOID SAPONINS FROM INDIGENOUS MEDICINAL PLANT CLEMATIS NAPAULENSIS DC. USED BY CHAKESANG TRIBE FOR TREATMENT OF RHEUMATOID ARTHRITIS

Thesis submitted to the Department of Botany, Nagaland University Lumami, Nagaland in partial fulfillment for the requirement of Degree of Doctor of Philosophy in Botany

By

Pangwan M Konyak

DEPARTMENT OF BOTANY

NAGALAND UNIVERSITY, LUMAMI – 798627

NAGALAND, INDIA

2024

नागालैण्ड विश्वविद्यालय

Nagaland University

(A Central University Established by the Act of Parliament No.35 of 1989)

Department of Botany, Lumami Nagaland: 798627
E-mail: botanynagauniv@gmail.com

Prof. Talijungla
Department of Botany

Phone: 7005639570
Email: lkr.talijungla@gmail.com

CERTIFICATE

This is to certify that the thesis entitled **"Systematic analysis and extraction of triterpenoid saponins from indigenous medicinal plant *Clematis napaulensis* DC. used by the Chakesang tribe for treatment of Rheumatoid Arthritis"** submitted to Nagaland University, Lumami in partial fulfillment of the requirements for the degree of Doctorate in Philosophy in Botany is an original research work carried out by **Mr. Pangwan M Konyak,** bearing registration number **Ph.D./BOT/00157** dated 17/08/2018.

Further, it is certified that no part of this thesis has been submitted anywhere for any other research degree.

Place: Lumami
Date:

Prof. Talijungla
(Supervisor)

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DECLARATION

I, **Mr. Pangwan M Konyak**, bearing registration number **Ph.D./BOT/00157** dated 17/08/2018, hereby declares that the thesis entitled **"Systematic analysis and extraction of triterpenoid saponins from indigenous medicinal plant *Clematis napaulensis* DC. used by the Chakesang tribe for treatment of Rheumatoid Arthritis"** being submitted to Nagaland University, Lumami, for the degree of Doctorate of Philosophy in Botany is the original and independent research work carried by me under the joint supervision of Prof. Talijungla, Department of Botany, Nagaland University, Lumami.

I further declare that this thesis has not been previously submitted to any University or tertiary institutions for award of any other degree or diploma. My declaration/thesis henceforth forwarded by my Supervisor and Head of department.

Pangwan M Konyak

Head of Department, Botany
Nagaland University

Prof. Talijungla
(Supervisor)

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PANGWAN M KONYAK

CONTENTS

Abbreviations and Symbols Used

1. RA : Rheumatoid Arthritis
2. DC. : De Candolle
3. WHO : World Health Organization
4. STDs : Sexually Transmitted Diseases
5. C = C : Carbon Double Bond
6. CVD : Cardiovascular Disease
7. iNOS : Inducible Nitric Oxide Synthase
8. BV-2 : Bacterial Vaginosis
9. MALME-3M : Malignant Human Melanoma Cell Line
10. MCF-7 : Human Breast Cancer Cell Line
11. MIC : Minimum Inhibitory Concentration
12. NO : Nitric Oxide
13. RAW 264.7 cells: Macrophage cell line that was established from a tumor in a male mouse induced with the Abelson murine leukemia virus.
14. IC50 : Half Maximal Inhibitory Concentration
15. IPP : 3- Isopentenyl Pyrophosphate
16. GPP : Geranyl Pyrophosphate
17. DMAPP : Dimethylallyl Pyrophosphate
18. P_{450} : Cytochrome P450 are Heme cofactor containing enzymes functioning as Monooxygenases
19. SFE : Supercritical Fluid Extraction
20. PLE : Pressurized Liquid Extraction
21. MAE : Microwave Assisted Extraction
22. SPME : Solid Phase Micro-Extraction
23. UAE : Ultrasound Assisted Extraction
24. HPLC : High Performance or High Pressure Liquid Chromatography
25. MPLC : Medium Pressure Liquid Chromatography
26. TLC : Thin Layer Chromatography
27. HSCCC : High Speed Counter Current Chromatography
28. HPLC-UV : High Performance Liquid Chromatography- Ultraviolet Spectroscopy

29. HPTLC : High Performance Thin Layer Chromatography
30. HPLC-CAD : High Performance Liquid Chromatography- Charged Aerosol Detector
31. NMR : Nuclear Magnetic Resonance
32. COX : Cyclo-Oxygenase
33. TPC : Total Phenolic Content
34. TFC : Total Flavonoid Content
35. TTC : Total Triterpenoid Content
36. ROS : Reactive Oxygen Species
37. FRAP : Ferric Reducing Anti-oxidant Power
38. TPTZ : 2,4,6-Tris(2-pyridyl)-s-triazine / $C_{18}H_{12}N_6$
39. MPO : Myeloperoxidase
40. EC : Enzyme Commission
41. EM : Electromagnetic
42. DPPH : 2,2-diphenyl-1-picrylhydrazyl ($C_{18}H_{12}N_5O_6$)
43. NSAIDs : Non-Steroidal Anti-Inflammatory Drugs
44. PLA2 : Phospholipase A2
45. ASA : Acetyl Salicylic Acid (Aspirin)
46. B.P : Boiling Point
47. Rf : Retention factor
48. GAE : Gallic Acid Equivalent
49. QSE : Quercetin Standard Equivalent
50. OAE : Oleanolic Acid Equivalent
51. C_A : Control absorbance
52. S_A : Sample absorbance
53. PBS : Phosphate Buffer Solution
54. Mol : molarity
55. N : normality
56. MW : Molecular weight
57. BSA : Bovine Serum Albumin
58. λ : Wavelength
59. R^2 : Regression Value
60. ml : milliliter

61. nm : nanometer
62. Kg : kilogram
63. g : gram
64. mg : milligram
65. µg : microgram
66. ºC : Degree Celsius
67. % : Percent
68. w/w : weight/weight
69. w/v : weight/volume
70. v/v : volume/volume
71. NaOH : Sodium Hydroxide
72. H_2SO_4 : Sulphuric Acid
73. HCl : Hydrochloric acid
74. CH_3OH : Methanol
75. C_2H_5OH : Ethanol
76. H_2O_2 : Hydrogen Peroxide
77. O_2^- : Superoxide Anion

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CHAPTER 1

INTRODUCTION

The term ‘Medicinal plants’ includes various type of plants used in herbal medicine remedies against numerous health problems. These days the term ‘Alternative medicine’ has also been used in the western culture with the focus on idea of medicinal properties. The current beliefs also comes to light that we have trust and uses only medicine that comes in shape of pills and tablet. Even so, many of these medicines are the extract of plants active ingredients as per their intended usage. These plants have vast source of ingredients that could be used in drug development and synthesis (Rasool Hassan, 2012). Considering the fact that, about 3.3 billion people in developing countries are dependent on these medicinal plants on regular basis (Davidson, 2000). Medicinal plant could act synergistically where it acts to neutralize the negative health condition. It could also act in support of official medicine for many dreadful diseases like cancers. It could also be recognized by its ability to prevent the appearances of some diseases or reduces the usage of chemical remedies if in case the disease is already present (Singh, 2015 and Rasool Hasaan, 2012). However, due to increased destructive harvesting there are many described medicinal plants that are endangered, threatened or vulnerable (Hamilton, 2008 and Larson *et al*., 2007).

Medicinal plants contain compounds that could provide a physiological response to the human body against diseases and the compounds includes tannins, alkaloids, carbohydrates, terpenoids, steroids and flavonoids (Edoga and Okwu *et al*., 2005). The study was focused on analyzing systematically, the triterpenoid saponins that was present in a medicinal plant *Clematis napaulensis* DC., found in Nagaland indigenously. It was also based on the fact gathered from local Chakesang tribe of Nagaland who has been using the plant for treatment of Rheumatoid Arthritis (RA). Scientifically relating the plant to RA, it has been known that triterpenoid saponins are present in higher concentration in many medicinal plants exhibiting properties against RA. Therefore the study was focused on the efficacy of plant against the symptoms exhibited by RA followed by the extraction of triterpenoid saponins from the plant.

Preliminary screening of phytochemicals in medicinal plants has been an essential step in the detection of bioactive compounds present in any medicinal plant and could subsequently lead to the drug discovery and development (Yadav *et al*., 2014). Exogenous chemicals and endogenous metabolic processes produces high amount of free radicals, especially oxygen derived radicals causing cellular and tissue damages. Anti-oxidants are compounds that inhibits or slow down these processes of oxidation chain reactions (Halliwell and Gutteridge, 1989). Oxidative damage plays a key role in many progressive physiological disorders and diseases such as artheroclerosis, rheumatoid arthritis, gastritis, ageing, nervous system injury, inflammatory response, respiratory and liver diseases, cancer etc. (Pourmorad *et al*., 2006).

CHAPTER 2

LITERATURE REVIEWS

Medicinal plants has been the most common reservoir of life saving drugs found in the world and has been considered as the most efficient way to treat many diseases and ailments since the dawn of civilization. The popularities in its usage were being supported by the fact that the organic compounds from herbal medicine has rare side effects otherwise very common in chemical therapies (Pritam and Munim *et al*., 2017). WHO had estimated about 50,000 of higher plants being used in medicinal purposes (Schippman, 2002) and about 75-80% of the world population were dependent on plant derived drugs for health care and cosmetics obtained from traditional healers (Martin *et al*., 2008). Traditional folk treatments from wild species have always been a guiding destination for the research community to search for novel medicine in the quest of healthy life for humans and animals (Achterberg, 2013). The phyto-medicines could be derived from the bark, leaves, flowers, roots, fruits and seeds (Craigg and David, 2001). Knowledge of the chemical constituent of plants has been desired as it would be valued in the synthesis of complex chemical substances (Parekh and Chanda, 2007).

Infectious diseases have always been an important health hazards all over the world, both in developing and developed countries (Sasikumar *et al*., 2003). In the last three decades, new antibiotics and chemicals have been produced by the pharmaceutical industries against the microbes causing these diseases. However, drug resistances are being developed by the microbes (Nascimento *et al*., 2000). Therefore, there is an urgent need for the alternative medicine to the ailments. The interest in the traditional ethnomedicine may lead to the discovery of novel therapeutic agents. Medicinal plants have been finding their way to the pharmaceutical, neutraceutical, cosmetics and food supplement industry (Mohanasundari *et al*., 2007).

There were reported 300,000 species of documented higher plants on the planet synthesizing enormous amount of chemicals of diverse structure and class. These compounds, about 200,000 of them have been identified and classified further into primary metabolites and secondary metabolites. The primary metabolites include sugars, fatty, amino and nucleic acids.

It also includes compounds found in all plants that are being needed for growth and development (Feihn, 2002 and Wu *et al*., 2008).

The genus *Clematis* was first started by Linnaeus in 1753 with only nine species. It has increased in number since and now the genus *Clematis* has about 355 species within the eudicot family. The genus consists of woody, vigorous climbling vines distributed mainly in the temperate zone of north earth's equator (Wang and Li, 2005). Through molecular phylogenetic and taxonomic study, it was revealed that Yunnan, China is the place where most *Clematis* were being found in the world and the Hengduan mountain of Northwestern Yunnan is considered the center of origin, differentiation and endemism of the genus (Jiang *et al*., 2007).

Clematis are a sources for various conventional medicines used since the beginning of Chinese civilization. Root, rhizomes and stems of some *Clematis* have been used to disperse wind damp, unclogged channels and ease pain. It have also been used orally to treat STDs, Podagra, RA, Chronic skin disease and as a diuretic. In folk medicine it have been used for treatment of blister infection and ulcers (Sun and Yang *et al*., 2009). Hao *et al*., in 2013 reported that to date, more than 30 species of *Clematis* have been characterized for their chemical components. *Clematis flammula*, which is commonly found in the Mediterranian, were also being used in Spain, Italy, Turkey and Algeria where the dried leaves of the species were being used to treat arthritis, superficial burns and even cancer (Boloued, 1998). *Clematis lasiandra* Maxim. a perennial herbaceous plant, in which the entire plant and rhizome have long been used as folk medicines in China for treatment of dehygrosis, antitoxic, diuretic, analgesic and antipyretic etc. (Zhang *et al*., 2010).

According to pharmacophylogeny, plants with close genetic relationship were also similar in their systematic classification and chemical compositions (Xiao *et al*., 2006). Different types of phytochemical classes were being reported in the genus *Clematis* such as saponins, flavonoids, alkaloids, coumarins and anthocyanins having various bioactivities (Chang *et al*., 2017). Hai *et al*., in 2013 that since 2009, 19 novel triterpenoid saponins have been isolated from *Clematis* plants., more than 50 oleanolic type prototype saponins, more than 40 hederagenin type prototype saponins and two gypsogenin saponins have been found in *Clematis* plants. Flavonoids were the major compounds in extract prepared from *Clematis*

terniflora. *Clematis brachiata* as well as *Clematis rehderiana* showed anti-inflammatory response in rat supposedly due to flavonoids (Du *et al*., 2010). Twenty-four lignans were isolated from *Clematis* mainly eupomatene lignans, cyclolignans, monoepoxylignans, bi-sepoxylignans and lignanolides. Six coumarins were isolated. Alkaloids isolated fell under two categories mainly – aporphine and terpenoids of which the terpenoid alkaloid exhibited anti-fungal activity (Li *et al*., 2009).

Clematis napaulensis DC. (syn. *Clematis forestii*) also commonly known as Nepal clematis is a species of flowering plant belonging to the buttercup family of Ranunculaceae. The nodding cream-coloured flowers are up to 3 cm (1.2 in) across and scented. Notable features in the plants include ribbed stems, ternated leaves, which are followed by handsome large fruit clusters. The leaves are stalkless and clasp to the stem. The plant does not survive harsh winter climates. This remarkable, unique, and extremely rare plant from Nepal and parts of southern China, loses most of its foliage in late spring or early summer and remains dormant throughout the hottest months of the year, then in autumn it puts on lots of lush new foliage. Suddenly, large clusters of buds erupt, which open into flowers in early winter and are quite spectacular being pendulous and scented, resembling greeny-yellow bells filled with attractive, protruding red-purple stamens, which finally ripen into large, fluffy seeds densely clustered in a 1.5 cm head head which last for several months. It is perfect for a warm, sheltered courtyard garden or cool conservatory (Costin *et al*., 2000). The seeds are usually wind dispersed. The Chakesang tribe of Nagaland, India has been using this plant for generations in the treatment of Rheumatoid Arthritis, however in a crude manner.

Fig 2.1: *Clematis napaulensis* bloom

Fig 2.2: *Clematis napaulensis* matured leaves

Fig 2.3: *Clematis napaulensis* immatured seeds

Fig 2.4: *Clematis napaulensis* matured seeds

Secondary metabolites are compounds that had showed curative properties against several ailments occurring in man and could explain their traditional usage in treatment. The compounds in this group such as phenolics, alkaloids, terpenoids, steroids, quinones and saponins have more complex structure and are more restricted in their distribution. But they are despensible even though their metabolic functions remain still at large for most (Li *et al*., 2006).

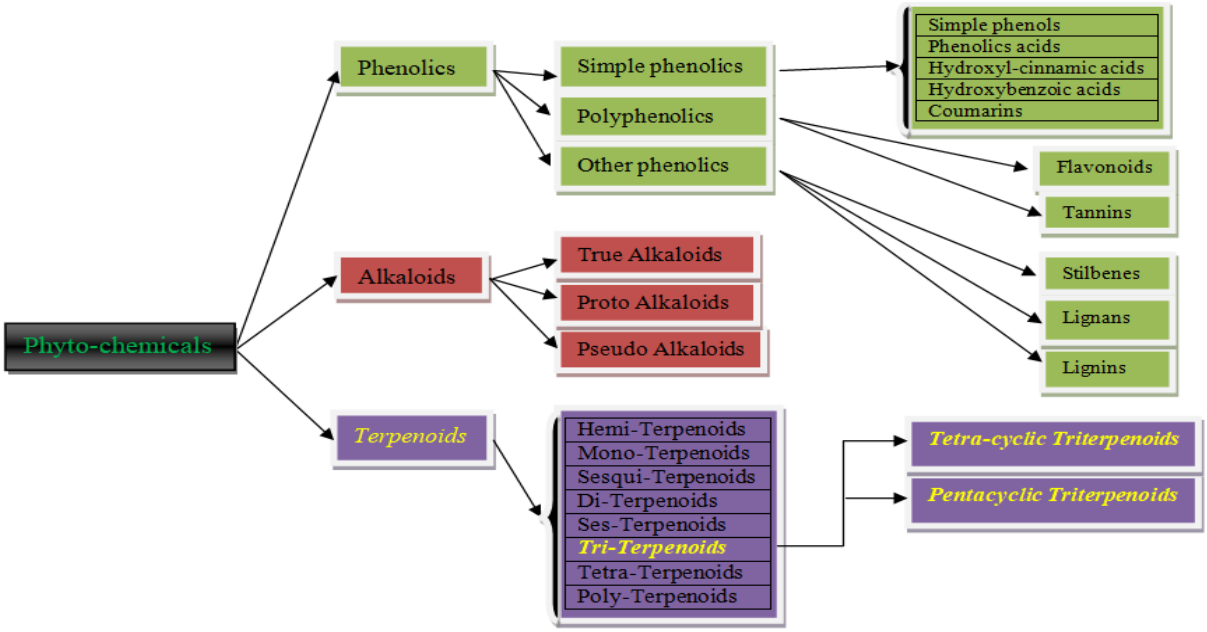


Fig 2.5: Generalized classification of phytochemicals

Phenolic compounds are vast, diverse, ubiquitous and widespread in nature. The compounds are known to exhibit various biological activities such as anti-oxidant, anti-microbial and anti-inflammatory properties (Al Mamari, 2021). Large group of Phenolics contains simple phenols (catechol, resorcinol, hydroquinone etc.), phenolic acids, stilbene (resveratrol, etc.), flavonoids (quercetin, cyanidin, etc.), biflavonoids (ormocarpine, etc.), proanthocyanidins (epicatechin), tannins, coumarins and anthraquinones (Wollenwaber, 1993).

- **Simple Phenolics Compounds**

Simple phenols – The compounds are hydroxyphenols or dihydroxybenzenes. Examples are catechol (1,2-dihydroxybenzene), resorcinol (1,3-dihydroxybenzene), and hydroquinone (1,4-dihydroxybenzene). Other simple substituted phenol compounds can also be dihydroxy-phenols or trihydroxybenzenes. Examples are pyrogallol (1,2,3-trihydroxybenzene), hydroxyquinol (1,2,4-trihydroxybenzene), and phloroglucinol (1,3,5-trihydroxybenzene).

Phenolics acids – Phenols that contain a carboxylic acid are termed as phenolic acids. If the carboxylic acid functional group is directly bonded to the phenol ring, the phenolic compound is termed as hydroxybenzoic acid. When carboxylic acid functional group

and the phenol ring are separated by two doubly bonded carbons (C=C bond), phenolic compounds are termed as hydroxycinnamic acids.

Hydroxyl benzoic acids – Hydroxyl benzoic acids are benzoic acids substituted with a hydroxyl group. Alternatively, they can be viewed as phenols that are substituted with a carboxylic acid functional group that is directly bonded to the phenol ring.

Hydroxycinnamic acids – When the carboxylic acid functional group is separated from the phenol ring by a C=C bond, phenolic acids are described as hydroxycinnamic acids.

Coumarins – Hydroxycoumarins are hydroxyl-substituted coumarins. They are examples of phenolic compounds. Examples of hydroxycoumarins are scopoletin and auraptene.

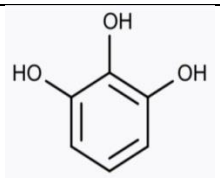
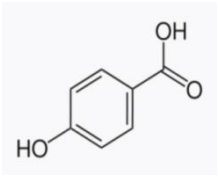
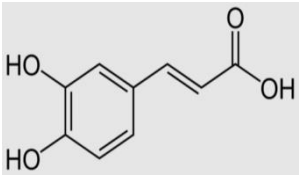
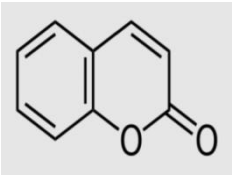
 Pyrogallol		 Caffeic acid	
Simple Phenolics	Hydroxy/Hydroxyl benzoic acid	Hydroxycinnamic acid	Coumarin

Fig 2.6: Skeletal structure of simple phenolic compounds

- **Polyphenolics:** Phenolic compounds that contain more than one phenol unit are considered "polyphenol". Polyphenolic compounds have C15 general skeleton representation.

Flavonoids – Flavonoids represent a highly diverse class of polyphenolic secondary metabolites, which are abundant in gymnosperms and angiosperms. It has also been reported from primitive taxa, such as bryophytes, pteridophytes and algae (Oh *et al*., 2004 and Imperato, 2008). Overall, about 10,000 flavonoids have been recorded which represent the third largest group of natural products following the alkaloids (12,000) and terpenoids (30,000). Flavonoids mostly act as communicators in plants with their environment due to their colours, symbiotic nature with the pollinators etc. (Havsteen, 2002). Tapas *et al*., 2008 and Alzand *et al*., 2012 have independently showed flavonoids has pharmacological activities, including antioxidant, cytotoxic, anticancer, antiviral, antibacterial, anti-inflammatory,

antiallergic, antithrombotic, cardioprotective, hepatoprotective, neuroprotective, antimalarial, antileishmanial, antitrypanosomal and antiamebial properties.

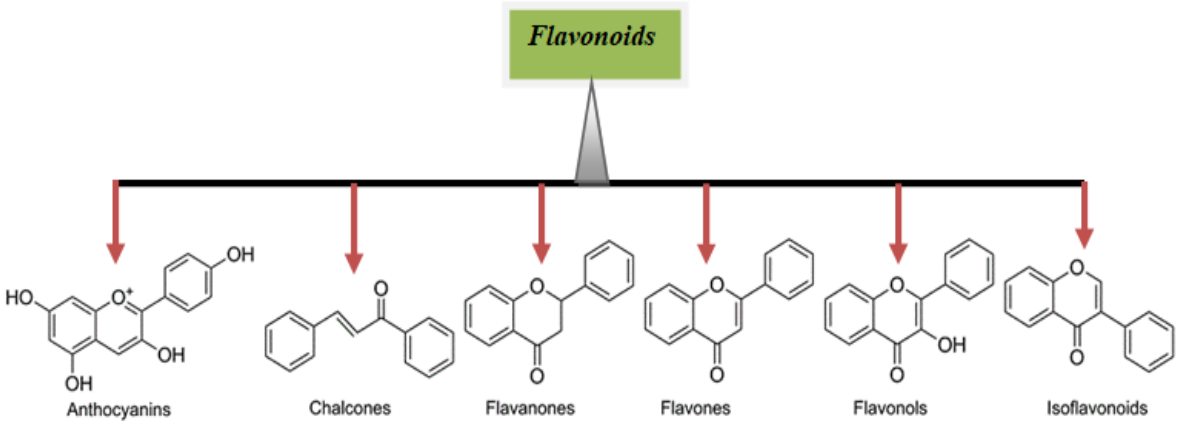


Fig 2.7: General Structural classification of Flavonoid classes

Tannins – Any phenolic compounds of sufficiently high molecular weight ranging from 500 to 3000 (Smith and Swaain, 1962) containing sufficient hydroxyls and other suitable groups e.g. carboxyls to form effectively strong complexes with proteins and other macromolecules (Ashok and Upadhyaya, 2012). They are known to bind to and precipitate proteins and amino acids. Tannins are subdivided into three types – Hydrolysable (e.g. Gallic acid), Condensed (e.g. Flavones) and Phlorotannins (e.g. Phloroglucinol). Hydrolyzable tannins can be gallotannins or ellagitannins. Gallotannins are polyols that are substituted with gallic acid units. The galloyl units in gallotannins are linked by depside (ester) linkages. Commonly the polyol core is a D-glucose that is substituted with gallic acid units. Tannic acid is an example of gallotannins. Condensed tannins are polymeric phenolic compounds that consist of catechin units. When depolymerized, they give anthocyanidin. Thus condensed tannins are called proanthocyanidins. Complex tannins are gallotannins or ellagitannins bonded to a catechin unit.

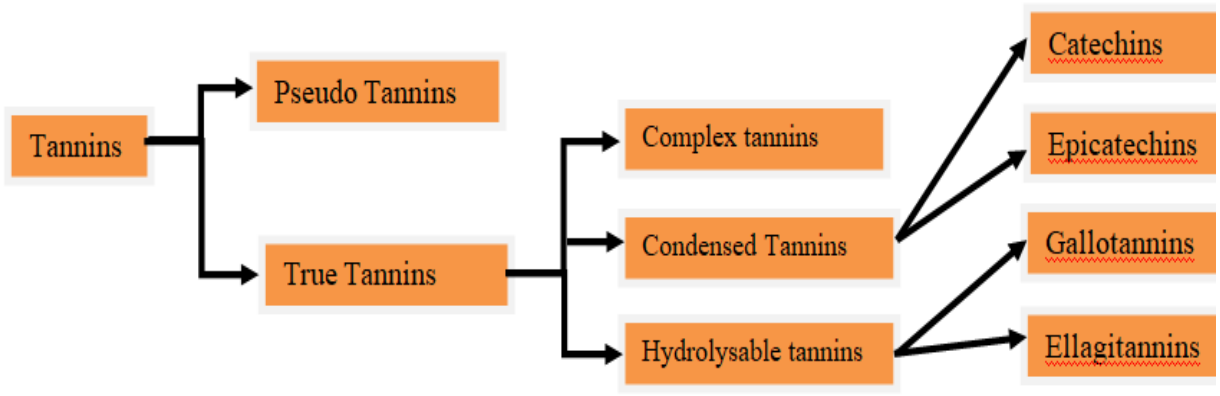


Fig 2.8: Generalized classification of tannins (Khanbabaee and Ree, 2001)

- **Other Phenolics**

Stilbenes – They are phenolic compounds in which two phenol units are linked by two doubly bonded carbons. Stilbenes are synthesized by plants from phenylalanine/ polymalonate route, using key enzyme as stilbene synthase (Roat and Saraf, 2015). The skeletal structure of stilbenes contains two benzene ring joined by an ethylene bridge as C_6-C_2-C_6. From this relative simple structure, a large array of compounds are being substituted in varying numbers and location of hydroxyl groups, the substitution being done by sugars, methyl, methoxy and other residues (Waffo-Teguo *et al*., 2008).

Among the stilbenes, resveratrol is highest studied with consideration for potential medicinal use due to its wide range of biological activities. It has reported properties such as lipolysis stimulation, anti-inflammatory and modulation of cell proliferation. These properties among many are suggested to provide protection against chronic diseases such as cancer, cardiovascular and neuro-degenerative pathologies (Khawand *et al*., 2018).

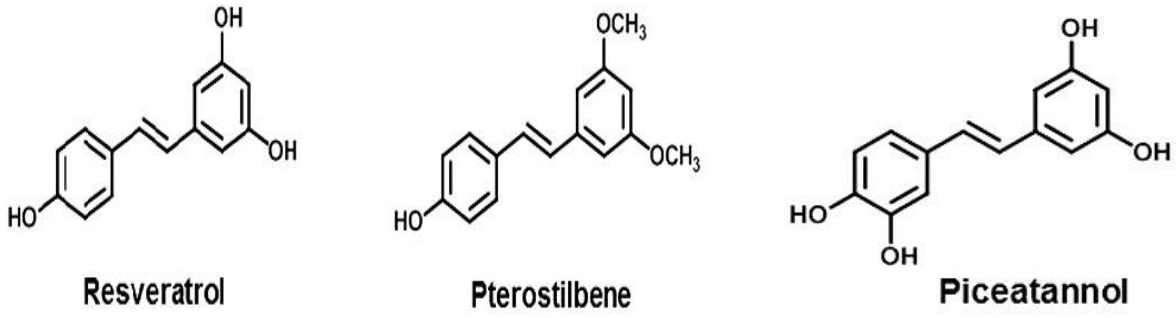


Fig 2.9: Structures of common stilbenes

Lignans – They are a large group of polyphenols forming a building block of plant cell wall. There are reported 450 types based on structures distributed in over 70 plant families. Lignans consist of two phenol units linked by four carbons. Lignans major role with respect to their function in plants are antifungal, antimicrobial, antiviral, and even insecticidal properties which may have arisen during their co-evolution of plants and insects and from the natural defense mechanisms providing protection against diseases (Gang *et al*., 1999). Examples include matairesinol, secoisolariciresinol and pinoresinol. Lignans have shown a spectrum of important biological and health-promoting activities such as cancer chemo-prevention, antioxidant and anti-inflammatory properties, lipid-lowering effects, cardiovascular disease (CVD) protection, obesity, diabetes management and anti-microbial. Investigation of dietary lignans for human health is challenged however, by differences in bioavailability from varied food sources, the effects of food processing, and having accurate estimates of total consumption in human populations to assess the contribution on health outcomes (Tham *et al*., 1998).

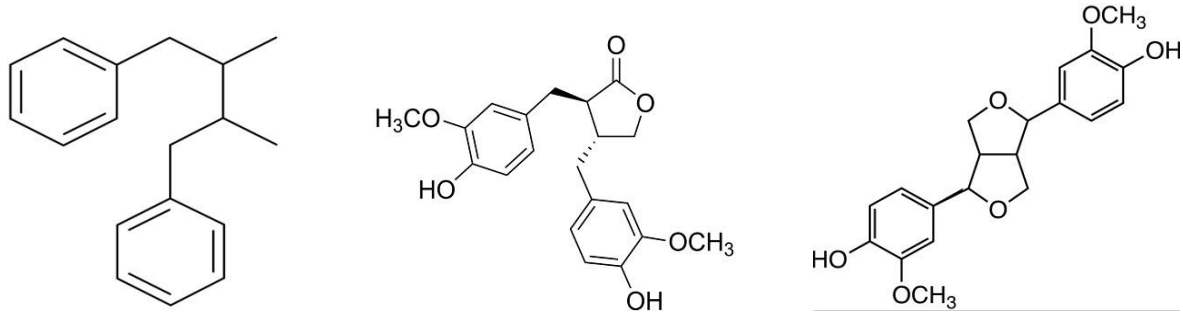
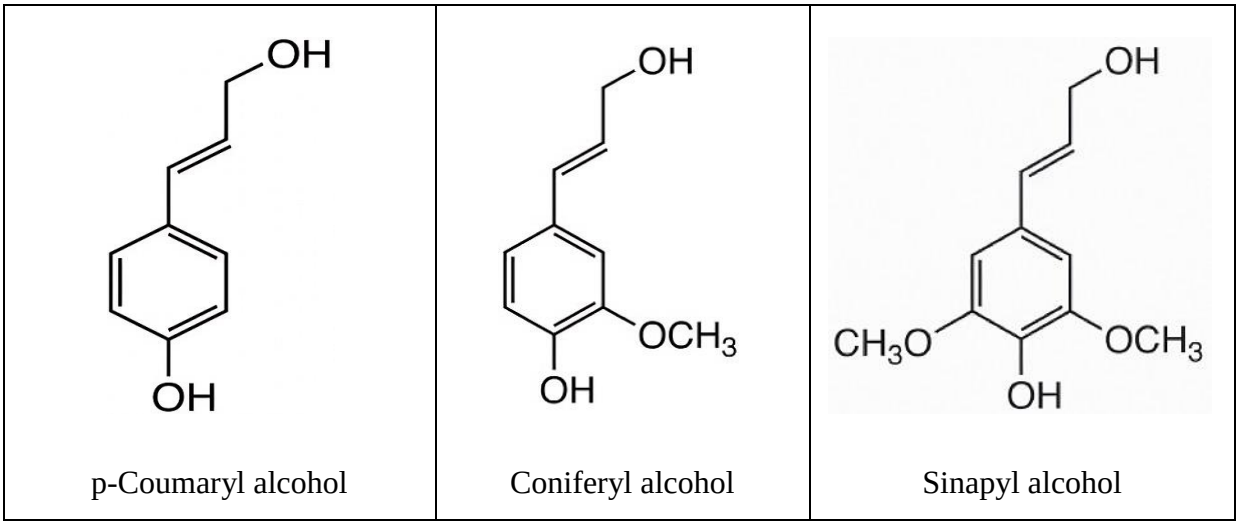


Fig 2.10: Lignans basic structure (L), Matairesinol (C) and Pinoresinol (R)

Lignins – They are basically found in the plant cell wall alongwith cellulose, hemicellulose and pectin. The three basic building blocks of lignin are p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol which are synthesized via the phenylpropanoid pathway in plants and differs in the extend of methoxylation as 0, 1, 2 (Boerjan *et al*., 2003). Lignins consist of phenol units or phenolic compounds that are linked with each other by carbon chains to form a 3D network. Lignins are high molecular weight polymers of average ~20,000 (Parasuraman, 2007) and are the second most abundant biomolecule after cellulose with composition ranging between 15-40%. However many of the taxononomic groups of non-flowering plants are unable to synthesize lignins owing to evolution (Stern *et al*., 2000).



p-Coumaryl alcohol	Coniferyl alcohol	Sinapyl alcohol

Fig 2.11: Basic branched aromatic monomers of lignin biosynthesis (Durazzo *et al*., 2019 and Mark *et al*., 2019).

Alkaloids are complex and diverse with atleast one nitrogen containing biomolecules found in bacteria, fungi, animal and plants. Their classifications by structure are rather non-reliable and hence more often they are classified by source of origin or by biochemical origin such as Shikimic, ornithine, lysine and nicotinic pathway, the histidine and purine pathway and the terpenoid and polyketide pathways (Gutiérrez-Grijalva *et al*., 2020). Alkaloids are a very wide group of natural compounds derived from secondary metabolites about 40,000 as per reported in dictionary of Alkaloids (Buckingham *et al*., 2010) and found in 25% of the plants. Many alkaloid structures remained unknown until well into the twentieth century when X-ray spectroscopy became widely available, and after organic chemistry had advanced to the point where these molecules could be produced synthetically (Hesse, 2002). Their main characteristic feature is the presence of a basic atom of nitrogen in any position of the molecule not including nitrogen in an amide bond or peptide (Lu *et al*., 2012). Nowadays, research has led to identification of several types of alkaloids in over 4000 different plants. Some plant families have been known to harbour high content of alkaloids, such as the Papaveraceae, Ranunculaceae, Solanaceae, and Amaryllidaceae families (*Encyclopædia Britannica*, 2018).

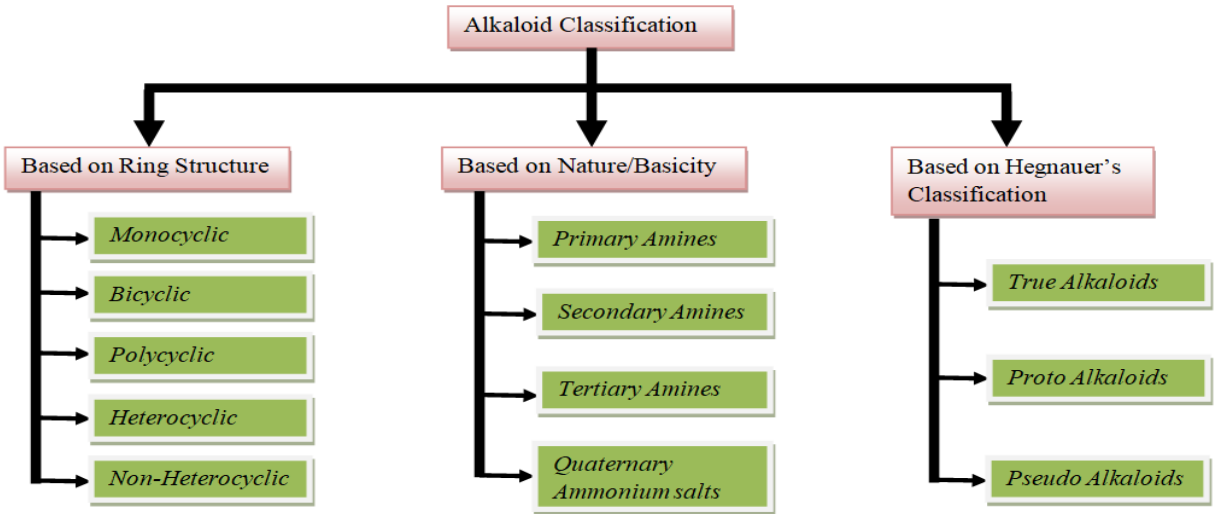
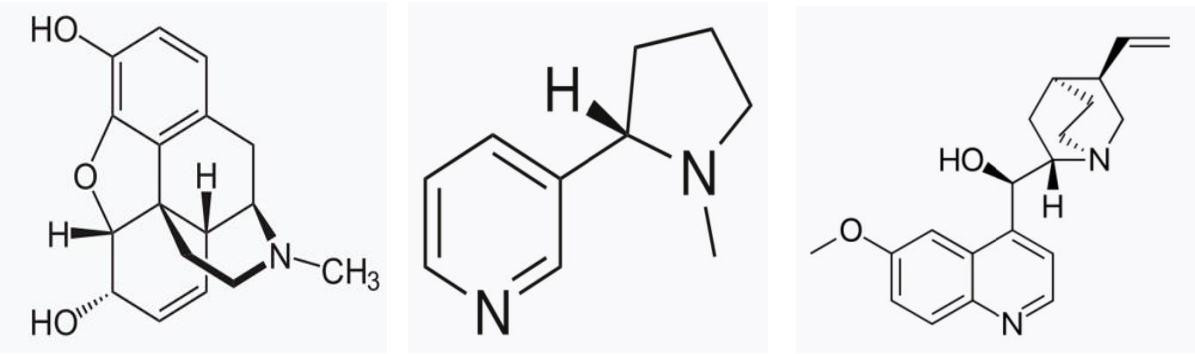


Fig 2.12: Different classification of Alkaloids

Alkaloids common in our day to day live are being derived from plant such as Morphine from *Papaver somniferum*, Caffeine from *Camillia sinensis*, Nicotine from *Nicotiana tobacum*, Cocaine from *Erythroxylum coca*, Atropine from *Atropa belladonna* and Quinine and *Chincona pubescens* etc. (Gutiérrez-Grijalva *et al*., 2020). In the light of history, alkaloids containing plant species were being used since very ancient times and mention in historical evidences, moreover associated with some important characters like Socrates (death by *Conium maculatum*), Cleopatra (used *Hyoscyamus maticus* for pupil dilation) and *Atropa belladonna* being used by medieval women for their beauty purposes (Ncube *et al*., 2015). The biological significance of alkaloids depends on their significant relation with health benefits. Alkaloids are now medically known anesthetics, stimulants, anti-bacterials, anti-malarials, analgesics, anti-hypertensive agents, spasmolysis agents, anti-cancer drugs, anti-asthma therapeutics, vasodilators, anti-arrhythmic agents, etc. These properties, as well as their toxicity continue to be an important research field (Kuete, 2014). An important widely used alkaloid is Morphine, a strong opiate found naturally in Opium as dark brown resin from its poppies. It can be used as pain medication and is also used for recreational purpose or used to make other illicit opioids. Other important alkaloids are like Quinine (used in treatment of malaria) and nicotine found in nightshade family of plants (used for recreational purposes).



Morphine Nicotine Quinine

Fig 2.13: Structures of common alkaloids

Terpenoids are modified class of terpenes which are found in root, stem, leaves, flowers and other part of numerous plant species. Terpenoids were known as volatile substances which gave plants and flowers their fragrance and usually an identity of higher plants soft tissues (Mabou *et al*., 2021). The buiding block isoprene unit has general formula $(C_5H_8)_n$. They could be grouped into classes according to the number of isoprene units (n) in the molecule: hemiterpenes (C_5H_8), monoterpenes ($C_{10}H_{16}$), sesquiterpenes ($C_{15}H_{24}$), diterpenes ($C_{20}H_{32}$), triterpenes ($C_{30}H_{48}$), tetraterpenes ($C_{40}H_{64}$), and polyterpenes (Mabou and Yossa, 2021). They are the largest and most widespread secondary metabolites in nature, about 30,000 with different physical, chemical and biological properties. A wide variety of biological activities shown by terpenoids, augments our knowledge continually as new molecules are being investigated, mostly for practical importance as a source of pharmacologically interesting agents (Rohmer, 1999). Terpenoid alkaloids are a group of Pseudo-alkaloids (without amino acid origin) or false alkaloids whereby, a Nitrogen atom is inserted late on a biosynthetic stage.

Some of the important studied terpenoids are mention below as per their isoprene unit as:

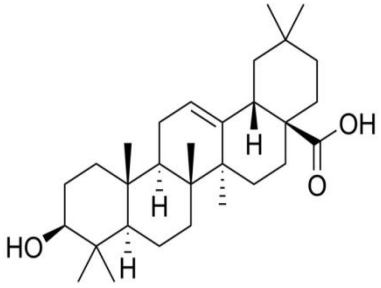
- Hemiterpenoids (5C) – Ioprene, Prenol, Tiglic acid, Isovaleric acid, Caffeic acid
- Monoterpenoids (10C) – Linalool, Citronellol, Thymol, Carvacrol, longifone, α-Ionone
- Sesquiterpenoids (15C) – Driminin, Selaterpene A, Tatridin A, Parthenolide, Tanachin

- Diterpenoids (20C) – Phytol, Sclareol, Carnosic acid, Gibberillin A1, Casbene
- Sesterterpene (25C) – Sesterstalin, Heliocide H, Ophiobiolin K, Peruvin, Leucosceptrene
- Triterpenoids (30C) – Oleanolic acid, Bitulinic acid, Ursolic acid, Sitosterol, α-amyrin
- Tetraterpenoids (40C) – Lycopene, β-Carotene, Lutein, Crocin, Canthaxanthin
- Polyterpenoids (>40C) – Natural rubber (cis) and gutta percha or balata (trans)

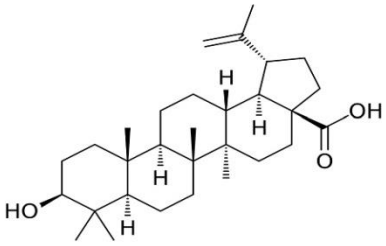
The above few listed terpenoids have shown numerous pharmacological uses. Hemiterpenes Cibotiumbaroside B and F were found to suppressed osteoclast formation and hepatocyte protective property respectively in dose dependent manners (Senhua *et al*., 2018). Thymol from Thymus vulgaris showed anti-cancer property of the gastric cell (Kang *et al*., 2015), Tatradin A from Oncosiphon piluliferum showed significant in vitro antiplasmodial activity against the D10 Plasmodium falciparum strains (Pillay, 2007). Xiaocong and Feng in 2018, reported that Nudiflopene F (a diterpene from Callicarpa nudiflora) have strong interactions with the iNOS protein by targeting residues of the active cavities of iNOS n BV-2 cells (IC50 28.1 and 23.3 μg/mL). Sesterterpenes Thorectandrol A and B isolated from marine sponge Thorectandra sp. inhibited the growth of MALME-3M (melanoma) and MCF-7 (breast) cancer cell lines in the range 30-40 μg/mL (Charan, 2001). Micona sp. extracted Ursolic acid and Oleanolic acid mention before, displayed the antibacterial effect, with MIC values ranging from 30μg/mL to 80μg/mL against the following microorganisms: Streptococcus mutans, Streptococcus mitis, Streptococcus sanguinis, Streptococcus salivarius, Streptococcus sobrinus, and Enterococcus faecalis (Wolska *et al*., 2010). Tetraterpene Crocin from Crocus sativus induces apoptosis of MCF-7 cells by activation of caspase-8 (Lu *et al*., 2015). An irregular terpenoid, Amestolkolides B from Talaromyces amestolkiae YX1, exhibited anti-inflammatory activity in-vitro by inhibiting nitric oxide (NO) production in lipopolysaccharide activated in RAW264.7 cells with IC50 values of 30 ± 1.2 and 1.6 ± 0.1 μM, respectively (Georgeiv *et al*., 2013).

Triterpenoid saponins were known to be present widely throughout the plant kingdom, marine organism, phytoplankton, algae, sponges, mollusks, mangroves and microorganisms. The chemical and structural diversity of saponins with useful bioactivities,

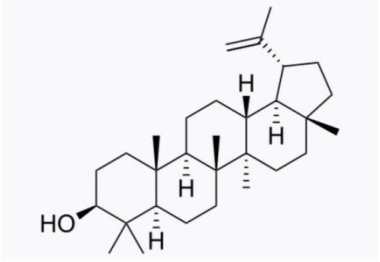
challenges of identification, unusual biosynthetic process and chemical synthesis for agricultural, industrial, and pharmaceutical applications have generated greater interest for further exploration of saponin chemistry and biology (Garai, 2014). They have been ubiquitous to plants helping in their defense against biotic stresses. To date, more than 120 saponins have been identified from *Clematis* plants, including about 70 oleanolic saponins, 50 hederagenin saponins, and two gypsogenin saponins (Lin *et al*., 2021). Significantly, the aglycones of them are all oleanane pentacyclic triterpenoids.



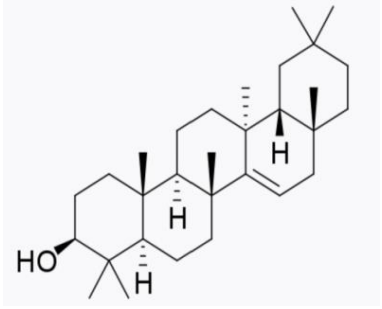
Oleanolic acid



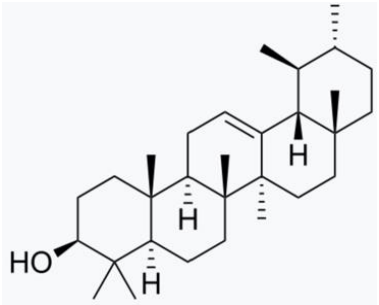
Betulinic acid



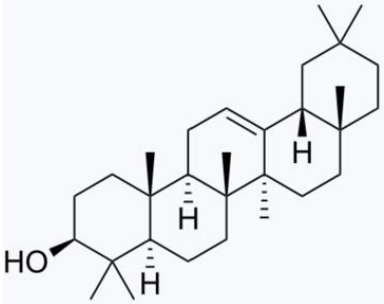
Lupeol



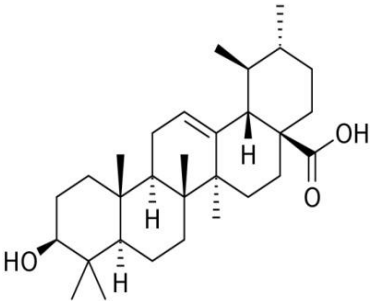
Tarexerol



α-Amyrin



β- Amyrin



Ursolic Acid

Fig 2.14: Structures of commonly known Triterpenoids

The common precursor is a Squalene (30C molecule), which via cationic intermediates is oxidized to 2,3-oxidosqualene. The synthesis of triterpenoids occurs basically in the cytosol utilizing IPP (3-isopentenyl pyrophosphate) and its isomer DMAPP

(dimethylallyl pyrophospahte), both 5C isomers to form a monoterpene GPP (Geranyl Pyrophosphate, 10C) by enzyme Prenyl transferase. GPP condenses with one more IPP to give FPP (Farnesyl Pyrophosphate, 15C) by same enzyme. Next two molecules of 15C FPP condenses to form a 30C triterpene Squalene by enzyme Squalene synthase. The pathway results with cyclization to give 2,3-oxidosqualene which is also the precursors for phytosterols and steroid saponins (Netala *et al*., 2014). After cyclization, the basic triterpenoid cyclic structure undergoes oxidation by monooxygenases and glycosylations of hydroxyl groups. Chemical synthesis of saponins recapitulates the main biosynthetic steps (Yendo *et al*., 2014). Triterpenoid saponins are synthesised via the isoprenoid pathway by cyclization of 2,3-oxidosqualene to give primarily oleanane (β-amyrin) or dammarane triterpenoid skeletons. The triterpenoid backbone then undergoes various modifications (oxidation, substitution and glycosylation), mediated by cytochrome P450-dependent mono-oxygenases, glycosyltransferases and other enzymes (Haralampidis *et al*., 2002).

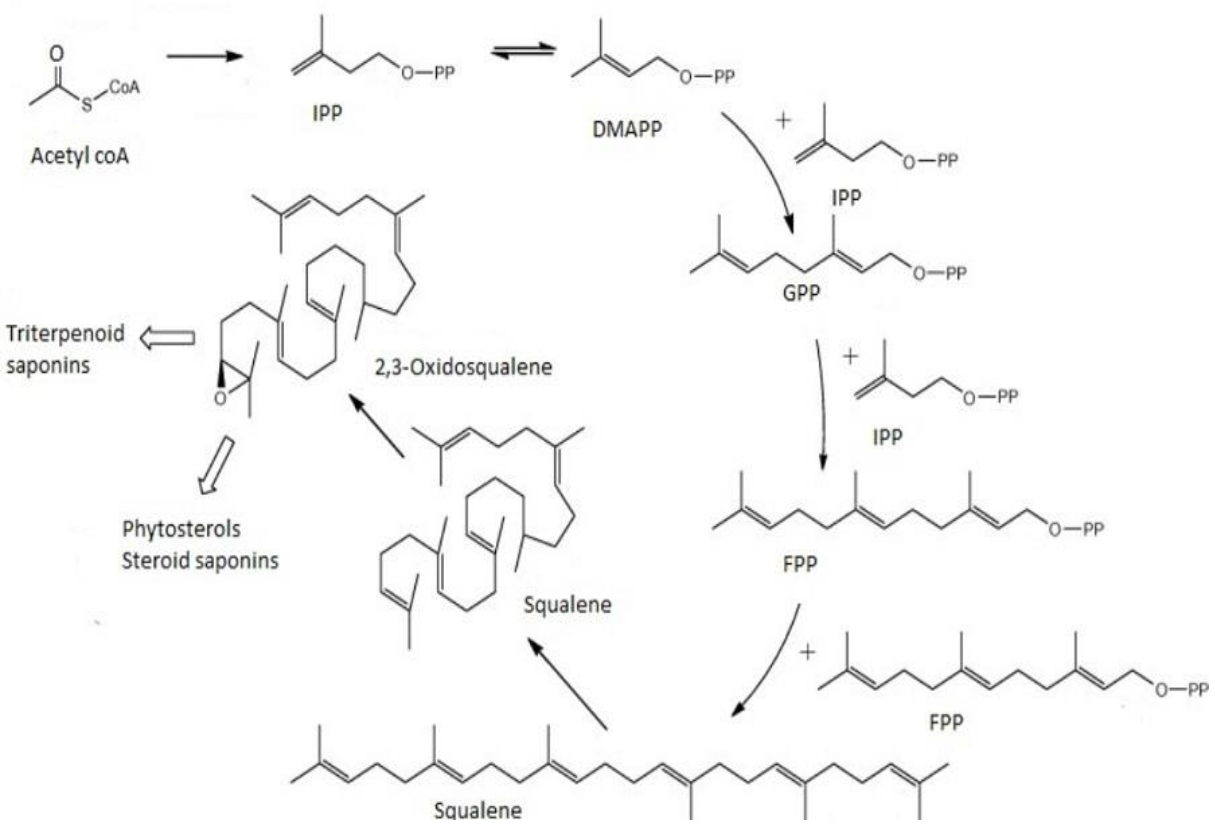


Fig 2.15: Pathway of Triterpenoid saponins Biosynthesis (Netala *et al*., 2014)

The different extraction techniques that have been employed for separation and isolation are supercritical fluid extraction (SFE), pressurized liquid extraction (PLE), microwave-assisted extraction (MAE), solid-phase microextraction (SPME), ultrasound-assisted extraction (UAE), superheated liquid extraction, and extraction with supercritical or subcritical water. The recent advances in separation methods are semi preparative HPLC, medium pressure liquid chromatography (MPLC), high speed countercurrent chromatography (HSCCC) and flash chromatography (Sticher, 2008). HPLC-UV, HPTLC, HPLC-CAD allows structural elucidation of the extract and Mass Spectrometry and NMR spectrometry are vital for multi-dimensional 2D, 3D and 4D structures development (Yeom *et al*., 2012). Previous works on triterpenoid saponins extraction from related clematis species have revealed presence of oleanane type compounds (Kizu *et al*., 1995).

Saponins could be classified into groups based on the nature of the aglycone skeleton. The first group consisted of the steroidal saponins, which has been almost exclusively found in the monocotyledonous angiosperms. The second group consists of the triterpenoid saponins, which have been most common and occured mainly in the dicotyledonous angiosperms (Vincken, 2007). The biologically active compounds responsible for the medicinal use of the plant are triterpenoid saponins (2.5-6%): the bidesmosidic glycosides of hederagenin and oleanolic acid (hederacoside C, B, D, E, F, G, H and I) and the monodesmoside α-hederin. Other groups of the identified compounds were represented by phenolics (flavonoids, anthocyanins, coumarins, and phenolic acids), amino acids, steroids, vitamins, volatile and fixed oils, β-lectins, and polyacetylenes (Yulia *et al*., 2012). Triterpenoid saponins mainly with oleanolic acid and hederagenin as the aglycones have been reported as the primary components in *Clematis parviloba*. These saponins have been found to display significant anti-tumour, anti-pyretic, analgesic, anti-bacterial, anti-inflammatory and diuretic effects (Yan *et al*., 2009). *Hedera helix* L. extract containing triterpenoid saponins have been reportedly used to treat acute inflammation with coughing and symptomatic treatment of chronic inflammatory bronchial diseases (Hussain and Awad, 2014). The roots and rhizomes of *Clematis mandshurica* Rupr. was the main source of traditional Chinese medicine “Weilingxian”, widely used as an anti-inflammatory and anti-tumor agent (Li *et al*., 2013). Twenty-seven triterpenoid saponins have been isolated from the roots and rhizomes of

Clematis chinensis, and some of these compounds show inhibitory activities against COX-1 and COX-2 enzymes, which were crucial in inflammatory response (Wu *et al*., 2010).

Species of Clematis	**Triterpenoid saponins (structural nomenclature)**
Clematis tangutica Maxim.	1) 3-O-β-D-xylopyranosyl-(1→2)-α-L-arabinopyranosyl hederagenin 28-O-α-L-rhamnopyranosyl-(1→4)-β-Dglucopyranosyl-(1→6)-β-D-glucopyranoside 2) 3-O-{β-D-xylopyranosyl-(1→2)-[β-D-glucopyranosyl- (1→4)]-α-L-arabinopyranosyl} hederagenin 28-O-α-Lrhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-Dglucopyranoside 3) 3-O-β-D-ribopyranosyl oleanolic acid 28-O-α-Lrhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-Dglucopyranoside (Zhang *et al*., 2012)
Clematis chinensis Osbeck.	1) 3-O-beta-[(O-alpha-L-rhamnopyranosyl-(1→6)-O-beta-D-glucopyranosyl-(1→4)-O-beta-D-glucopyranosyl-(1→4)-O-beta-D-ribopyranosyl-(1→3)-O-alpha-L-arabinopyranosyl)oxy]olean-12-en-21alpha hydroxyl 28-oic acid-O-alpha-L-rhamnapyranosyl –(1→4)-O-beta-D-glucopyranosyl-(1→6)-glucopyranosyl ester (Liu *et al*., 2009)
Clematis argentilucida H.Lev & Vaniot.	1) 3β-O-[β-D-Xylopyranosyl-(1→3)-α-L-rhamno pyranosyl-(1 → 2)-β-D-glucopyranosyl]-28-hydroxy-18αH -ursan-20-en 2) 3β-O-[β-D-Xylopyranosyl-(1 → 3)-α-L-rhamno pyranosyl-(1 → 2)-β-D-glucopyranosyl]-28-hydroxytaraxeran-14-en 3) 3b-O-[β-D-xylopyranosyl-(1/3)-a-L-rhamnopyranosyl-(1/2)-β-D-gluco-pyranosyl]-12-oleanene-3β,28-diol (Zhao *et al*., 2012)
Clematis tibetana Kuntz.	1) 3-O-(2-O-caffeoyl)-b-D-glucopyranosyl-(1→4)-b-D-glucopyranosyl- (1→4)-b-D-ribopyranosyl-(1→3)-a-L-rhamnopyranosyl-(1→2)-a-L-arabinopyranosyl hederagenin 28-O-a Lrhamnopyranosyl-(1→4)-b-D-glucopyranosyl-(1→6)-b-D-glucopyranoside 2) 3-O-a-L-rhamnopyranosyl-(1→2)-a-L-arabinopyranosyl gypsogenin 28-O-a-L-rhamnopyranosyl-(1→4)-b-D-glucopyranosyl-(1→6)- b-D-glucopyranoside 3) 3-O-b-D-ribopyranosyl hederagenin 28-O-a-L-rhamnopyranosyl-(1→4)- b-D-glucopyranosyl-(1→6)-b-D-glucopyranoside. (Kawata *et al*., 2001)

Clematis lasiandra Maxim.	1) Hederagenin 3-O-β-D-g lucopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside (kalopanax saponin H) 2) 3-O-α-L-rhamnopyr anosyl-(1→2)-α-L-arabinopyranosyl hederagenin 28-O-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl ester (hederasaponin C) 3) Hederagenin 3-O-α-L-rhamnopyranosyl-(1→2)-β-D-xylopyranoside 4) 3-O-α-L-rhamno pyranosyl-(1→2)-α-L-arabinopyranosyl hederagenin 28-O-β-D-glucopyra nosyl-(1→6)-β-D-glucopyranosyl ester (dipsacoside B) 5) 3-O-β-D-glucopyranosyl-(1→2)-β-D-xylopyranosyl oleanolic acid 28-O-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl ester (flaccidoside) (Li *et al*., 2022)
Clematis mundsurica Rupr.	1) queretaroic acid **OR** 3-O-β-D-glucopyranosyl-(1→4)-β-D-ribopyranosyl-(1→3)-α -L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside 2) 21α- hydroxyoleanolic acid **OR** 3-O-β-D-glucopyranosyl-(1→4)-β-D-ribo pyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside 3) 3-O-β-D-glucopyranosyl- (1→4)-β-D-glucopyranosyl-(1→4)-β-D-ribopyranosyl-(1→3)- α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl hederagenin 28-O-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside (Qiang *et al*., 2017).
Clematis grata Wall.	1. 3-O-β-D-glucopyranosyl-3-β-hydroxyl-olean-12-en-28-oic acid (Sati *et al*., 1990).

Table 2.1: Triterpenoid saponins in some related species of genus *Clematis*

The method of phytochemical crude extract has been essential in obtaining maximum product extract from the plant. The traditional method includes Maceration and Soxhlet extraction. Modern techniques such as Microwave Assisted Extraction (MAE), Ultrasound Assisted Extraction (UAE) and Supercritical Fluid Extraction (SFE), in which the ultimate aim were to obtain the extract at less cost (Vongsak *et al*., 2013). Powdered samples are more homogenized and offer more contact of the analytes with the extraction solvent medium used. For this the sample powder size has to be less than 0.5 mm for an ideal extraction. However to obtain the powdered sample the plant sample have to be moisture free and the process could be done at a controlled temperature to avoid phytochemicals denaturation such as oven drying at 44.5°C (Mediani *et al*., 2013), Lyophilization or Freeze drying, Microwave drying (Kaufmann and Christen, 2002).

Researchers have become convinced that the compound derived from plants for instance, phenolics, flavonoids and antioxidants compounds, does more in preventing different diseases (Fahey, 2005). Phytochemicals are natural occurring in plants in medicinal plants and are synthesized from plant primary metabolites such as carbohydrates, amino acids, chlorophyll, lipids etc. to give secondary metabolites such as tannins, flavonoids, terpenoids, alkaloids, glycosides, steroids, saponins, phenolics etc. (Ghahi, 1990 and Dobelius, 1993). The medicinal value of a plant lies in the chemical substances that produce definite physiological responses on the human body. Most important among these were the flavonoids, tannins, flavonoids and phenols derivatives (Dhandapani and Sabna, 2008). Therefore basic phytochemical identification has been vital before proceeding with the work of isolation and therapeutic application (Morton, 1991). Methanol was used as the solvent for the initial extraction as it could solubilized most of the active phytochemicals present in the plant due to its high polarity. However some standard screening analysis could be done at the powdered state even before extraction owing to the fact that some of them are liable to denature if followed under same solvent system (Balamurugan *et al*., 2019).

Phenolic compounds are the most widely distributed among plants though their concentration may depend on the types of plants but they are uncommon among bacteria, algae and fungi. It have been considered to have a key role as defense compounds when environmental stress such as high light, low temperature and deficiency in nutrients (Lattanzio, 2013). Phenolics could be divided into two classes: Benzoic acid derivatives (Gallic acids) and Cinnamic acid derivatives (coumaric, caffeic and ferulic) (D'Archivio *et al*., 2007). Broadly the definition of phenolics is concerned with the term 'Phenol' that contains a Phenyl ring bearing one or more hydroxyl groups. However the definition is misleading in case of some case due to the fact that the compounds such as gossypol, the phenolic carotenoid 3-hydroxyisorenieratene (I) or the phenolic female sex hormone oestrone (II), which are principally terpenoid in origin (Harbourne, 1989). The Total Phenolic Content (TPC) could be estimated by spectrophotometric method described by Singleton *et al*., 1999 using Folin-Ciocalteu reagent.

Flavonoids are a group of natural phytochemicals with variable phenolic structures. They are sub-grouped as flavones, flavovones, flavonols, isoflavonols, catechin,

anthocynains and chalcones (Panche *et al*., 2016). In plants they have the role of biotic and abiotic stress, acts as UV filters, signal molecules, antimicrobial, detoxifying agent, seeds and spores germination, colour and aroma (Takahashi, 2004 and Griesbach, 2005). Flavonoids have been mentioned to have an immense medicinal value in improving health having antioxidant properties (Pietta, 2000), reducing the risk of cardiovascular diseases (Majewska, 2012), anti- cancer properties (Chahar *et al*., 2011, Cutler *et al*., 2008, Dong *et al*., 2010 and Majewski *et al*., 2012), effective against neuro-degenerative diseases such as Alzheimer's and Parkinson's by modulating neuronal functions (Macready *et al*., 2012 and Prasian et. al., 2010).

Terpenes and Terpenoids are the primary constituent of the plant flower and leaves in the form of essential oils. The compounds class belongs to the isoprenoid groups, even though isoprenoid themselves are not found in nature, its polymers are wide spread as terpenic hydrocarbons and their oxygen derivatives (Ghorai *et al*., 2012). The total terpenoid content (TTC) could be estimate by the method described by Ferguson (1956).

For a plant to be considered as a medicinal plant it has to have properties such as anti-oxidant and anti-inflammatory mainly as the free radicals and inflammation are associated with most of the diseases and disorders. Other specified uses are anti-cancer, pain-relievers, anti-microbial and coagulant etc. Medicinal plants have become more popular to the world of research as natural antioxidant sources because of their ability to detoxify free radicals from the biological system. Free radicals are capable of attacking the healthy cells of the body, causing them to lose their structure and function. Cell damage caused by free radicals appears to be a major contributor to aging and to degenerative diseases such as cancer, cardiovascular disease, cataracts, immune system decline and brain dysfunction (Cadenas, 2000). Free radicals are electrically charged molecules i.e they have an unpaired electron, which causes them to seek out and capture electrons from other substances in order to neutralize themselves (Muthiah, 2012). Many herbal plants contains antioxidant compounds and these compounds protect cells against the damaging effects of reactive oxygen species (ROS), such as singlet oxygen, superoxide, peroxyl radicals, hydroxyl radicals and peroxynitrite (Dasgupta 2004 and David *et al*., 2004). Following are some anti-oxidant test reviewed:

- **Reducing Power Assay (FRAP assay)**

Benzie and Strain (1996) initially developed the assay as Ferric Reducing Ability of Plasma (FRAP) to measure the Anti-oxidant power. The Ferric Reducing Anti-Oxidant Power (FRAP) Assay is one of the most considered assay for total anti-oxidant capacity (Bolanos de la Torre, 2015). Kumar and Hemalatha (2011) have described the procedure for determining the reducing power of the extract. The FRAP assay is based on reduction of a colorless Fe^{3+}-TPTZ complex into intense blue Fe^{2+}-TPTZ once it interacts with a potential anti-oxidant. The method showed to be useful for screening of antioxidant capacity at low cost in a comparing efficiency test (Chen *et al*., 2015). The change in color would correspond to the maximum absorbance wavelength at 700nm in UV-Vis spectroscopy and the standard comparison was done by use of Ascorbic acid (Maisarah, 2013).

Potassium ferricyanide + Ferric chloride $\rightarrow$ Potassium ferrocyanide + Ferrous chloride

The Half Maximal Inhibitary Concentration (IC_{50}) is a measure of potency of a substance in inhibiting a specific biological function. It indicates how much of a particular inhibitory substance e.g. a drug can be able to initiate inhibition in vitro, a biological process by 50%. The term is comparable to Effective Concentration (EC_{50}) (Caldwell *et al*., 2012).

- **Hydrogen Peroxide Scavenging Assay:**

Reactive oxygen species (ROS) is a collective term used for free radicals or molecular species that can generate free radicals. Most of these reactive oxygen species including superoxide anion (O^{-}_{2}), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH) are naturally produced as byproducts of normal aerobic metabolisms and are increased by infections, exercise, stress conditions, radiations etc (Winterbourn, 2008). Amongst the ROS, H_2O_2 is an important molecule as although it is not toxic by itself, but can be converted to other even more toxic radicals such as OH by Fenton reaction or hypochlorous acid by the enzyme myeloperoxidase (MPO, EC 1.11.2.2) (Droge, 2002). The generation of H_2O_2 by activated phagocytes is known to play an important role as bactericidal and antifungal since it also acts as mediators of inflammation by activation of signal transduction pathways (Schreck and Baeuerle, 1991).

Uncontrolled generation of ROS is known to cause redox imbalance and oxidative stress which is harmful and responsible for various diseases including cancer,

neurodegenerative disorders, autoimmunity etc. Ruch *et al*. (1989) had developed a method to determine the Hydrogen Peroxide scavenging activity. Chemically, the assay has been the measure of H_2O_2 being converted to H_2O and O_2 due to the scavenging by antioxidant. The reaction is measured at the UV spectrum of 230nm wavelength.

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

- **DPPH Scavenging Assay**

DPPH Scavenging is a rapid, simple and inexpensive method to determine the antioxidant potential of a medicinal plant extract or a food sample. 2,2–Diphenyl-1-picrylhydrazyl (DPPH) is a widely used free radical as it is stable unlike most of the free radicals that are almost non-existent in its active state unless sharing of electron occur or losing of electron. This property of free radicals is also the root cause for making them injurious during cellular metabolism (Kirtikar and Basu, 2006). The DPPH assay method is based on the reduction of stable DPPH, to the state where it contains an odd electron, giving a maximum absorption at 517nm wavelength (purple colour). When Antioxidants react with DPPH, which is a stable free radical becomes paired off in the presence of a hydrogen donor (e.g., a free radicalscavenging antioxidant) and is reduced to the DPPHH and as consequence the absorbance's decreased from the DPPH. Radical to the DPPH-H form, results in decolorization (yellow colour) with respect to the number of electrons captured. More the decolorization more is the reducing ability (Harborne and Ghosh, 1998).

Inflammation is a physiological response that protects the body from tissue injury. Acute inflammation, with exudation of fluid and plasma proteins as its main features, occurs very rapidly, and the process can last for few or several minutes to several days. Chronic inflammation occurs when the acute inflammatory process occurs repeatedly or continuously, with the process lasting for several weeks to months and even years (Paramita *et al*., 2017). The migration of leukocytes from the venous systems to the site of damage, and the release of cytokines, are known to play a crucial role in the inflammatory response. These chemicals cause widening of blood capillaries (vasodilation) and the permeability of the capillaries. This will lead to increased blood flow to the injured site (Hollman, 2004). Although inflammation is a physiological process within the body, it can manifest as symptoms such as severe pain, rheumatoid arthritis, and asthma. Standard anti-inflammatory drugs have been used to

alleviate these symptoms, such as non-steroidal anti-inflammatory drugs (NSAIDs) and Corticosteroids that inhibit the enzymes cyclooxygenase (COX) and phospholipase A2 (PLA2), respectively. However, cases have been reported that these COX inhibitors are associated to increased risk of heart attack and stroke. (Oyekachukwu *et al*., 2017).

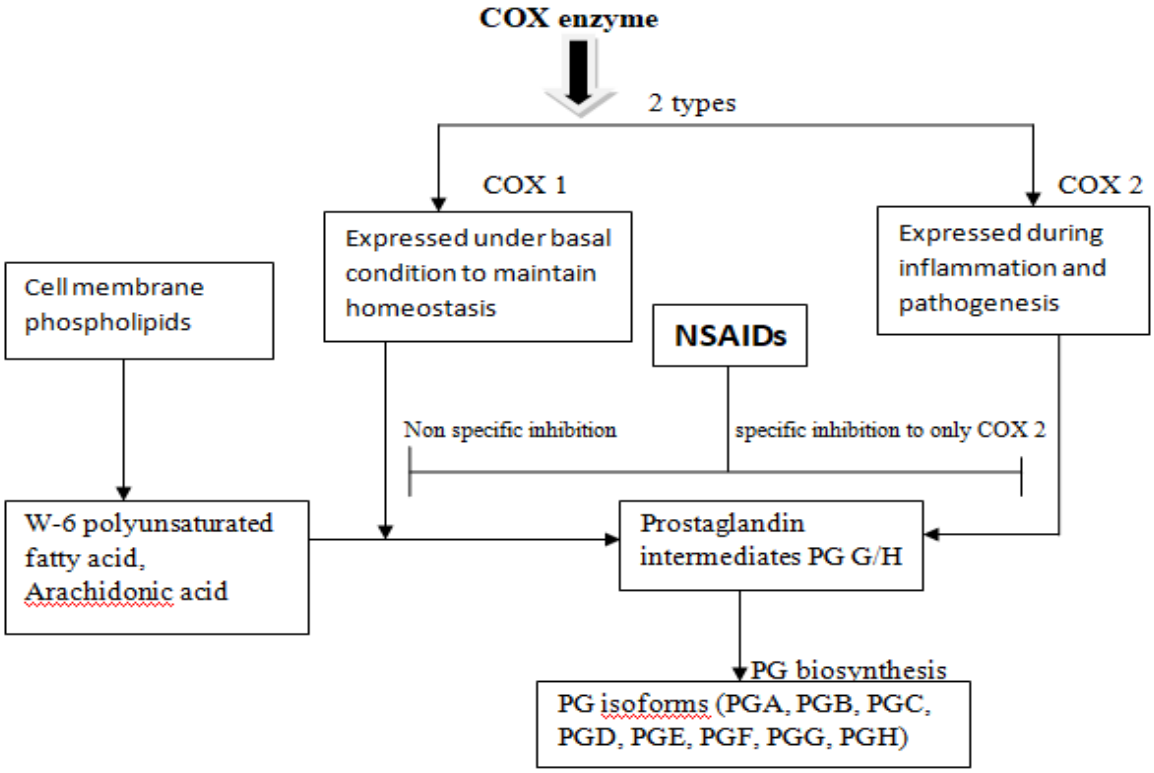


Fig 2.16: Targeted inhibition pathway of COX enzyme action by NSAIDs

Inhibition of Protein Denaturation Assay

Protein denaturation is defined as the loss of biological properties of a protein molecule. It has been found to be co-related to many disorders and diseases where inflammation occurs, such as rheumatoid arthritis, cancer and diabetes. The ability of a substance to prevent the protein denaturation could also lead to the prevention of inflammatory disorders (Sangeetha and Vidhya, 2016). The *in vitro* method had been adapted from the method of Mizushima and Kobayashi (1968). Here Bovine Serum Albumin (BSA) or Egg Albumin were used as a standard protein which was taken at different concentration. Acetyl Salicylic Acid (ASA) a common anti-inflammatory drug could be used as a positive

control. So the efficacy of the medicinal plant as an anti-inflammatory agent was a comparative result to ASA.

Phytochemicals are the organic chemicals present naturally in plants. It is with these chemicals that a plant could be used as a medicinal source, whether in crude or for extraction purpose. Plants have been used traditionally in raw, crude form for medical treatment since as long as civilization. The purpose of systematic analysis of a medicinal plant is to profitably extract a targeted compound from the plant for an intended use. However, the process would be subjected to multiple sequential steps, where each steps is an integral part that needs to be followed and developed after much review so that the end product is profitable.

- **Collection of plants:** Plants under consideration have been collected either from wild forests or from cultivation. When plants are collected from wild, there is a risk that they have been incorrectly identified. The major advantage of wild plants is that it does not contain any pesticides. After the plants have been collected from wild, it have to be processed for cleaning in order to prevent the deterioration of phytochemicals present in plants.

- **Cleaning of plants:** After plants collection, it has to be cleansed properly. The cleansing process could involved the following steps - Cleaning, washing, peeling or stripping leaves from stems. Cleaning has to be done by hands in order to get better results.

- **Drying:** The main purpose of drying is to remove the water content from plants so that the plants could be stored. Plants have to be dried immediately as soon as the plants collection or it would lead to spoilage of plant materials. Drying could be done either by natural process or by artificial process.

a) Natural drying: Natural process includes sun drying. Sometimes plants have to be placed on drying frames or on stands, to be air-dried in barns or sheds. But this may take few weeks for complete drying. The duration depends on temperature and humidity.

b) Artificial drying: Artificial drying could be done with the help of artificial driers. The process would reduce the drying time to several hours or minutes. The common method that have been employed in drying medicinal plants is warm-air drying. In this process, plants would be placed in the plates of drier on which warm air was blown. This method have been

mainly applicable to fragile flower and leaves and would required large number of workers since loading and unloading of plants has to be done manually. (Banu and Cathrine, 2015).

- **Powdering:** Lowering particle size could increase surface contact between samples and extraction solvents. Grinding resulted in coarse smaller samples meanwhile, powdered samples have a more homogenized and smaller particle, leading to better surface contact with extraction solvents. This particular pre-preparation is important, for efficient extraction to occur, as the solvent should make contact with the targeted analytes and particle size smaller than 0.5 mm is ideal for efficient extraction (Technical note, 2013).

Crude Extraction of the phyto-constituent have been pre-requisite for any phyto-extraction processes, after which only the targeted compound could be extracted from the crude extract. The process would be dependent on factors such as:

Choice of solvent – A property of a good solvent in plant extractions includes

- Low toxicity
- Ease of evaporation at low heat
- Promotion of rapid physiologic absorption of the extract
- Preservative action
- Inability to cause the extract to complex or dissociate

The factors affecting the choice of solvent -

- Quantity of phytochemical to be extracted
- Rate of extraction
- Diversity of different compounds extracted
- Diversity of inhibitory compounds extracted
- Ease of subsequent handling of the extracts
- Toxicity of the solvent in the bioassay process
- Potential health hazard of the extractants

Water	Ethanol	Methanol	Chloroform	Ether	Acetone
Anthocyanins	Tannins,	Anthocyanins	Terpenoids	Alkaloids	Phenol
Starches	Polyphenols	Terpenoids	Flavonoids	Terpenoids	Flavonols
Tannins	Polyacetylene	Saponins		Coumarins	
Saponins	Flavonols,	Tannins		Fatty acids	
Terpenoids	Terpenoids	Xanthoxyllins			
Polypeptides	Sterols	Totarol			
Lectins	Alkaloids	Quassinoids			
		Lactones			
		Flavones			
		Phenones			
		Polyphenols			

Table 2.2: Solvents used in some important phytochemicals extraction

Soxhlet extraction has been used where the desired compound has a limited solubility in a solvent, and the impurity is insoluble in that solvent. If the desired compound has a high solubility in a solvent then a simple filtration could be used to separate the compound from the insoluble substance. The advantage of this method was that instead of many portions of warm solvent being passed through the sample, just one batch of solvent is recycled. This method should not be used for thermo-labile compounds as prolonged heating could lead to degradation of compounds (Sutar *et al*., 2010).

Microwave Assisted Extraction (MAE) is a process that improves solvent extraction at higher temperature and isolates the required compounds from sample matrix through microwave energy. The application of microwave energy for sample preparation was first applied in the gossypol from cottonseed in 1986. Microwave energy is a non-ionizing radiation, which can penetrate into certain materials and interact with the polar components to generate heat. Heating of microwave energy acts directly on molecules by ionic conduction and rotation of dipoles. One of the main advantages of using MAE is the reduction of extraction time, which can mainly be attributed to the difference in the heating performance of microwave and conventional heating. Since then MAE has attracted growing interest, and it is called Green extraction technology because of its high efficiency, low consumption and less pollution (Li *et al*., 2012).

Other extraction techniques are Maceration, Sonication, Decoction, Infusion, Digestion, Serial Exhaustive Extraction, Plant Tissue Homogenization and Percolation. All these techniques are used as per the need depending on solvent volatility and polarity, extract volatility, final extract amount etc. (Handa *et al*., 2008).

Fractionation is a separating process that involves preferential solubility of the solute of interest among the two immiscible liquid phases. It could be considered as an alternative energy saving procedure at the initial stage of extraction in which, most of the non-essentials could be being removed depending on their physical properties. In many case the solvent used could also be efficiently recovered back.

Methanol is a polar solvent that could dissolve most of the phytochemicals. Also the choice of methanol over other potential solvent is it boiling point (BP) at which the extraction has to be made. The BP is not too high so as to disintegrate the phytochemicals of choice and also not too low so that the solvent is unable to penetrate the plant cell. However, the choice of methanol has certain disadvantage in the process in that it cannot be used directly for fractionation and has to remove completely before the crude extract could be used again. The recovery process could be done using the Vacuum Rotary Evaporator, where the extract and methanol were separated out profitably or a lyophilizer (Freeze drying) was used on a smaller scale. The ultimate step of fractionation starts with using double distilled water as water is polar but immiscible with many solvents.

- **Plant pigments removal:** Plants were known to be associated with many pigments such as Chlorophyll, Xanthophyll and Carotene etc. that are present, depending on the parts and functions associated. Chlorophyll however was the major pigment that needs to be removed from the plant extract. Acetone has always been a probable solvent for pigment but it is volatile (BP-56ºC) and miscible with water at all proportion. Therefore Ethyl Acetate could be taken into consideration, as pigment extraction solvent owing to its favorable properties that its Boiling point is 77ºC and more importantly immiscible with water. Therefore all the pigments could be separated out from water fraction.

- **Fat removal:** Fats were found to be present both in animal and plant products. It has also been considered as phytochemicals known as sterols and stereolins, commonly known

as oils. They could be either profitably extracted from plant or removed from extract by use of petroleum ether (BP-40º to 60ºC). Di-ethyl ether could have been another probable fat extracting solvent however, it has very low volatility (BP-35ºC) and tends to evaporate before fat extraction.

- **Recovery of targeted sample:** Important phytochemicals of consideration, that were present in the water fraction could be recovered back using any of the solvent immiscible with water yet, could dissolve the targeted phytochemical. For example, studies have shown that n-butanol is an important solvent that could dissolve triterpenoid saponins. It has low volatility (BP-125ºC), immiscible with water and less polar alcohol. Therefore, it could be used as end storage for raw triterpenoid saponins reservoir.

Chromatography is a separation technique used to separate components in a mixture. The components of the mixture could be dispersed in a liquid solution known as the mobile phase, which holds it through a structure containing another substance known as the stationary phase. Component separation requires differential partitioning between the mobile and stationary phases. The analytical goal of chromatography was to determine the qualitative and quantitative chemical makeup of a sample, and its primary purpose was to purify and extract one or more components of a sample (Sayed, 2021). Chromatography means color-writing and the more specific definition is that, it is a physical process of separation at which a mixture of compounds could be separated and isolated, purified into different molecules that depend on different distribution rates based on **s**olubility of sample in mobile phase, affinity (if polar or non-polar molecules) and interaction with fixed material i.e., the stationary phase (Mcnaught and Wilkinson, 1997).

Retention factor (R_f) or Rate flow is a qualitatively determination and identifier to the new separated components, and it is a standard value in a range of 0 to 1. When the value of Rf is close as possible to 0, it refers to the good interaction that occurs between the sample components and the stationary phase due to high polarity of both stationary and mobile phase.

$$\text{Retention Factor } (R_f) = \frac{\textbf{Distance moved by solute}}{\textbf{Distance moved by solvent}} \quad \text{(Nielson, 2010)}$$

We could classify and summarize the chromatographic method technique into three different ways as - Depending on the shape of the stationary phase it could be planar and

column chromatography, depending on the physical state of both stationary and mobile phase it could be gas and liquid chromatography and depending on the interaction between stationary and mobile phase it could be affinity, ion exchange, partition, adsorption, size exclusion chromatography.

- **Thin Layer Chromatography:** Thin layer chromatography (TLC) has been a quick, sensitive, and inexpensive technique, used to determine the number of components in a mixture, verify the identity and purity of a compound, monitor the progress of a reaction, determine the solvent composition for preparative separations and analyzing the fractions obtained from column chromatography (Cai, 2014). The first partition chromatography on a planar surface was paper chromatography, first used in 1940. But later it was replaced by TLC and is widely which has become one of the most routinely used chromatography techniques (Ettre and Kalasz, 2001).

TLC is also a liquid-solid adsorption technique where the mobile phase ascends the thin layer of stationary phase coated onto a backing support plate. The coated adsorbents are like silica gel, alumina, or cellulose on a flat, inert substrate. Compared to paper, it has the advantage of faster runs, better separations, and the choice between different adsorbents. Thin layer chromatography (TLC) has been among the most useful tools for following the progress of organic chemical reactions and for assaying the purity of organic compounds in phytochemistry and Biotechnology. Like all chromatographic methods, TLC takes advantage of the different affinity of the analyte with the mobile and stationary phases to achieve the separation of complex mixtures of organic molecules. Finding the mobile phase solvent system have been the most crucial and difficult task. Usually a solvent consist of three to five component having varying polarities. However the number of solvents should not affect the homogeneity of the system or its transparency.

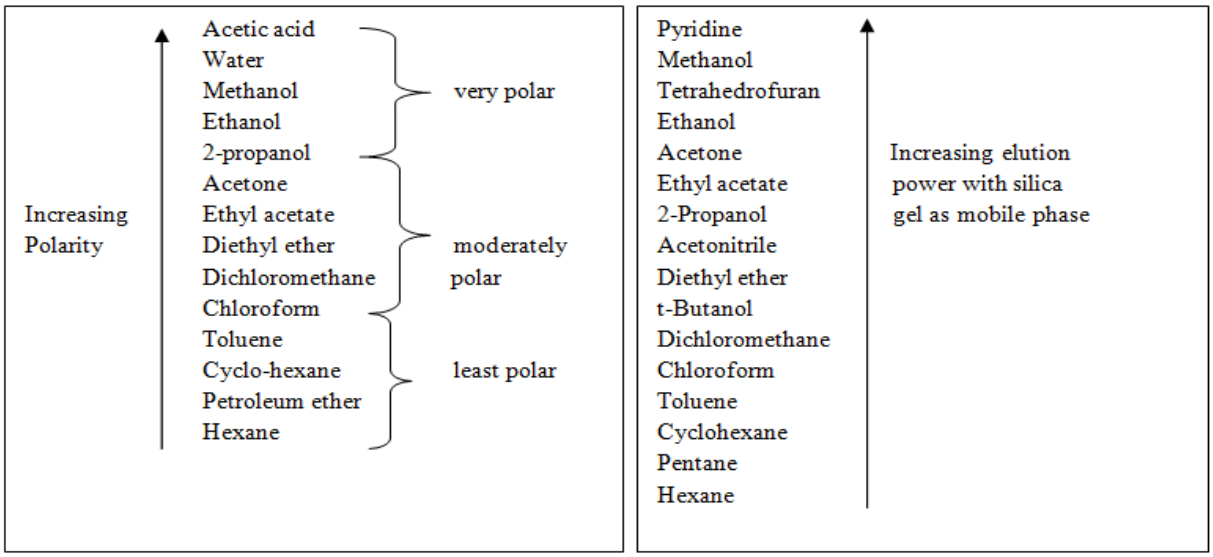


Fig 2.17: Polarity of commonly used mobile phases (L) and elution power with silica gel (R)

- **Column Chromatography:** Column chromatography is a ubiquitous technique in separation involving three basic components viz., the solid stationary phase, mobile phase and the sample and it has been used very commonly in organic chemistry (Harward *et al*. 1989). Its principles are same as TLC however larger quantities of sample could be effective separated out, thus it could be used to quantify the isolated compound. Another difference was that TLC works in opposite direction i.e. against gravity whereas Column chromatography is toward gravity. Column chromatography could be used on both a large and small scale. The applications of the technique have been wide reaching and cross many disciplines including biology, biochemistry, microbiology and medicine.

In column chromatography, the stationery phase is solid and the mobile phase is liquid. The packing could be achieved by two ways, either by directly pouring the dry silica gel directly and then wetting it to pack or by preparing the slurry of silica gel and then pouring it into the glass tube, whereby in both case a suitable solvent is used (Oslemcozkun, 2016). The compound mixture moves along with the mobile phase and separates out depending on the difference in affinities to the stationary phase which was based on the polarity of the compound. The most polar compound would elude the last and the least polar would first exit the column. Secondly, the factors considered were time and volume factor, since if we were not aware of the duration, the samples would get accumulated at the bottom or mixed with one another (Das and Dasgupta, 1998). The column chromatographic technique has been one of

the most convenient and widely used methods for purifying compounds. Column chromatography have been an extremely valuable technique, when synthesizing or isolating novel compounds, as very little needs to be known about a compound and its physical properties prior to the purification process. The pharmaceutical industry routinely uses column chromatography to purify compounds as part of its early stage drug development process (Silverman and Holladay, 2014).

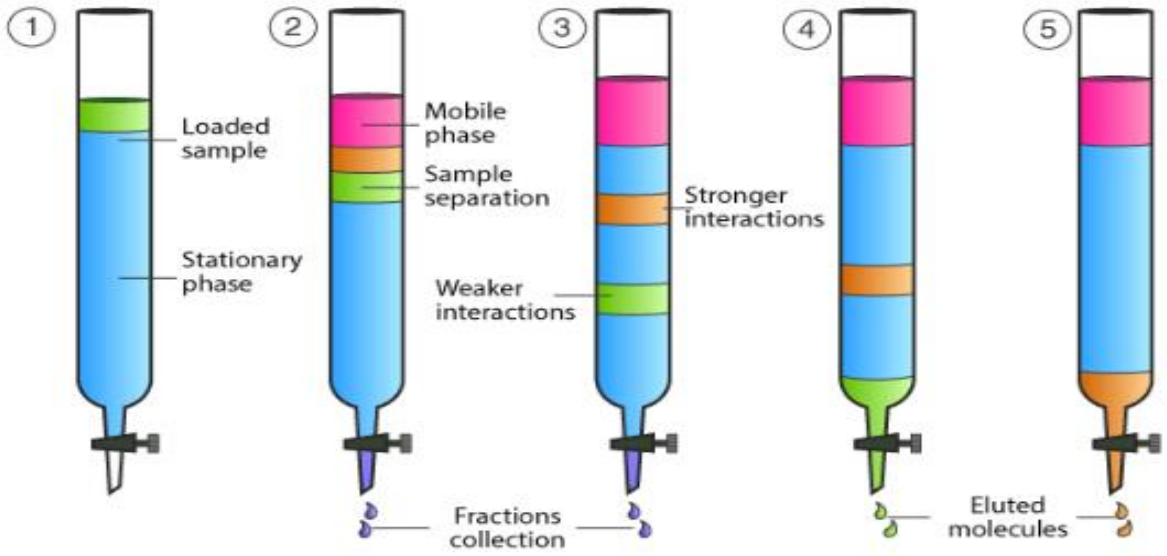


Fig 2.18: Diagrammatic view of Column separation (Sireesha *et al*., 2023)

Some of the important factors to be considered before performing experiment by column chromatographic technique include:

- The packing of column must be absolutely air tight without any bubble. The packing must be homogenous which could be achieved while packing by gentle tapping.
- Choice of mobile phases – Depending upon the targeted elute the ratio of the mixture of mobile phase were being designed. However, to obtain this choice, preliminary test were being performed so as to know the best ratio that gives the maximum elution.
- The time of elution has been vital for collection as well as to calculate the Retention value. Practically, if the distance travelled is not a discrete quantity then a series of collection could be made for an analyte. The choice of stationary phase material and the particle size – Usually silica gel (for polar functional group compounds) or Alumina (less polar) is being used (Cellulose was also considered previously). The materials should have

good adsorption property, inert with the analytes as well as mobile phases, uniform size (50-200µm) and inexpensive.

Spectroscopy is the study of the properties of matter through its interaction with various types of radiation (mainly electromagnetic radiation) of the electromagnetic spectrum. Spectrometry and spectrometric methods refer to the measurement of the intensity of radiation with a photoelectric transducer or other types of electronic device. The UV-Vis spectrophotometry has been one of the oldest instrumental techniques of analysis and has been the basis for number of ideal methods, for the determination of micro and semi-micro quantities of analytes in a sample. It concerns with the measurement of the consequences of interaction of Electromagnetic radiations in the UV and/or visible region with the absorbing species like, atoms, molecules or ions. The principle could be represented by two equations below as

- Bohr's model

$\Delta E = E_2 - E_1 = hv = hc/\lambda$

Where,

h – Planck's constant (6.62×10^{-34} Js)

c – Velocity of light (2.99×10^{8} m/s)

v – Frequency

λ – Wavelength

E1 and E2 are initial and final energies,

- Beer-Lambert's Law

$A = \text{Ɛ}bc = -\log(1/T)$

Where,

A – Absorbance

Ɛ – Molar absorptivity or molar absorption co-efficient

b – Thickness of the sample

c – Concentration

T – Transmittance (Picollo *et al*., 2018)

UV- Vis Spectrophotometer are basically designed to include both UV spectrum (λ= 180-400 nm) and Visible spectrum (λ= 400-780 nm). The technique have been instrumental in numerous chemical analysis based on their optical behavior where certain

group of compound responds to a particular wavelength in the EM spectrum, that can be exploited for their quantitative as well as qualitative analysis.

Basically, during analysis of a certain compound, the pre-determined wavelength for the compound is being set in the spectrophotometer. A standard graph in the increasing or decreasing concentration could be plot for a known sample, against which the unknown sample was to be plotted and compared. Some of the common samples to be analyzed with the absorption spectra are given as:

Sl no	Types of analysis	Absorption spectra	Standard used
a.	Total Phenolics Content	760 nm	Gallic acid (a phenolic)
b.	Total Flavonoids Content	415 nm	Quercetin (a flavonoid)
c.	Total Terpenoids Content	548 nm	Oleanolic acid (a triterpenoid)
d.	DPPH Scavenging assay	517 nm	Ascorbic acid (an antioxidant)
e.	H_2O_2 Scavenging assay	230 nm	Ascorbic acid (an antioxidant)
f.	FRAP Assay	700 nm	Ascorbic acid (an antioxidant)
g.	Anti-inflammatory Assay	660 nm	ASA (an anti-inflammatory agent)

Table 2.3: UV-Vis analysis and their absorption spectra

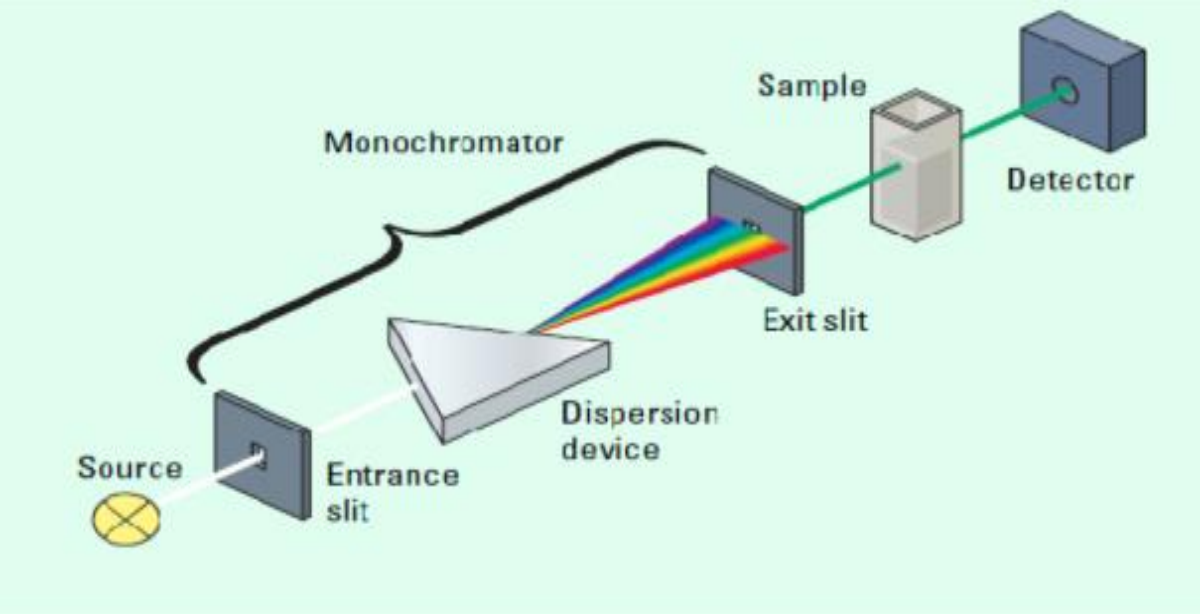


Fig 2.19: Schematic representation of a Spectrophotometer pathway (Gohain, 2008)

CHAPTER 3

MATERIALS AND METHODS

3.1. Plant Sample Harvesting:

Plant sample refers to leaves of *Clematis napaulensis* DC. as the foliage of the plant was the main interest, to harvest the targeted phytochemicals owing to the fact that it is the part of the plant being used by the Chakesang tribe in the treatment of Rheumatoid Arthritis. The selection of foliage as plant sample was also supported by the fact that the stem and branches were very thin and insignificant compared to leaves which were significant in quantity. The floral parts were also very limited in both quantity as well as duration of blooming.

Material Required:

a) Plant sample (leaves)
b) Dao/ cutter
c) Vacuum hot air oven [Biocraft Scientific Systems]
d) Electric grinder/ blender
e) Sieve (1.0mm pore size)
f) Dry storage

Procedure:

a) The leaves of *Clematis napaulensis* DC. were harvested in the month of February and March from Khonoma village, Kohima Nagaland.
b) Without delay to prevent the decaying, the leaves were washed of dirt and dried of unwanted debris, soil sediments and particulates.
c) The overall leaves harvested, were dried in the Vacuum hot air oven for 72 hours at 50ºC to completely remove any moisture from the sample.
d) The dried moisture free samples were subjected to intense grinding using Electric blender for 5 minutes or until reduced to powdery form.
e) The reduced powdered samples were then filtered through a fine pored sieve to separate out the unwanted remains.
f) The overall powdered samples were then weight, packed air-tight in equal quantities and stored in moisture free environment for further analyses.

3.2. Solvent Extraction

Material Required:

a) Powdered sample
b) Methanol 99% (Molychem)
c) Three Soxhlet apparatus units (Borosil)
d) Triple heating mantle for Soxhlet (Coslab)
e) Water chiller (Innovative Technologies)
f) Vacuum rotary evaporator (Equitron Roteva)

Procedure:

a) Accurately, 200g of the sample powder was weigh and packed for extraction.
b) The sample was subjected to soxhlet extraction using methanol at 70ºC and condensed at temperature of 5ºC to prevent loss of solvent.
c) The process of soxhlet extraction was continued for at least 4 hours or until the residue is free from any soluble phytochemical.
d) The filtrate was oven dried for 24 hours and weighed.
e) The solvent extract obtained was subjected to concentrating in vacuum rotary evaporator at 50ºC and 150mbar pressure.
f) The extract was sealed and stored in cold storage (4ºC-10ºC) whereas, the methanol recovered from the concentration process was stored for further process of extraction.

3.3. Preliminary phytochemical screening

Following preliminary phytochemical screening test were performed on the extract sample based on standardized test.

Test for Phenolic compounds

Small amount of extract was dissolved in 10 ml distilled water in a test tube. After complete dispersion the solution was filtered through Whatmann's paper. The filtrate was used for the following analysis**.**

A) Ferric chloride test: The filtrate was treated with 5% of Ferric chloride solution. Appearance of black precipitate confirms the presence of phenolic compounds.

B) Lead acetate test: Few ml of the filtrate were treated with lead acetate solution. The formation of white precipitate indicates the presence of phenolics compounds (Raaman, 2006).

Test for Alkaloids

Small amount of the extract was mixed with dilute Hydrochloric acid (dil. HCl).

A) Mayer's test: To the small amount of filtrate, few drops of Mayer's reagent were added. Formation of white precipitate indicates the presence of Alkaloids. Mayer's reagent is prepared by combining Solution A (1.358gm HgCl + 60ml dist. water) + Solution B (5gm KI + 10ml dist. water) + final volume with dist. water to 100ml (Rondon *et al*., 2017).

B) Wagner's test: To a small amount of filtrate a small amount of Wagner's reagent was added. A brown precipitate formation indicates the presence of alkaloids. Wagner's reagent is prepared by combining 1.27gm Iodine + 2gm potassium iodide + Dist. water upto 100ml (Rimjhim *et al*., 2014).

Test for De-oxy Glycosides (Keller-Killiani's test)

To 5ml of extract, glacial acetic acid, 1-2 drop of 5% Ferric chloride and concentrated sulphuric acid was added. Appearance of reddish brown color at the junction of the two liquid layers and bluish green upper layer indicates the presence of de-oxy sugars (Singh and Kumar, 2017).

Test for Anthraquinone Glycosides (Bontranger's test)

To 5ml of extract, dilute sulphuric acid was added. It was then boiled and filtered. To the cold filtrate, equal volume of benzene or chloroform was added, shaken well and organic solvent was separated. Upon addition of Ammonia, appearance of ammonical layer turning pink or red indicates the presence of anthraquinone glycoside (Njoku and Obi, 2009).

Test for Flavonoids

A) Ferric chloride test: To small quantity of extract a small quantity of neutral ferric chloride was added. Blackish red colour formation indicates the presence of flavonoids. (Silva *et al*., 2017).

B) Lead acetate test: To a small quantity of the extract a small quantity of Lead acetate solution is added. Formation of yellow precipitate indicates the presence of flavonoids (Tiwari *et al*., 2011).

Test for Saponins (Foam test)

Small amount of extract was taken in a test tube and 5ml of distilled water was added and shaken vigorously. Formation of foam indicates the presence of Saponins (Rauf *et al*., 2013).

Test for Steroids & Triterpenoids (Salkowski's test)

Small amount of the extract is diluted with 5ml chloroform followed by few drops of sulphuric acid. The solution is mixed well and allowed to stand at room temperature. Red colour formation at the chloroform layer indicates the presence of Steroids (Singh and Kumar, 2017).

Test for Tannins

A) Catechic test: Measure 1ml of aqueous extract and add 5ml distilled water in a test tube and filtered 0.1% $FeCl_3$(ferric chloride) is added to the filtered samples and observed for brownish green or a blue black coloration which shows the presence of tannins.

B) Braymer's Test: To 1ml extract, 3ml distilled water was added followed by adding of 3 drops of 10% $FeCl_3$. Appearance of Blue-green colour indicates the presence of tannins in the sample. (Uma *et al*., 2017).

C) Gelatin Test: 1ml of extract was dissolved in 5ml of distilled water. It was then dissolved in 1% gelatin solution and added with 10% NaCl solution. White precipitate formation indicates presence of tannins (Pandey & Tripathi, 2014).

Test for Proteins

A) Biuret test: To 5ml of extract solution, 4% Sodium Hydroxide and 1% Copper Sulphate was added. Appearance of yellow to pink colour indicates the presence of proteins (Gahan, 1984 and Silva *et al*., 2017).

B) Xanthoprotic test: To 1ml of extract solution add few drops of concentrated Nitric acid (HNO3). Appearance of yellow coloration indicates the presence of aromatic amino acids. On the addition of an alkali, the colour changes to orange due to formation of salt of the tautomeric form of the nitro compounds (Tiwari *et al*., 2011).

Test for Gums (Fehling's test)

The test solution was hydrolysed using diluted HCl. Fehling's test was performed on the hydrolysed solution. The formation of red colour indicates the presence of gum.

Fehling A: 34.66gm copper sulphate + distilled water to make final volume 100ml.

Fehling B: 173gm potassium sodium tartarate + 50gm NaOH + distilled water to make 100ml.

Test for Reducing Sugars (Fehling's test)

1ml Fehling A + 1ml Fehling B solution was mixed and boiled for 1 minute. An equal amount of substrate solution was added and kept in water bath for 8 minutes. Yellow precipitate followed by brick red indicates the presence of reducing sugars (Joseph *et al*., 2013).

Test for Non-Reducing Sugars (Iodine test)

5ml of the substrate solution was mixed with few drops of dilute iodine solution (Lugol's iodine). Appearance of blue colour that disappears on heating but reappears on cooling indicates the presence of non-reducing sugars.

Lugol's iodine: 10gm Potassium Iodide + 5gm iodine + in 100ml distilled water.

3.4. Phytochemical Estimation

3.4.1 Total Phenolic Content (TPC)

The TPC of the *Clematis napaulensis* DC. sample extract was estimated as per the procedure described by Bhalodia *et al*., 2011 and Patel *et al*., 2010 with slight modification in the concentration sequences.

Materials and Reagents required:

a) Methanolic extract of *Clematis napaulensis* DC.
b) Gallic Acid (Phenolic standard)
c) Methanol 99% (Molychem)
d) Folin Ciocaltiu's reagent
e) Distilled water
f) 7.5% Sodium carbonate solution (75mg in 10ml, w/v)
g) Vortex

h) 1ml pipette and 100µg pipette.

i) Test tubes, glasswares and cuvettes

j) Spectrophotometer (Biospectrophotometer).

Procedure:

a) 100mg of gallic acid was weighed and dissolved in 100ml of methanol accurately (1mg/ml) as stock solution.

b) Five working concentrations were diluted with methanol viz., 0.20mg/ml, 0.40mg/ml, 0.60mg/ml, 0.80mg/ml and 1.00mg/ml and tagged as S1, S2, S3, S4 and S5.

c) To each working solutions, 250µl of Folin Ciocaltiu's reagent was added.The overall volume of each working solutions were made upto 1.5ml with de-ionized water.

d) 1ml of 7.5% Sodium Carbonate (Na_2CO_3) was added to each making the total volume upto 2.5ml. The five solutions were vortexed and kept in dark for 1 hour.

e) Meanwhile, the extract concentration of 0.40mg/ml was prepared and the reagents added similar to the Gallic acid standards with final volume of 2.5ml.

f) Absorbance reading was taken for Gallic acid standards after 1 hour at wavelength of 760nm. Also the absorbance reading of extract preparation was also taken at 760nm wavelength (λ).

g) Graphical analysis of Absorbance (Y) and Concentration (X) was plotted and using the slope equation, $Y = mX \pm$ error value. The Gallic Acid Equivalent (GAE) was obtained and the TPC can be calculated from the result. Regression value (R^2) was also calculated for accuracy.

3.4.2 Total Flavonoid Content (TFC)

The TFC estimation of *C.napaulensis* DC. crude sample extract was calculated using the procedure as described by Pallab *et al*., 2013, with slight change in the concentration of quercetin standards.

Material and Reagents required:

a) Quercetin (Flavonoid standard)

b) Methanol (CH_3OH)

c) Distilled water

d) 1% Aluminium chloride (10gm/L), w/v

e) 1M Potassium acetate (9.84gm/100ml, w/v) at pH 7.5

f) Vortex

g) Test tubes and Cuvettes

h) Pipettes (1ml & 100µl)

i) Spectrophotometer (Biospectrophotometer)

Procedure:

a) 20mg of Quercetin was weighed and dissolved in 20ml of methanol as standard stock solution (1mg/ml).

b) From the stock, standard working concentrations of 50µg/ml, 100µg/ml, 150µg/ml, 200µg/ml and 250µg/ml were prepared and tagged as A1, A2, A3, A4 and A5.

c) To each of the working solutions, 1.5ml methanol, 100µl of 1%Aluminium chloride and 100µl of Potassium acetate were added.

d) All the standards volume were made upto 5ml, vortexed and kept at room temperature (25ºC) for 30 minutes.

e) Working concentration of methanolic extract at 100µg/ml was prepared and added with 1.5ml methanol, 100µl of 1%Aluminium chloride and 100µl of Potassium acetate.

f) Extract working volume was also made upto 5ml using distilled water, vortexed and kept at room temperature (25ºC) for 30 minutes.

g) The absorption reading of all the standard samples and extract preparation was taken at 415nm wavelength (λ).

h) Graph of Quercetin Equivalent (QE) was plotted and slope was obtained as well as the Regression value (R^2).

i) Calculation of percentage flavonoid content in the extract was calculated.

3.4.3. Total Triterpenoid Content (TTC)

The TTC estimation for the crude sample extract of *C.napaulensis* DC. was performed as per the protocol followed by Ke *et al*., 2014 and Wei *et al*., 2015 with modification in the concentration.

Material and Reagents required:

a) Oleanolic acid (Triterpenoid saponins ṁ standard)
b) Methanol
c) 5% Vanillin- Glacial acetic acid solution (5gm/100ml, w/v)
d) Perchloric acid
e) Water bath
f) Vortex
g) Test tubes and cuvettes
h) Pipettes (1ml and 100µl)
i) Spectrophotometer (Biospectrophotometer)

Procedure:

a) 10mg of Oleanolic acid was weigh and dissolved in 10ml of methanol as standard stock solution (1mg/ml, w/v)
b) Five working concentrations were prepared in the order 50µg/ml, 100µg/ml, 150µg/ml, 200µg/ml and 250µg/ml of 1ml each and tagged as S1, S2, S3, S4 and S5 respectively.
c) The five standard concentrations were dried over the water bath set at the temperature of 60ºC.
d) After evaporation, 300µl of 5% Vanillin-Glacial Acetic acid solution was added to each standard followed by addition of 1ml Perchloric acid.
e) The standard solutions were then vortexed and heated over the water bath at 60ºC for 45 minutes.
f) 100µg/ml of sample extract solution in methanol was also prepared simultaneously and dried, added with 5%Vanillin-Glacial Acetic acid, Perchloric acid and incubated.
g) After incubation, both the standards and the sample extract volume were made upto 5ml volume and absorbance readings were taken for all the samples at 548nm wavelength (λ).
h) Graph was plotted for Oleanolic Acid Equivalent (OAE) and Regression value was obtained. Calculation of TTC was estimated using Oleanolic Acid Equivalent.

3.5. Anti-Oxidant Assays

3.5.1. DPPH Scavenging Assay

The reference for DPPH Scavenging assay was from the experiment performed by Reshma *et al*., 2014.

Materials and reagents Required:

a) Crude methanolic extract of *C. napaulensis* DC.

b) 0.1mM 2,2 Diphenyl-1-picylhydrazyl ($C_{18}H_{12}N_5O_6$) in methanol
1M DPPH is 394.32gm/l (w/v) = 39.432gm/100ml (w/v)
1mM is 0.394 gm/l (w/v) = 0.039432 mg/100ml (w/v)

c) Ascorbic acid ($C_6H_8O_6$)

d) Methanol (CH_3OH)

e) UV-Vis Spectrophotometer (Biospectrophotometer)

Procedure:

a) Working solution of 0.1mM DPPH was prepared.

b) 1ml each of sample extract at 100µg/ml concentration in methanol was prepared in triplet and tagged as I, II, and III.

c) A 1ml solution of Ascorbic acid in methanol at 100µg/ml concentration was also prepared.

d) To both Ascorbic acid and sample extract solution, 1ml each of 0.1mM DPPH was added and incubated at 37ºC for 15 minutes in the dark.

e) The absorbance readings of all the samples were noted at 517nm wavelength (λ) in the UV-Vis spectrophotometer.

f) The average absorbance of the triplet extract samples was taken and the percentage scavenging was calculated with respect to Ascorbic acid absorbance as:

$$\textbf{DPPH Scavenging } (\%) = \frac{\textbf{CA}-\textbf{SA}}{\textbf{CA}}\textbf{x100}$$

Where, C_A = Control absorbance and S_A = Sample absorbance

3.5.2. Hydrogen Peroxide Scavenging Assay

The Hydrogen peroxide scavenging assay is performed by protocol adapted in reference to the work done on *Trichodesma zeylanicumm* leaves by Ngonda, 2013 and by Ruch *et al*., 1989.

Materials and Reagents required:

a) Crude methanolic extract of *C. napaulensis* DC.

b) Hydrogen peroxide (50%, v/v)

C_1 = 500gm/l, C_2 = 34gm/l (MW = 34gm/mol)

V_2 = 0.1 litre,

$V_1 = \frac{34\frac{g}{l} * 0.1l}{500g/l}$ = 0.0068 litre = 6.8ml

Thus, 1M H_2O_2 = 6.8ml H_2O_2 + 93.2ml PBS = 100ml

c) Phosphate buffer tablets (Merck)

d) 1% Sodium Hydroxide solution

e) Ascorbic acid ($C_6H_8O_6$)

f) Methanol (Molychem)

g) UV-Vis Spectrophotometer (Biospectrophotometer)

Procedure:

a) Phosphate Buffer Solution (PBS) was prepared by dissolving buffer tablets in 100ml of distilled water and adjusting the pH 7.4 using dilute NaOH.

b) 10ml stock solution of methanolic leave extract was prepared by dissolving crude extract in methanol at the concentration of 1mg/ml.

c) Five working concentrations were prepared from the stock viz., 10µg/ml, 20µg/ml, 40µg/ml, 80µg/ml and 160µg/ml and tagged as A1, A2, A3, A4 and A5.

d) Ascorbic acid in methanol control solution was prepared at the concentration of 160µg/ml.

e) To 2.5ml each of 1M H_2O_2 solution, 500µl each of sample extract were added. Also to 2.5ml each of 1M H_2O_2 solution, 500µl of Ascorbic acid solution was added.

f) The overall samples were left standing at room temperature (25±1ºC) for 10 minutes.

g) Taking PBS without H_2O_2 as blank, the absorbance reading of all the samples were taken at 230nm wavelength (λ_{UV}).

h) Graph was plotted between Concentration (X) and Absorbance (Y) to confirm the scavenging activity with increasing concentration.

i) Percentage inhibition was calculated using the absorbance of sample at 160µg/ml and ascorbic acid at 160µg/ml as

$$\textbf{\% } H_2O_2 \textbf{ Scavenging activity} = \frac{\text{Acontrol} - \text{A5}}{\text{Acontrol}} \times 100$$

Where, Acontrol = Absorbance of ascorbic acid control (160µg/ml)

A5 = Absorbance of A5 sample at 160µg/ml

3.5.3. Reducing Power Assay

The Reducing Power assay or also known as **Ferric Reducing Anti-Oxidant Power Assay (FRAP assay)** was performed as per the protocol of Maisarah *et al*., 2013 and Hemalatha *et al*., 2016 with slight change in concentration. Here the basic principle was to measure the reduction of Fe^{3+} to Fe^{2+} by any compound.

Materials and Reagents required:

a) Phosphate Buffer Solution (pH 6.6) – It is prepared by dissolving buffer tablets and adjusting the pH using dilute NaOH.
b) 1% Potassium Ferrocyanide solution (1gm/100ml, w/v)
c) 10% Trichloro-acetic acid (1gm/10ml, w/v)
d) 0.1% Ferric Chloride solution (1gm/l, w/v)
e) Ascorbic acid stock solution (1mg/ml, w/v)
f) Sample extract solution of *Clematis napaulensis* DC. in distilled water.
g) Water bath
h) Centrifuge
i) Vortex/shaker
j) UV-Vis Spectrophotometer (Biospectrophotometer)

Procedure:

a) From the sample stock solution, four working concentrations were diluted out viz., 100µg/ml, 200µg/ml, 300µg/ml and 400µg/ml and tagged as S1, S2, S3 and S4 respectively.
b) Following standard Ascorbic acid solutions were also prepared viz., 20µg/ml, 40µg/ml, 60µg/ml, 80µg/ml and 100µg/ml and tagged as A1, A2, A3, A4 and A5 respectively.
c) Both the sample and standard preparations were mixed with 2.5ml of PBS (pH 6.6) and 2.5ml of 1% Potassium ferrocyanide.

d) The mixtures were incubated at 50ºC for 20 minutes followed by rapid cooling.

e) 2.5ml of 10% Trichloro-acetic acid was added to each, followed by centrifugation at 3000rpm for 10 minutes.

f) About 2.5ml of supernatant were recovered carefully from each in new test tubes and added with 2.5ml distilled water and 0.5ml of 0.1% Ferric chloride.

g) A control was also prepared simultaneously starting with PBS and does not have ascorbic acid or sample preparation added to it.

h) The overall mixtures were allowed to stand for 10 minutes and absorbances reading were taken for all at 700nm wavelength (λ) using control as blank reading.

$$\% \text{ Reducing Power} = \frac{\textbf{Ascorbic acid IC50}}{\textbf{Sample extract IC50}} \times 100$$

i) Inhibitory Concentration or IC_{50} of both sample extract and ascorbic acid were calculated using graphical analysis.

j) Percentage Reducing Power of Sample extract was calculated with respect to Ascorbic acid.

3.6. *In vitro* Anti-inflammatory Assay

Protein denaturation is one of the chief mechanisms for the cause of inflammation. In disorders such as Rheumatoid arthritis, diabetes and cancer, it has significance in contributing to the extent of severity of disease development. An anti-inflammatory agent or a medicinal plant should have or expected to contain certain phytochemicals that can inhibit the denaturation of inflammatory protein and prevent the progress of disease.

The procedure for inhibition of protein denaturation was adapted from work of Sakat *et al.*, 2010 and also used by Sangeetha and Vidhya in 2016.

Materials and Reagents required:

a) Sample extract of *C. napaulensis* DC.

b) Bovine Serum Albumin (BSA) protein

c) Phosphate Buffer Solution (PBS)

d) Distilled water (as negative control)

e) Water bath (Innovative Tech)

f) Acetyl Salicylic Acid (Aspirin)

g) Spectrophotometer (Biospectrophotometer)

Procedure:

a) 1ml each of sample extract solutions were prepared at the concentration of 50µg/ml, 100µg/ml, 200µg/ml, 400µg/ml, and 800µg/ml in distilled water and tagged as A1, A2, A3, A4 and A5.

b) 1ml of 800µg/ml acetyl salicylic acid was prepared as positive control.

c) 450µl of 5%BSA in distilled water (w/v) was prepared and added to all sample extract solutions and positive control.

d) All the extract samples and positive control preparations were mixed with 1.4ml of PBS buffer.

e) All the mixtures were incubated at 35ºC for 15 minutes in water bath followed by heating at 70ºC for 5 minutes.

f) The mixtures were then rapidly cooled down under tap water and immediately their absorbance readings were taken at 660nm wavelength (λ).

g) The graph of absorbance versus concentration was plotted for the sample extract solutions to show the effect with increasing concentration. The Regression value (R^2) was calculated using the plotted graph.

h) The sample extract solution absorbance at 800µg/ml was taken in triplicate to obtain the average as the final absorbance. Percentage inhibition as compared as compared to positive control (ASA) inhibition was calculated by the relation.

$$\textbf{\% Inhibition of BSA denaturation} = \frac{\textbf{Abs. of sample solution of 800µg/ml}}{\textbf{Abs. of positive control of 800µg/ml}} \textbf{ x 100}$$

3.7. Systematic Analysis of extraction

Before proceeding with the extraction and separation of triterpenoid saponins from *Clematis napaulensis* DC, the plant was studied in a span of time and observations were made regarding its vegetative season, dormancy and availability.

3.7.1. Sample collection and preparation

Materials required:

a) Dao/ Cutter

b) Vacuum Hot Air Oven (Biocraft)

c) Electric Grinder/ Blender

d) Sieve

e) Dry storage

Procedure:

a) The leaves of *Clematis napaulensis* DC. were harvested in the month of February and March from Khonoma village, Kohima Nagaland.

b) Without delay to prevent decay, the leaves were washed of dirt and dried of unwanted debris, soil and particulate.

c) The overall harvests were dried in the vacuum Hot Air Oven for 72 hours at 50ºC to completely remove any moisture from the sample.

d) The dried moisture free samples were subjected to intense grinding using Electric blender for 5 minutes or until reduced to powdery form.

e) The reduced powdered samples were then filtered through a fine pored sieve to separate out the unwanted remains.

f) The overall powder samples were weight, air-tight packed in equal quantities and stored in moisture free environment for further analyses.

3.7.2. Crude Extraction

Materials and reagents required:

a) Powdered sample

b) Methanol (Molychem)

c) Three Soxhlet apparatus units (Borosil)

d) Triple heating mantle for Soxhlet (Coslab)

e) Vacuum Hot Air Oven (Biocraft)

f) Water Chiller (Innovative Technologies)

g) Rotary Vacuum evaporator (Evator)

Procedure:

a) Accurately, 200g of the sample powder was weigh and methanol was used to wet the powder and mixed with glass rod to make it into slurry.

b) The slurry was subjected to Microwave radiation at 80ºC for 3 minutes at the interval of 1 minute each. If required, the amount of methanol was maintained enough with constant stirring and prevents cake formation (Microwave Assisted Extraction).

c) Using filter paper, the methanol solvent was recovered and the residue of MAE was dried in Hot Air Oven and packed in three pouches

d) The residue were subjected to Soxhlet Extraction using methanol at 70ºC and condensed at temperature of 5ºC by supplying chilled water from water chiller.

e) The process of Soxhlet extraction was continued until the residue is freed from any soluble phytochemicals.

f) The residue was oven dried and weighed whereas the solvent extract obtained was subjected to concentrating in Vacuum Rotary Evaporator at 50ºC and 150mbar pressure.

g) The extract was sealed and stored in cold storage (4-10ºC) whereas, the methanol recovered from the concentration process was stored for further process of extraction.

3.7.3. Fractionation

Materials Required:

a) 1000ml Separating Flask (Borosil)

b) 100ml Measuring Cylinder (Borosil)

c) Rotary Vacuum Evaporator (Evator)

d) Magnetic Stirrer

e) Distilled water

f) Ethyl Acetate (Qualigens)

g) Petroleum Ether

h) n-Butanol

i) Crude extract of *Clematis napaulensis* DC.

Procedure:

a) 47.62ml of crude extract out of 100ml concentrated extract was separated (0.84gm/ml, w/v) and dried in Hot Air Oven for 24 hours at 60ºC.

b) The crude extract was weigh and suspended in 100ml of pure distilled water.

c) The suspension was stirred continuously for 20 minutes in magnetic stirrer or until the extract is completely dissolved in the aqueous phase.

d) The aqueous phase was partitioned successfully with Petroleum Ether using Separating flask for the purpose of removing fat and pigments.

e) The procedure was repeated (3 times), each time recovering the aqueous phase.

f) The aqueous phase is again partitioned with Ethyl acetate (3 times) for other organic material removal after which the aqueous phase was recovered.

g) The remaining aqueous phase is partitioned with n-Butanol for the recovery of triterpenoid saponins from the aqueous phase.

h) The procedure was repeated three times, each time recovering the n-Butanol phase.

i) The n-Butanol phase was subjected to approx. 550 mbar at 80°C to obtain concentrated extract and recovery of n-Butanol using Rotary evaporator (B.P of n-Butanol = 118°C at 25°C and 1 atm).

j) The concentrated extract was further dried in hot water bath at 80°C for 2 hours to obtain slurry.

3.7.4. Column Chromatography

Materials Required:

a) Chromatographic columns (30mm diameter, 60cm height)

b) Hot Air Oven

c) Silica gel (60-20 particle size)

d) Stretchable rubber tube.

e) Non-absorbent cotton

f) Test tubes and Racks

g) Distilled water

h) High purity methanol (CH_3OH)

Procedure:

a) About 5gm of dry silica gel (60-120 particle size) was weigh and mixed with the n-butanol slurry extract.

b) Mixing of the slurry was done evenly in a beaker using a clean spatula and the mixture was dried overnight completely in hot air oven.

c) Chromatographic Column (3.5cm diameter, 60cm height) was set up maintaining absolute perpendicularity.

d) Initially dried 100gm of silica gel was measured and wet with 100ml of methanol followed by continuous stirring

e) The wet silica gel was transferred to the column with continuous stirring and continuous gentle tapping with rubber tube to avoid formation of air space.

f) The silica gel was filled up to 3/4 of the column height and methanol covering about 1cm above it.

g) The column was allowed to stand for 15 minutes to pack properly.

h) The dried silica gel containing n-Butanol fraction of the extract was loaded and let the dry silica gel absorbed the methanol in the column.

i) A small quantity of non-absorbent cotton was placed above the packing to prevent air transit.

j) 50ml each of mobile phases containing Chloroform: Methanol: Water was prepared in the ratio of 85:15:1.5, 80:20:2, 75:25:2.5, 70:30:3 and 65:35:3.5 (A, B, C, D, E) respectively in five conical flask.

k) The first mobile phase was taken and filled slowly in the column sidewise without disturbing the silica in the column.

l) Twenty-five test tubes were taken and marked as A1 to A5, B1 to B5, C1 to C5, D1 to D5 and E1 to E5. In each test tube about 10ml of the eluted solvent was collected sequentially.

m) From the change in colour approximately 100 ml of the elution was collected excluding the methanol present already in the stationary phase.

n) The elutes were concentrated and extract was obtained.

o) The procedure was repeated for all the other mobile phases.

p) The extract were dried on water bath completely and about 500-1000µl of methanol was added to each and stored in cold storage for further analysis.

3.7.5. UV-Vis Spectrophotometric analysis

The procedure is undertaken to know about the concentration of triterpenoid extracted and to know about the best combination of the mobile phase that is feasible for the isolation of triterpenoid from the butanol.

Materials and Reagents required:

a) Column eluted samples

b) Hot Water Bath

c) Cuvettes

d) UV-Vis Spectrophotometer

e) 5%Vanillin-Glacial acetic acid

f) Perchloric acid

Procedure:

a) From the determination of Total Triterpenoid Content (TTC) the derivation of the standard absorbance graph was referred, where Oleanolic acid (A triterpenoid saponin) was used.

b) Column elutes marked as A3, B3, C3, D3 and E3 were the samples considered for quantifying analysis.

c) 100µg/ml of each of the mention samples were taken and dried over water bath at 60ºC.

d) 300µl of 5%Vanillin-Glacial acetic acid was added to all the five tubes followed by addition of 1ml Perchloric acid and heated in water bath for 45 minutes at 60ºC.

e) The overall volumes of each were made upto 5ml and absorbance measured at 548nm.

f) The absorbances obtained by each sample were used to calculate the concentration of the isolate in each sample.

g) Finally, the deduction of best mobile phase for extraction was obtained from the calculated concentrations of the isolated samples.

3.7.6. Thin Layer Chromatography

Materials Required:

a) Silica coated TLC plate (Merck)

b) Oleanolic acid (A triterpenoid saponin)

c) n-Hexane, HPLC (Himedia)

d) Ethyl acetate (Qualigens)

e) Whatman paper

f) Metallic iodine

g) Chromatographic chamber

Procedure:

a) TLC plate at a dimension of 10x10 cm^2 was cut and mark a line with pencil 1cm in one of the corner.

b) 6 spots were marked with pencil (E, F, G, H, I and one standard) and sequentially 5 spots for five elutes and one standard (taken Oleanolic acid 100µg/100ml) were done and dried completely in hot air oven.

c) Mobile phase solvent system of Hexane: Ethyl acetate at the ratio of 7.2**:**2.8 (10ml) was prepared.

d) Mobile phase was poured into a beaker wetting a square cut Whatmann filter paper.

e) The TLC plate was then placed parallel to the whatman paper vertically and ran until the solvent front reached 7 cm from spot.

f) The plate was removed and placed in room temperature to dry for five minute.

g) Few crystals was taken along with cotton and placed in an iodine chamber.

h) After two minutes the plate was visualized and analyzed with compared to standard to obtain the result.

i) Among the spots, the elution of G (80:20:2) was chosen for further analysis since it shows the strongest spotting as compared to standard.

CHAPTER 4

RESULTS AND OBSERVATIONS

4.1. Results obtained from phytochemical screening

Test for Phenolic compounds

3.7.6.1. Ferric chloride test: Appearance of black precipitate was observed

3.7.6.2. Lead acetate test: Appearance of white precipitate was observed

Test for Alkaloids

A) Mayer's test: Formation of white precipitate was observed

B) Wagner's test: Formation of brown precipitate was observed.

Test for Deoxy-sugar (Keller-Killiani test): Appearance of reddish brown color at the junction of the two liquid layers with bluish green upper layer was observed.

Test for Anthraquinone glycoside: Upon addition of ammonia, appearance of ammonical layer turning pink or red was observed.

Test for Flavonoids

A) Ferric chloride test: Formation of blackish red colour was observed.

B) Lead acetate test: Formation of yellow precipitate was observed.

Test for Saponins: Foamy layer was observed on vigorous shaking.

Test for Steroids and Triterpenoids (Salkowski's test): Formation of an interface with a reddish brown coloration was observed.

Test for Tannins

A) Catechic test: Brownish green coloration was observed.

B) Braymer's test: Bluish green coloration was observed.

Test for Proteins

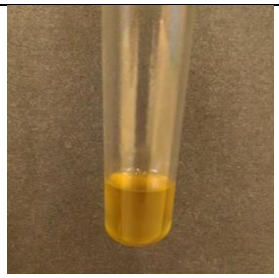
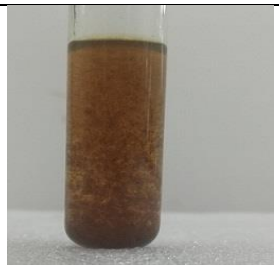
A) Biuret's test: Appearance of greenish-yellow colour was observed.

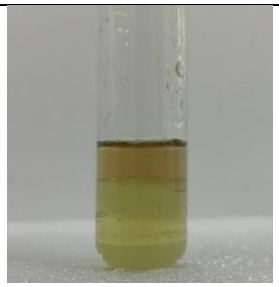
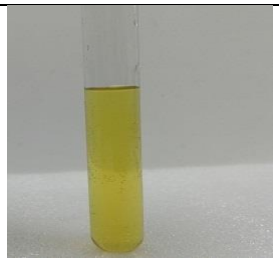
B) Xanthoproteic's test: Appearance of purple blue colour on addition of nitric acid.

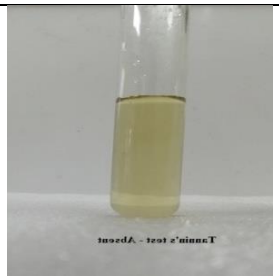
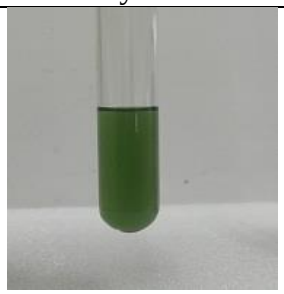
Test for Gums: Formation of red coloration was observed.

Test for Reducing sugars: Yellow precipitate followed by brick red formation was observed.

Test for Non-reducing sugars: Formation of greenish yellow colour was seen.

Sl no.	Compound Tested	Test performed	Observations	Inferences
1.	**Phenolics**	**Fig 4.1**: Ferric chloride test for phenolics	 Black precipitate	Present
		Fig 4.2: Lead acetate test for phenolics	 White precipitate	Present
2.	**Alkaloids**	**Fig 4.3:** Mayer's test	 Yellow-white precipitate	Present
		Fig 4.4: Wagner's test	 Brown precipitate	Present

3.	**Deoxy sugars**	**Fig 4.5:** Keller-Killiani's test	 Reddish-brown to bluish-green	Present
4.	**Anthra-quinone glycoside**	**Fig 4.6:** Bontranger's test	 Red coloration	Present
5.	**Flavanoids**	**Fig 4.7:** Ferric chloride test for flavonoids	 Black-red colour	Present
		Fig 4.8: Lead acetate test for flavonoids	 Yellow coloration	Present
6.	**Saponins**	**Fig 4.9:** Foam test	 White foam	Present

7.	**Steroids and triterpenoids**	**Fig 4.10:** Salkowski's test	 Red coloration	Present
8.	**Tannins**	**Fig 4.11:** Catechic test for tannins	 Gelatinous faint yellow	Present
		Fig 4.12: Braymer's test for tannins	 Bluish-green color	Present
9.	**Proteins**	**Fig 4.13:** Biuret test	 Greenish-yellow	Present
		Fig 4.14: Xanthoproteic Test	 Purple-blue colour	Present

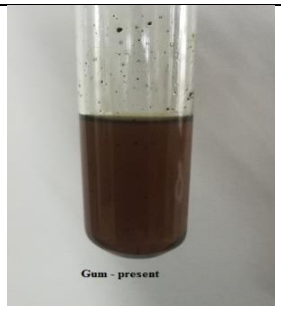
10.	**Gums**	**Fig 4.15:** Fehling test for gums	 Dark red colour	Present
11.	**Reducing sugar**	**Fig 4.16:** Fehling test for reducing sugar	 Dark red colour	Present
12.	**Non-reducing sugar**	**Fig 4.17:** Iodine test for non-reducing sugar	 Greenish yellow	Absent

Table 4.1: Observation and Inferences obtained from the phytochemical screening test.

4.2. Results from Phytochemical Estimation

4.2.1. Total Phenolic Content (TPC)

Samples	Concentration	Absorbance at 760 nm
S1	0.2µg/ml	0.108
S2	0.4µg/ml	0.207
S3	0.6µg/ml	0.346
S4	0.8µg/ml	0.462
S5	1µg/ml	0.583
Blank	00	00
Extract	0.4µg/ml	0.088

Table 4.2: Absorbance of Gallic Acid Equivalent (GAE) and sample extract

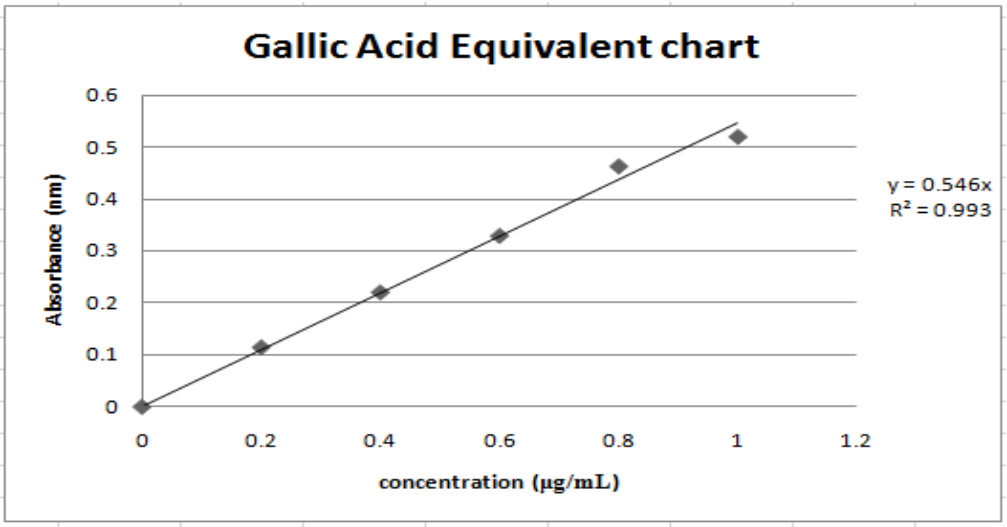


Fig 4.18: Gallic Acid Equivalent graph

$Y = mX \pm C$

Gallic Acid Eqivalent = 0.405 = m, Extract Absorbance (Y) = 0.088

From graph, $Y = 0.546X \pm$ and $R^2 = 0.993$

Therefore, $X = \frac{0.088}{0.546} = 0.1612$µg/ml

Therefore, the Total Phenolic Content (TPC) in *Clematis napaulensis* DC. extract was 0.1612µg/ml of GAE.

4.2.2. Total Flavonoid Content (TFC)

Samples	Concentration (µg/ml)	Absorbance at 415nm
Blank	0.00	0.00
S1	0.10	0.070
S2	0.20	0.120
S3	0.30	0.199
S4	0.40	0.261
S5	0.50	0.326
Extract	0.20	0.090

Table 4.3: Absorbance of Quercetin Equivalent (QE) and sample extract

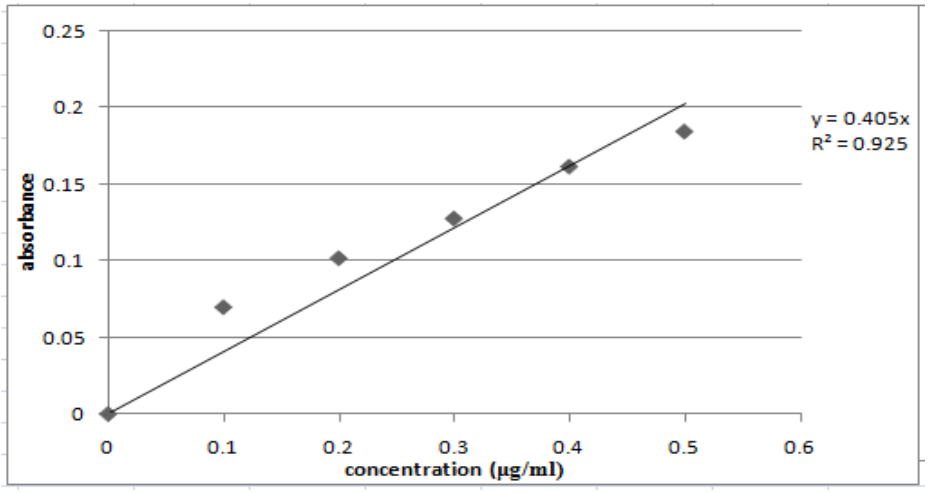


Fig 4.19: Quercetin Equivalent graph

Since, Y = mX ± C

Quercetin Equivalent (QE) = 0.405 = m and Extract Absorbance (Y) = 0.090

From graph, Y = 0.405X ± 0 and R^2 = 0.925

Therefore, X = $\frac{\mathbf{0.090}}{\mathbf{0.405}}$ = 0.2221µg/ml

The Total Flavonoid Content (TFC) in Clematis napaulensis DC. is estimated to be 0.2221µg/ml of Quercetin Equivalent.

4.2.3. Total Triterpenoid Content (TTC)

Sample	Concentration (µg/ml)	Absorbance at 548nm
Blank	0.0	0.0
S1	1.0	0.097
S2	2.0	0.136
S3	3.0	0.195
S4	4.0	0.289
S5	5.0	0.391
Extract	2.0	0.052

Table 4.4: Absorbance of Oleanolic Acid Equivalent (OAE) and sample extract

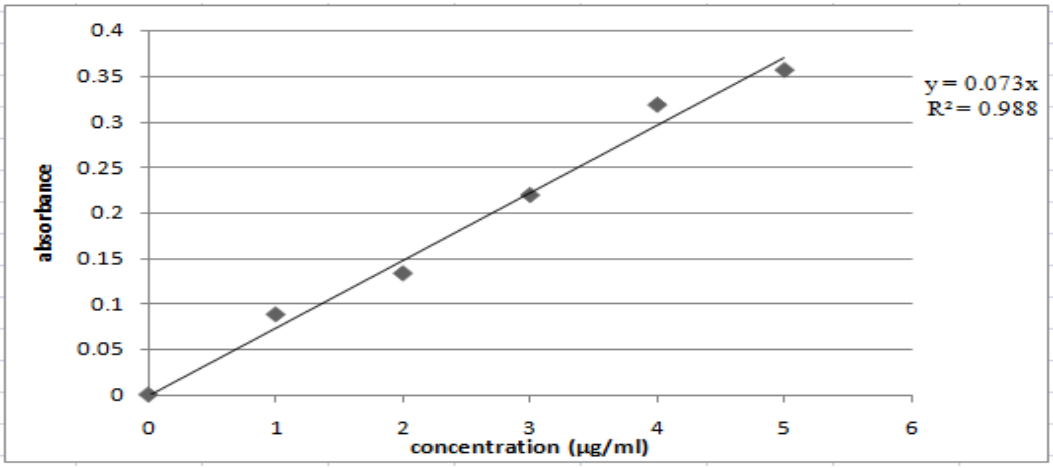


Fig 4.20: Oleanolic Acid Equivalent (OAE) Graph

Since, $Y = mX \pm C$

Oleanolic Acid Equivalent (OAE) = 0.073 = m and $R^2 = 0.988$

Extract Absorbance = 0.052 = Y

$X = \frac{0.073}{0.052} = 1.404$µg/ml

Therefore, the Total Triterpenoid Content (TTC) in *Clematis napaulensis* DC. is estimated to be 1.404µg/ml of Oleanolic Acid Equivalent.

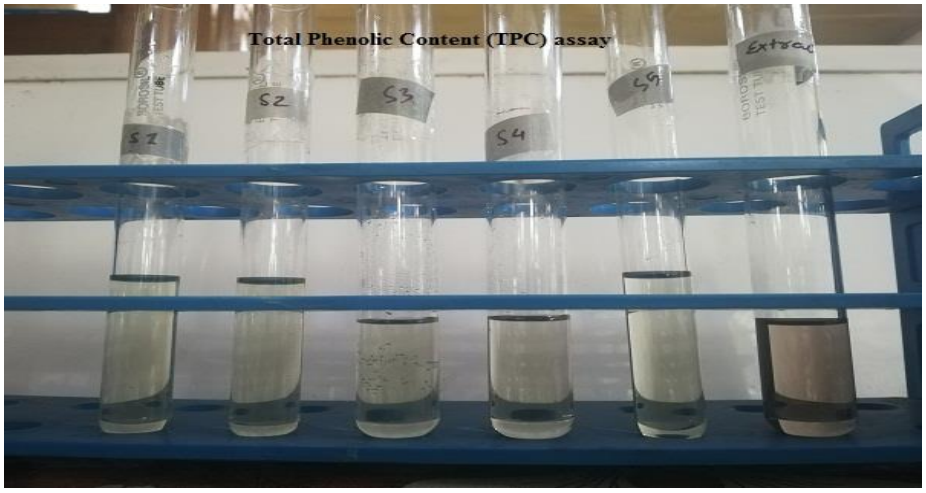


Fig 4.21: Total Phenolic Content assay

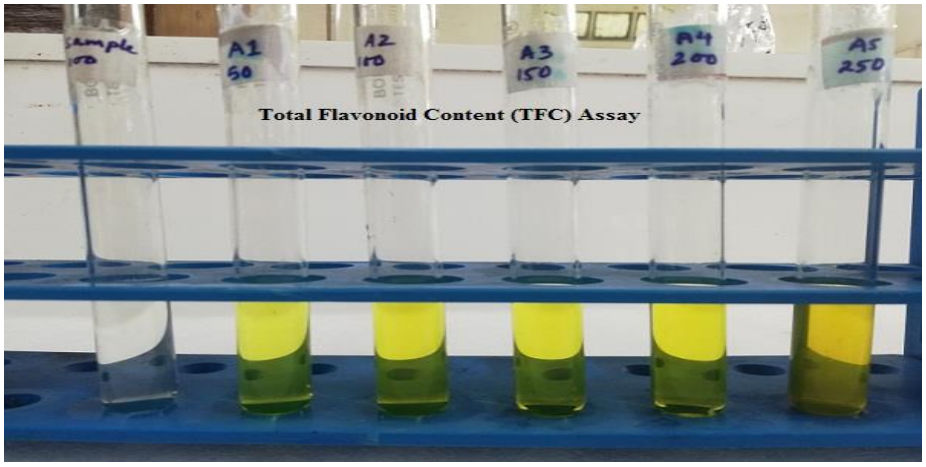


Fig 4.22: Total Flavonoid Content assay

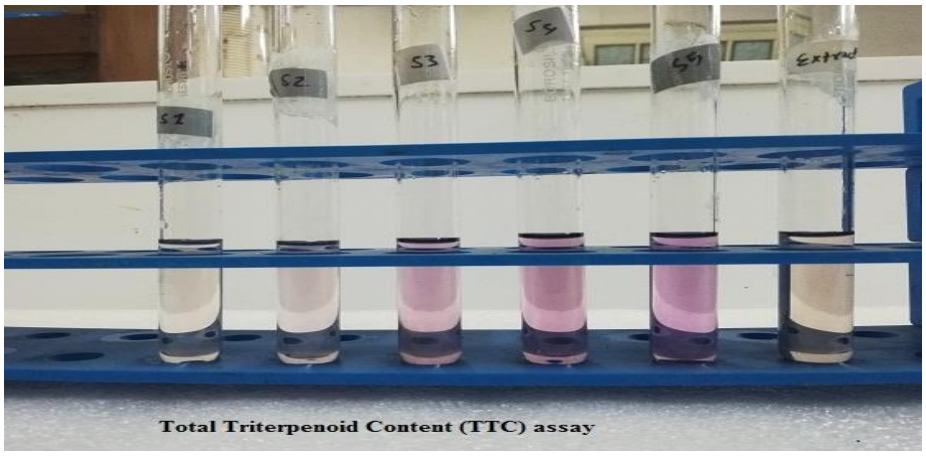


Fig 4.23: Total Triterpenoid Content assay

4.3. Results from prospecting of *Clematis napaulensis* DC. for medicinal properties:

4.3.1. DPPH scavenging Assay:

Sample	Concentration (µg/mL)	Absorbance (517nm)
Blank (Methanol)	00	00
Sample I	100	0.092
Sample II	100	0.035
Sample III	100	0.134
Ascorbic acid	100	0.054

Table 4.5: Triplet absorbance of sample extract and ascorbic acid

Average absorbance of samples = (AbsI + AbsII + AbsIII) / 3

= (0.092 + 0.035 + 0.134) / 3

= 0.087

Ascorbic acid absorbance = 0.054

Percentage Inhibition = {(0.087-0.054)/0.087} x 100

= 37.93% w/w

4.3.2. Hydrogen Peroxide Scavenging Assay:

Sample	Concentration (µg/mL)	Absorbance (230nm)
Blank	00	00
A1	10	0.223
A2	20	0.153
A3	40	0.121
A4	80	0.101
A5	160	0.091
Control(Ascorbic acid)	160	0.173

Table 4.6: Absorbances of H_2O_2 Scavenging Assay

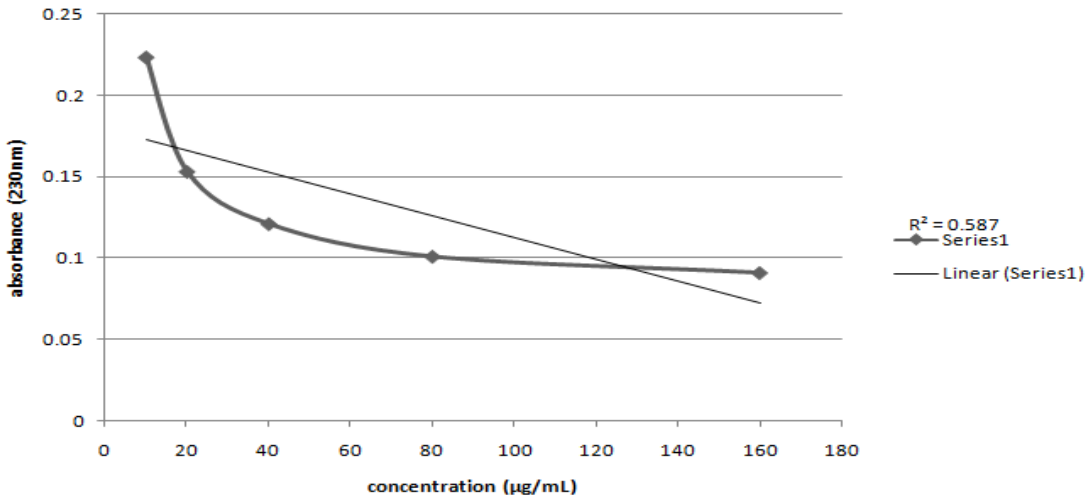


Fig 4.24: H_2O_2 Scavenging Assay Graph

Percentage inhibition was calculated by taking absorbance at of both sample and standard at 160µg/mL.

$$\text{Percentage inhibition} = \frac{\textbf{Standard Abs.} - \textbf{Sample Abs.}}{\textbf{Sample Abs.}} \times 100$$

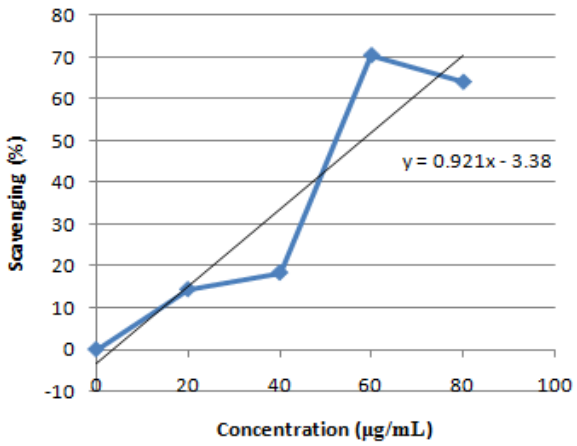
$$= \left\{\frac{\mathbf{0.173 - 0.091}}{\mathbf{0.173}}\right\} \times 100$$

= 47.40% w/w of Ascorbic acid

4.3.3. Ferric Reducing Anti-oxidant Power (FRAP) assay:

Sample	Conc. (µg/ml)	Absorbance (700nm)	Scavenging (%)	*IC50*
Control	**PBS**	**0.298**		
S1	20	0.043	14.4%	**= 57.96µg/ml**
S2	40	0.055	18.4%	
S3	60	0.210	70.5%	
S4	80	0.191	64.1%	
A1	100	0.180	60.4%	**= 215.53µg/ml**
A2	200	0.206	69.1%	
A3	300	0.225	75.5%	
A4	400	0.168	56.4%	
A5	500	0.162	54.3%	

Table 4.7: Absorbances and IC50 of sample and ascorbic acid



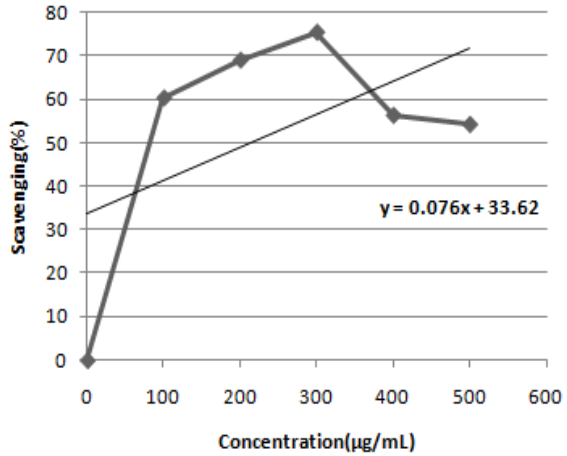


Fig 4.25: Ascorbic acid absorbance (FRAP assay) **Fig 4.26:** Sample absorbance (FRAP assay)

Ascorbic acid IC50: Y = 0.921X - 3.38

Taking Y = 50,

$$X = \frac{50+3.38}{0.921} = 57.96\mu g/ml$$

Sample extract IC50: Y = 0.076X + 33.62

$$X = \frac{50-33.62}{0.076} = 215.53\mu g/ml$$

$$\% \text{ Reducing power of extract} = \frac{\text{IC50 of Ascorbic acid}}{\text{IC50 of Sample Extract}} \times 100$$

= 26.89% w/w IC50 of ascorbic acid

4.3.4. Anti-inflammatory assay (Inhibition of protein denaturation assay):

Sample	Concentration (µg/mL)	Absorbances at 660nm
A1	50	0.055
A2	100	0.063
A3	200	0.092
A4	400	0.233
A5	800	0.337
Control (ASA)	800	1.220

Table 4.8: Absorbance of protein denaturation assay

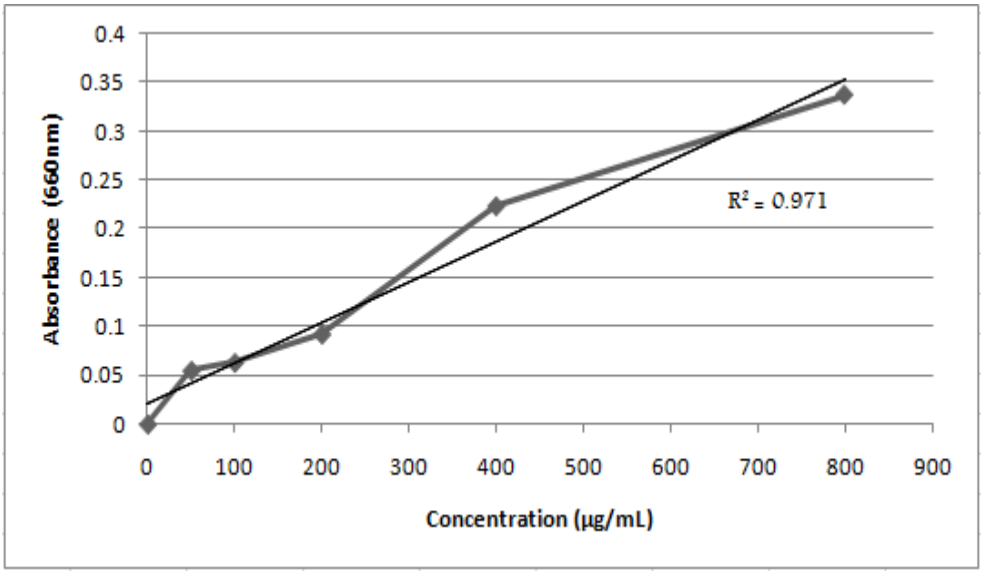


Fig 4.27: Absorbance graph of anti-inflammatory action

Absorbance of protein denaturation by sample at 800µg/ml = 0.337

Absorbance of protein denaturation by ASA at 800µg/ml = 1.220

Percentage inhibition by sample extract = $\frac{1.220-0.337}{1.220}$ x100

= 72.38% w/w of ASA

Therefore, the inhibition of protein denaturation of methanolic crude extract of *Clematis napaulensis* DC. is 72.38% of Acetyl Salicylic Acid.

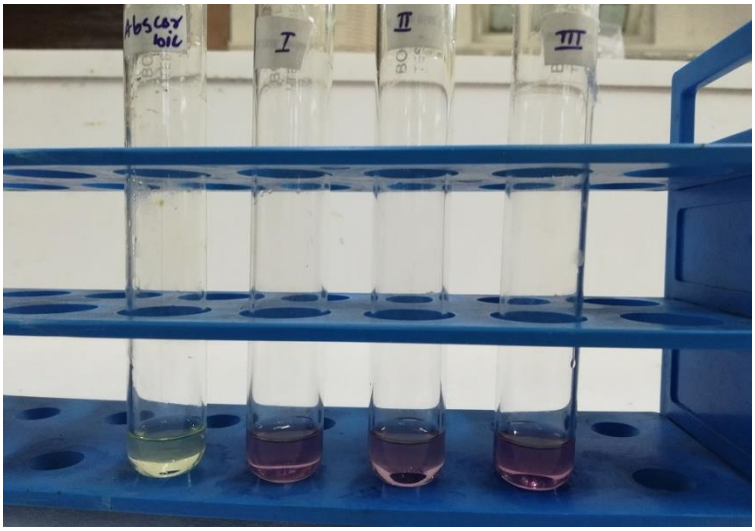


Fig 4.28: DPPH Scavenging Assay

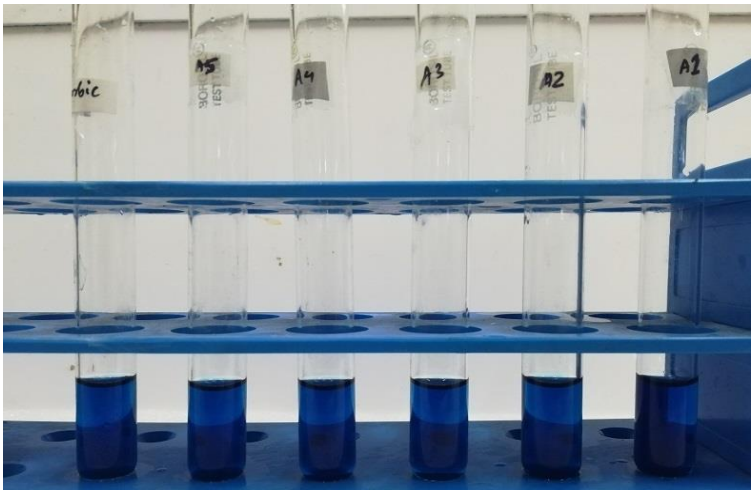


Fig 4.29: Hydrogen Peroxide Scavenging Assay

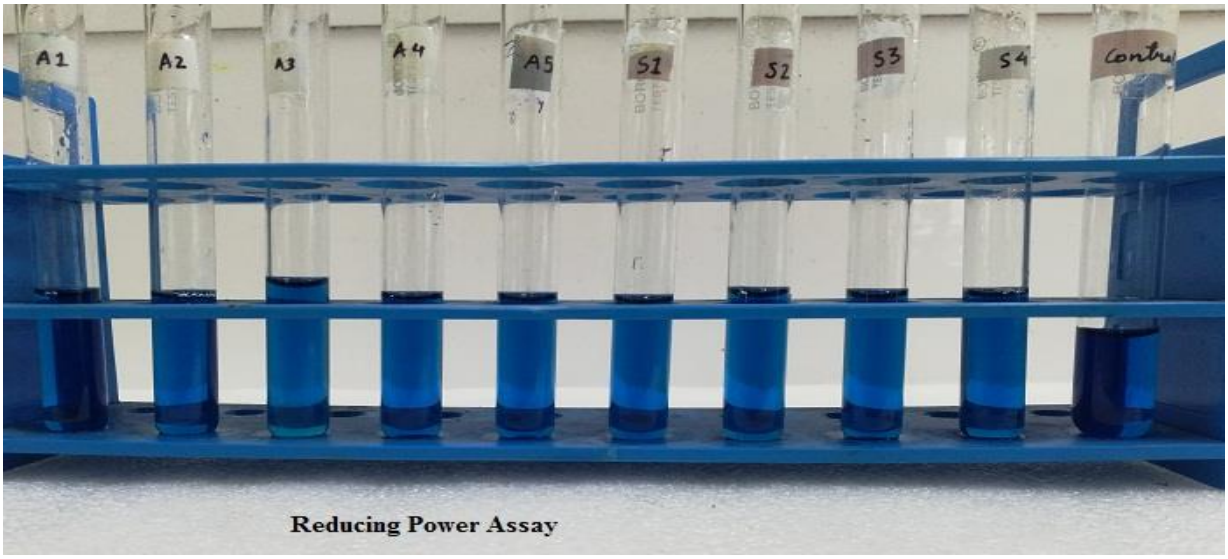


Fig 4.30: Ferric Reducing Anti-Oxidant Power (FRAP) Assay

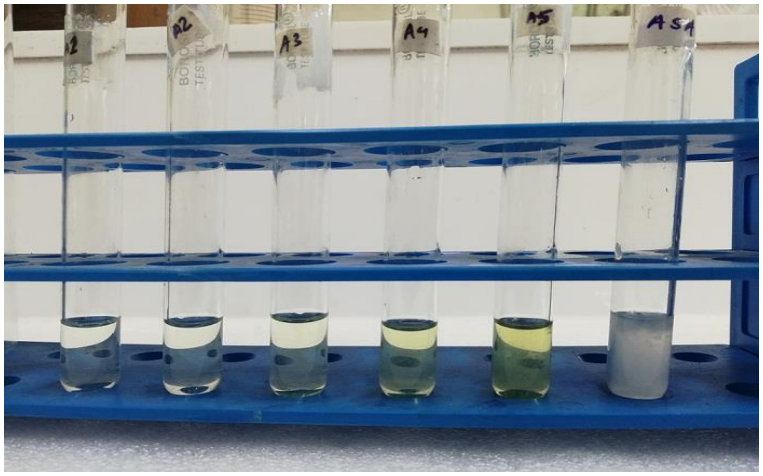


Fig 4.31: Inhibition of Protein denaturation assay (Anti-inflammatory Assay)

4.4. Result from crude phytochemical extraction

Weight of fresh leave sample harvested from the field = 4kg

Weight of dried leave sample = 0.2kg

Reduction in weight (Moisture content) = $\{\frac{\mathbf{4-0.2}}{\mathbf{4}}\}$ x 100

= 95% w/w

4.5. Result from extraction of Triterpenoid saponins

4.5.1. Crude Extraction :

For the process of separation and isolation the procedure of crude extraction was started with 200gm of the dried sample powder (Soxhlet Extraction).

Residue remained after drying = 158gm, w/w

Weight of the methanolic extract = 42gm, w/w

The extract is concentrated to the volume of 50ml,

Overall concentration of the extract = 42gm/50ml

Hence, concentration of extract = 0.84gm/ml, w/v

Fig 4.32: Dry and powdered form of *Clematis. napaulensis* DC. leaves

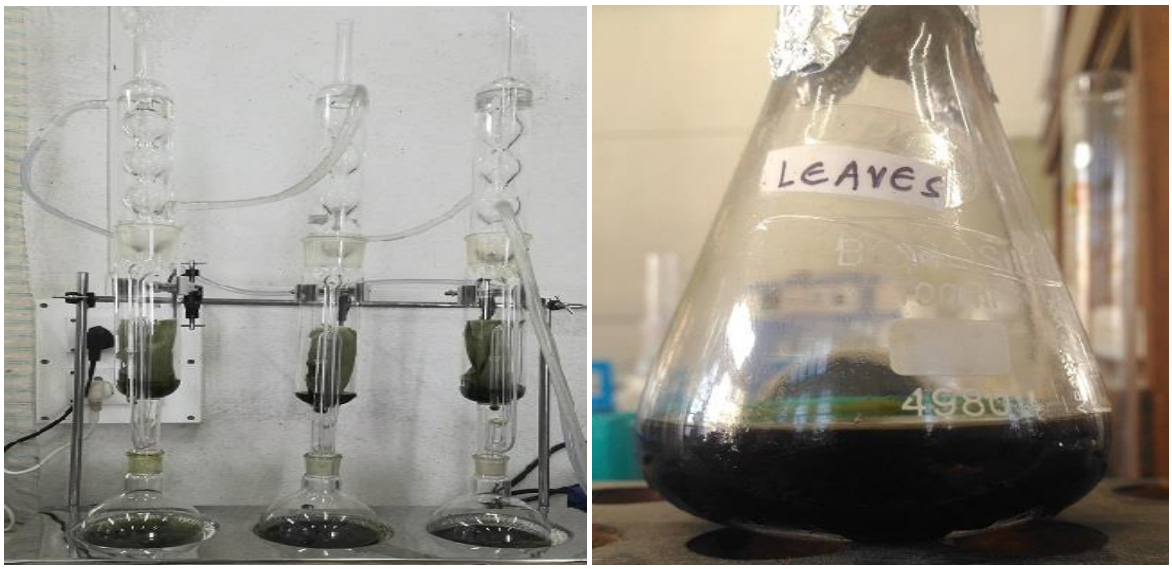


Fig 4.33: Soxhlet extraction (L) and Concentrated extract (R) of *Clematis napaulensis* DC. leave powder

4.5.2. Fractionation :

Concentration of the extract = 0.84gm/ml (w/v) or 42gm/50ml

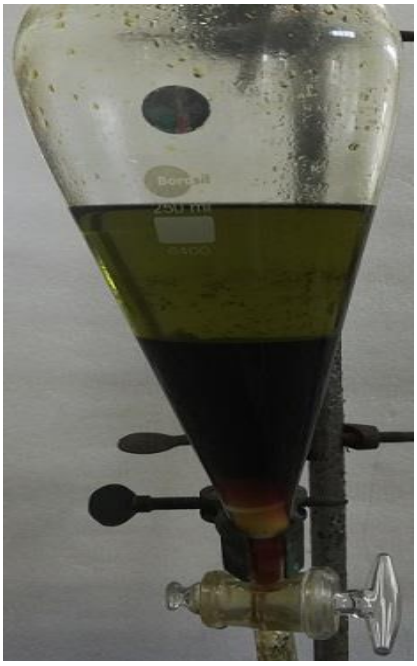
Weight of the extract taken = 20gm

Corresponding volume, 42gm/50ml = 20gm/V

V = $\{\frac{50*20}{42}\}$ml = 23.809ml of extract is dried to 20gm

Concentration of extract in distilled water = (20/100) gm/ml = 0.20gm/ml

100ml of extract in aqueous phase (0.20gm/ml, w/v) was used for fractionating with petroleum ether (100 ml), ethyl acetate and n-butanol.



4.34

4.35

4.36

4.37

Fig 4.34: Fractionation by petroleum ether (upper) with aqueous solution (lower)

Fig 4.35: Fractionation by ethyl acetate (upper) and aqueous solution remain (lower)

Fig 4.36: Fractionation by n-butanol (upper) and aqueous layer (lower).

Fig 4.37: Concentrating of n-butanol fraction containing triterpenoid saponins.

4.5.3. Column chromatography analysis

Sample	Absorbance (548nm)
Blank (methanol)	00
A3	0.096
B3	0.092
C3	0.093
D3	0.088
E3	0.090

Table 4.9: Absorbance of chromatographic elutes

The concentration of triterpenoid saponins in the five samples can be calculated by using the relation for standard Oleanolic acid in Total Triterpenoid Content analysis as,

Standard absorbance (m) = 0.102 {obtained from the graph}

From the relation, $Y = mX$

$$X \text{ (sample concentration)} = \frac{\mathbf{Y(OA\ absorbance)}}{\mathbf{m(Sample\ absorbance)}}$$

For Sample A3: Concentration = $^{0.102}/_{0.098}$ = 1.041µg/ml, w/v

For sample B3: Concentration = $^{0.102}/_{0.092}$ = 1.109µg/ml, w/v

For sample C3: Concentration = $^{0.102}/_{0.093}$ = 1.097µg/ml, w/v

For sample D3: Concentration = $^{0.102}/_{0.088}$ = 1.159µg/ml, w/v

For sample E3: Concentration = $^{0.102}/_{0.090}$ = 1.133µg/ml, w/v

Hence, the concentrations of elutes obtained are in the order D3 > E3 > B3 > C3 > A3, whereby D3 shows highest amount of isolate per unit volume. From the initial crude extract taken for isolation the amount of Triterpenoid saponins can be determined. From 4.5.2, the concentration of sample extract prepared was 0.20gm/ml, w/v and the extract concentration was 1.159µg/ml, w/v (D3). Hence the percentage of Triterpenoid saponins content and its overall isolate obtained made from the Total crude extract is as followed –

$$\text{Percentage of Triterpenoid saponins} = \frac{\mathbf{1.159}\mu g/ml}{\mathbf{0.20}mg/ml} \text{ x } 100$$

$$= \frac{\mathbf{1.159}}{\mathbf{0.20\ x\ 1000}} \text{ x } 100$$

$$= {}^{\mathbf{1.159}}/_{\mathbf{2.0}}$$

= 0.5795% of Triterpenoid saponins content per gram of crude extract was made from the powdered leave sample of *C.napaulensis* DC.

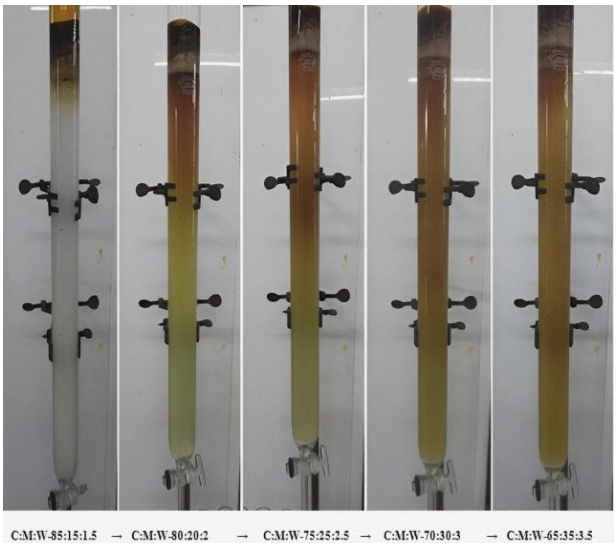


Fig 4.38: Column Chromatography setup

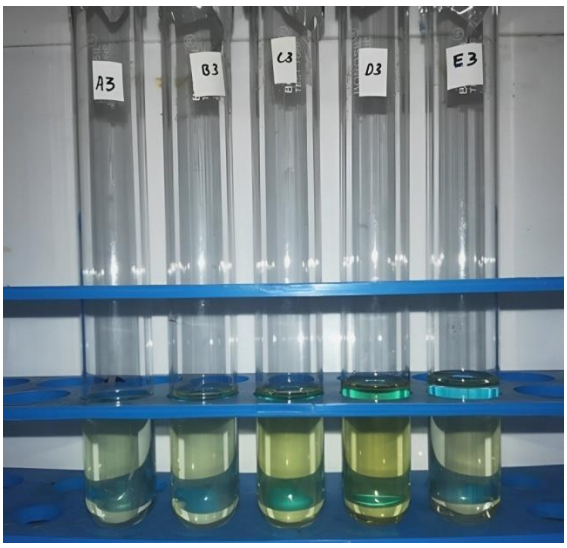


Fig 4.39: CC Samples for Absorbances

Fig 4.40: Overall Column Chromatographic elutes

4.5.4. Thin Layer Chromatographic analysis

The Thin Layer Chromatography (TLC) analysis showed that was overall presence of Triterpenoid saponins from elutes of column chromatography. However, there were varying in concentrations.

4.6. Overall Mass Flow:

Mass flow chart was an indicative diagram that showed the efficiency of extraction, comparative loss of masses at the process process to achieve the targeted substrate. Here the Triterpenoid saponins accounted for about 0.5795% of the total crude extract.

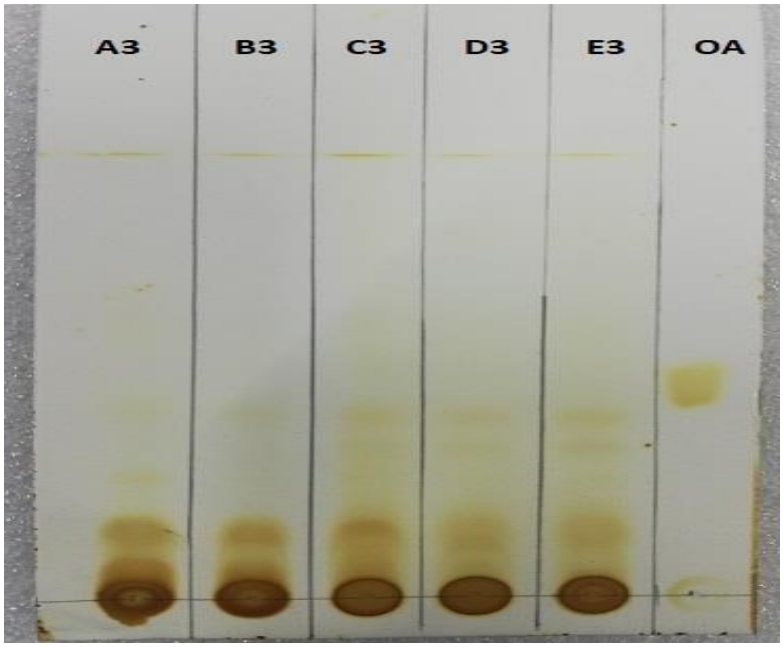


Fig 4.41: View of TLC under iodine vapour

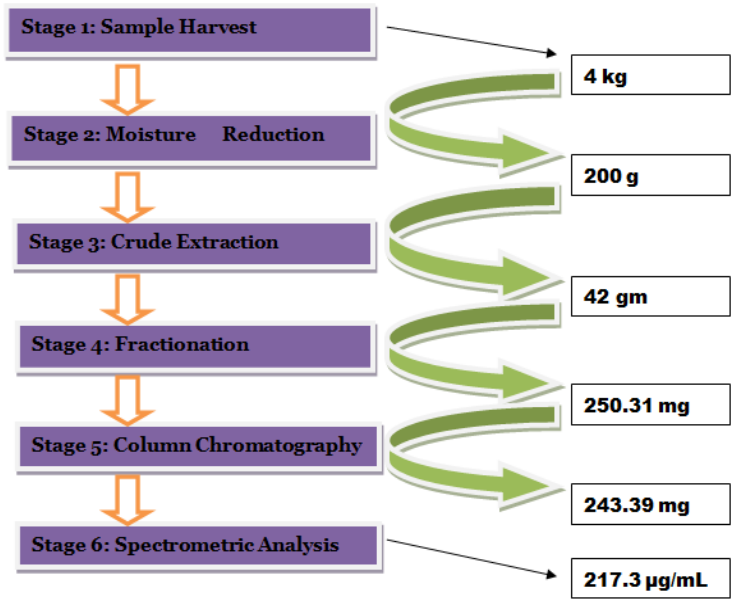


Fig 4.42: Mass Flow Diagram

CHAPTER 5

DISCUSSIONS

5.1. Preliminary studies

During the course of the research, *Clematis napaulensis* DC. was observed in its local environment. Unlike most plants the foliages of the plant were not abundant even in the vegetative period. The stem was semi-woody and dry therefore not an initial choice to be considered for analytical purposes.

Clematis napaulensis DC. period of vegetative growth have to be accurately known beforehand and in this regard, botanist of Kohima Science College Jotsoma have been instrumental. The knowledge had helped in gathering of foliage/leaves to an appropriate quantity to be analyzed.

5.2. Sample Harvesting

Part of the plant which was considered for analyses was leaves or foliage. Traditional uses have also suggested that leave portion as being used against Rheumatoid Arthritis (RA). The harvest was done alongwith other organic debris, but care has been taken to discard all debris and only leaves was taken for analysis. Initial weighing was done at this point.

The leaves have to be cleansed in running water to remove dust particulates apart from inorganic debris. It was first dried in open air to remove wet moisture overnight followed by drying in Hot Air Oven. The dried leave sample powdered by electric blender has to be sieve previously to remove non-powdery particulate after which only the second phase weighing was done. The quantity obtain here is taken for next analyses.

5.3. Phytochemical screening test in *Clematis napaulensis* DC.

Screening test for important classes of phytochemicals was done, however in a generalized but specific to the targeted phytochemicals. All the important class of phytochemicals found in most medicinal plants as well as those reported in some species of Clematis were found in *Clematis napaulensis* DC. as well (*viz*., phenolics, alkaloids, glycosides, flavonoids, saponins, steroids and triterpenoids, tannins, proteins, gums, reducing

and non-reducing sugars). Except for non-reducing sugar all the other phytochemicals that were tested were present.

It is an important point to be noted that most of the chemicals test performed are compounds of each other or bears the chemical as well as structural properties e.g. the compound that was studied in this research i.e. Triterpenoid saponins, which are C30 compounds (squalene) and saponin refers to compound with foamy quality if agitated, usually bitter taste. Chemically, saponins consist of an aglycone unit linked to one or more carbohydrate chains. These screening test were based mostly on the biochemical nature of the tested compound that give certain reaction, observation and hence the inferences. However, for more classified studies, higher and more advance physical methods have to be pursued.

5.4. Phytochemical Estimation in *Clematis napaulensis* DC.

In major class of Phytochemicals, there are hundreds or even thousands of individual compounds, classified based on their structural and functional groups. Therefore, the overall estimation has been based on the chemical reactivity of the targeted compound group to a certain reagent measured by spectroscopic measurement, taking into account that a certain compound group has specific wavelength to which it respond upon reaction.

From the literature, it has been studied that, Phenolics are large group of phytochemicals containing simple phenols phenolic acids, stilbenes, flavonoids, biflavonoids, proanthocyanidins, tannins, coumarins and anthraquinones all unified in their classification as phenol by containing a phenolic ring structure. Folin Ciocaltiu's reagent is a mixture of phosphomolybdate and phosphotungstate with additional lithium sulfate and bromine used for *in vitro* assay of phenolic anti-oxidants also called the Gallic Acid Equivalent (GAE) method. The reagent does not only measure phenols but would react with any reducing substances. It would therefore, measures the total reducing capacity of the sample. Folin-Ciocalteu method is thus based on electron transfer, and gives reducing capacity expressed as phenolic content. The resulting TPC vastly depends on the type of solvent used during extraction. Absorbance is measured in the infra-red spectrum (760nm).

It has been studied that flavonoids were a group of polyphenolics distinguished by 15 carbons structure, with two phenyl rings. Though widespread in nature in plants with numerous health benefits it hasn't been however, not an approved drug. The overall Total

Flavonoid Content (TFC) could be enhance however if the extraction could be done by petroleum ether rather than by methanol (Mathur and Vijayvergia, 2017). Formation of acid stable complexes with addition of Aluminum Chloride to C3 and C5 hydroxyl group of flavonoid groups is the basis of detection whereby Quercetin is the standard. Absorbance was measured at 415nm (visible spectrum).

It was necessary to optimize the extraction parameters for obtaining the highest amount of triterpenoid recovery. It was noted that several conventional methods involved solvent for extraction whereby there was loss of solvent and time. However in physical method both of the disadvantages could be excluded but, chances of altering the biochemical nature of phytochemicals could be higher. From the literature, it was noted that for TTC estimation, the initial crude extraction yields the highest triterpenoid with $CHCl_3$+H_2O followed by Ethanol.

In Vanillin-Glacial acetic acid method, the reaction of glacial acetic acid oxidizes triterpenoid saponins with vanillin, giving a distinctive purple colour. The intensity of the purple colour was proportional to the concentration of standard samples.

5.5. Prospecting of Clematis napaulensis DC.

Here, the prospecting of *Clematis napaulensis* DC. were done basically for its medicinal properties. The process has to be performed using some specific standard tests applicable to similar plants such as anti-oxidative, anti-cancerous and anti-inflammatory.

2,2–Diphenyl-1-picrylhydrazyl (DPPH) is a stable free radical that is dull black in colour. The chemical allows anti-oxidant test to be performed upon it unlike other free radicals that have unpaired electrons, whereby the test were not applicable. Compared to ascorbic acid (a standard antioxidant), the value of 37.93% (w/w) was higher than most, considering the fact that the experiment was being done on the crude extract of methanol. However it was an experimental evidence for presence of free radical scavenging property.

Hydrogen Peroxide (H_2O_2) is a pale blue liquid used as an oxidizer, antiseptic and bleaching agent all intended for external purposes depending on the concentrations. The extra oxygen act as electron acceptor from other substances thus it could oxidize the cell membrane of microbes. Similarly, the effect could be hazardous to individuals, unless proper care of

usage is not followed. The increasing concentration of sample crude extract has inversely proportional effect on H_2O_2 whereby the free oxygen were being scavenged and rendered into normal H_2O. The concentration of H_2O_2, when taken the absorbance at 230nm (λ_{uv}) showed a decreasing trend and as compared to ascorbic acid the value was 47.40% (w/w) at 160µg/ml. From the findings, it could be said that *Clematis napaulensis* DC. has free radical scavenging property.

FRAP assay is an electron transfer based method that measures the reduction of Fe^{3+} (ferric) to Fe^{2+} (ferrous) ions by antioxidant in acidic medium (pH 3.6) and temperature at 37ºC.The ferric ion is being supplied by the addition of Potassium ferrocyanide to both sample and ascorbic acid standard. The concentrations were prepared in arithmetic progression for both samples and standard and the change in the reduction after incubation can be measured at 700nm wavelength. IC_{50} (Half maximal inhibitory concentration) analysis showed the values of 57.96µg/ml for standard (ascorbic acid) and 215.53µg/ml for sample extract. Lesser the IC_{50} value, more potent can be the inhibitory substance. Hence, even in crude form of extract *C.napaulensis* DC. crude extract showed a potent IC_{50} value even though its lower than ascorbic acid in its ability to reduce Fe^{3+} to Fe^{2+}.

In our body, during physiological stress many symptoms also follow that cause severity of many diseases such as vasodilation, pain and arthritis etc. Protein denaturation is one of the chief factors whereby the protein loses its function due to change in structure. It could be due to external or genetic reason however a cascade of reaction, in which release of leukocytes and chemokines occurs. The effect could be long lasting in many inborn disorders or short lasting if it is a body response to external stimuli. Bovine Serum Albumin is a 583 amino acids long polypeptide, which has been used as a model serum protein and has 76% structural homology with human serum albumin (Topala *et al*., 2014). For the purpose of experiment, Aspirin (ASA) also an anti-inflammatory drug was being used as positive control. The sample concentrations were made in geometric increase starting with 50µg/ml to 800µg/ml to which the inhibition showed an increasing trend in buffered solution and Regression value was found to be 0.971. At 800µg/ml the inhibition strength of sample extract was 72.38% (w/w) of ASA which was a considerable yield for medicinal plant.

5.6. Crude Phytochemical Extraction

Many alternative solvents could have been a choice for crude phytochemical extraction in Soxhlet hot extraction, or even considered other physical and lesser time consuming methods. But Methanol was a solvent of choice for the extraction from *Clematis napaulensis* DC. due to its lesser volatility as compared to other solvent such as petroleum ether and chloroform which saves a lot of solvent in the extraction process meanwhile allowing the contents of the powder to exude out to the solvent. Toxic though it is by nature if consumed, however the polarity allows it to be efficiently handle during extraction. Most importantly, it does not react with targeted analyte to form new unwanted complexes, rather can be efficiently removed by evaporation and condensation.

5.7. Fractionating Techniques

Fractionation is based on the non-miscibility of two or three solvents in which the target analyte gets preferentially dissolved. The procedure follows in which complete extraction is required and unwanted/secondary solvents are removed effectively.

Though, the initial extraction was by methanol it was dried off from the crude extract and then dissolved in distilled water which is more polar than methanol. Water dissolved most of the dry extract including Triterpenoid saponins that are soluble in methanol when aided by magnetic stirrer. Petroleum ether successfully removed fat and pigments (e.g. chlorophyll, carotenoids and anthocyanins) from the extract. Fractionating with Ethyl acetate removed other organic that are not soluble with pet ether, leaving triterpenoid saponin in water. Finally, n-butanol separated out the triterpenoid saponins from water selectively. n-butanol is less polar than water but has higher B.P and volatility. Triterpenoid saponins were obtained by removing the n-butanol using vacuum rotary evaporator (550mbar pressure and at 80ºC). Also other phytochemicals like alkaloids, diterpenes, phlobatannins and tannins can still be at large in the n-butanol fraction.

5.8. Chromatographic techniques

The extract from n-butanol was again mixed with methanol for column chromatography. It is an important procedure in which the final extracts are separated out based on the colour concentration. But the mobile phase needs to have a best elution capacity w.r.t. the standard (Oleanolic acid in this case). The presence of Triterpenoid saponins as well

as the best solvent combination candidate is being determined by TLC. Triterpenoid saponins can however be only obtained in solution form and quantified by UV-Vis Spectroscopy.

Column Chromatography is a simple but effective procedure to isolate triterpenoid saponins from the fraction. Important factors during the procedure were considered for utmost separation such as absolute perpendicularity of column, usage of non-absorbent cotton to prevent loss of sample. Same quantity of mobile phase yield had to be recovered but in five sequential fractions for five columns. Each column have different ratio of mobile phase prepared, in increasing polarity. Result showed that, elute from column D3 tube having mobile phase ratio of 70:30:3 for Chloroform: Methanol: Water. It can be noted that in chromatographic procedures, polarity plays an important role. The n-butanol fraction contains the targeted triterpenoid saponins but it can also extract a fair amount of other metabolites. Therefore the most appropriate amount of the triterpenoid saponins can be estimated only by using the spectroscopic technique with a standard. Another important factor that can affect the extraction process is that the dried sample from n-butanol drying can be volatile like any other secondary metabolites. Therefore it has to be kept in polar solvent with a determined concentration.

In Thin Layer Chromatography analysis the natures of the mobile phases, the ratio of different phases taken in combination affects the subsequent separation depending on polarity which is an inherent property considered during TLC analysis. The result was indicative of the presence of triterpenoid saponins in all elutes of column chromatography however, it was dependent on the ratio of mobile phase and hence the polarities used in column. Further quantification by UV-Vis spectrometer revealed presence of triterpenoid saponins in level of secondary metabolites.

In the case of detection of triterpenoid saponins the coloration of the sample strength is vastly amplified by exposure to metallic iodine vapour kept in a glass beaker since there was only one standard (oleanolic acid). The detections were being shown by the elute having same coloration (brownish hue) as shown by standard in 10 minutes time. The strength of the hue reveals the level range of concentration. It was noted that the strength range of elutes was similar to that of the spectrometric result i.e. D3 have highest coloration and A3 lowest.

The research methodology applied for the extraction by chromatography were on a trial basis, as there were no concrete solvent mobile phase ratio best suited for the experiment. The most quantified mobile phase was chosen as a best mobile phase considering that all the column were fed with equal amount of the extract.

5.9. Efficiency of extraction

In process of extraction the mass flow chart construction gives a comparative idea of how much the extraction has been done and what amount of the sample are being rejected in the procedure. It was noticed in the overall procedure that most of the leave sample collected contains moisture (more than 90%). In powdered dry sample the non digestible crude fibers accounts for almost 80%. The final crude extract is only about 2% of the plant leave harvested.

The crude sample contains non-targeted extract such as pigments, fats, proteins as well as other numerous metabolites. It was found that after removal of bulk primary metabolites such as fats and pigments, the selective method of extraction was adopted. It is noted however, that during the process of fractionation, considerable amount of the extract are being lost. Analysis showed the amount of triterpenoid saponins to be 0.5795% of 42gm crude extract or 243.39mg of triterpenoid saponins was extracted. Therefore the concentration can be estimated as 5.795mg/g (w/w) of crude extract.

CHAPTER 6

SUMMARY AND CONCLUSION

Clematis species are widely studied and have been used in herbal medicine for their bioactive potent properties, many of which are targeted to similar kinds of ailments for exhibiting similar effect. However, *Clematis napaulensis* DC. was only being reported, but not studied in scientific aspect even though the case of being used indigenously for generations as medicine by Chakesang tribe of Nagaland, India to remedy against Rheumatoid arthritis, a bone related disorder in aged people. The topical applications suggest a potential anti-inflammatory property. It is also well known scientifically that, triterpenoid saponins as a class of compound, having anti-oxidant as well as anti-inflammatory properties.

The research study was mainly focused on strategic approaches towards crude extraction of bioactive compounds from *Clematis napaulensis* DC. from the foliage part of the semi-creeper plant, phytochemical screening, analyzing the extract for potential medicinal properties and finally develop an efficient extraction methodology for the extraction of triterpenoid saponins from the crude extract.

Identification of *Clematis napaulensis* DC. was done by Taxonomist from Kohima Science College, Jotsoma (KSCJ), Nagaland. The foliage of the plant was harvested from a specified location from Khonoma Village in Kohima, Nagaland. Literatures have shown that, among many organic extraction methods, Soxhlet hot extraction is the best considering the volatility of metabolites. The crude extraction of the plant sample powder has yielded 21% (m/m) of crude extract using conventional Soxhlet extraction with methanol at 70ºC. The crude extract was used for phytochemical screening which revealed the presence of phenolic compounds, alkaloids, glycosides (de-oxy and anthraquinone), flavonoids, saponins, steroids, terpenoids, tannins, proteins, gums and reducing sugars. Total Phenolic Compound (TPC) was estimated to be 0.1612µg/ml in the crude extract using Gallic Acid Equivalent, Total Flavonoid Content (TFC) to be 0.2221µg/ml using Quercetin Equivalent and Total Triterpenoid Content to be 1.404µg/ml using Oleanolic Acid Equivalent. DPPH scavenging assay estimated 37.93% w/w scavenging as compared to Ascorbic acid standard, Hydrogen peroxide scavenging assay as 47.40% w/w of Ascorbic acid standard and FRAP assay as

26.89% w/w IC50 of Ascorbic acid. Inhibition of protein denaturation assay, a test of anti-inflammatory potential revealed the potentiality of 72.38% w/w as compared to Acetyl salicylic acid (ASA) or commonly known as Aspirin.

The methanolic crude extract was dried in Vacuum rotary evaporator at 150 mbar pressure and 50ºC whereby the crude extract was dried, weighed and dissolved in distilled water before fractionation. The extract solution was fractionated using ethyl acetate (3 times), petroleum ether (3 times) to remove pigments and fats each time recovering water portion. Finally fractionation was done using n-butanol (3 times) recovering the butanol fraction. The butanol was removed at 80ºC and 550 mbar pressure which yielded 250.31mg of extract. Column extraction from fractionation yielded 243.39mg from overall 250ml mobile phase (972.12µg/ml). UV-Vis Spectrometric analysis showed the concentration of 217.03µg/ml using Oleanolic acid as standard (1mg/ml). Hence it was determined that about 0.5795% w/w of Triterpenoid saponins can be obtained per gram of crude extract, which was made from the powdered leave samples of *C.napaulensis* DC. Thin Layer Chromatography analysis from elutes of column were performed and the result revealed the presence of triterpenoid saponins in all elutes.

It has to be noted that even in the elutes of column the sample cannot be of absolute purity. Even if the consideration of n-butanol as best solvent media to extract triterpenoid saponins, it has to be noted that it is also a potential solvent to many other metabolites in the trickle down pathway of extraction. Hence UV-Vis spectroscopy has been instrumental in determining the actual triterpenoid saponins using the standard Oleanolic acid.

Based on the results it could be concluded that *Clematis napaulensis* DC. is indeed a plant of medicinal uses. The presence of many individual classes of compounds relevant to ethnomedicine suggests the potential remedy even for other ailments. In its crude form or indigenous usage, the bioactive compounds however were in very dilute concentration due to presence of many primary as well as secondary metabolites. It was indeed tedious but a rewarding effort to concentrate the targeted compounds. Strategic approaches to extract triterpenoid saponins have proven to be fruitful as both spectroscopic as well as extracted weight showed promising presence of the targeted bioactive compounds. The method

developed for the purpose of study can be used in future analysis of similar nature of work. The process of extraction can be adopted for other plants crude extract of interest as well.

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Paper Presentations

1. National Conference of "Stakeholders on Conservation, Cultivation, Resource Development and Sustainable Utilization of Medicinal Plants in North-East India", March 6^{th} – 7^{th} 2019 organized in Department of Botany, Nagaland University Lumami, Nagaland in collaboration with Society of Conservation and Resource Development of Medicinal Plants (SMP), New Delhi.
2. "1^{st} Virtual International Conference on Biotechnology and Bioinformatics", 26^{th} - 27^{th} February 2022, organized by Insight Biosolutions, France in Technical collaboration with Indian Science and Technology Foundation, India.
3. Online International Conference on "Novel Approaches in Life Sciences", 8^{th} – 9^{th} April, 2022 at Guru Nanak Khalsa College of Arts, Science and Commerce, Matunga Mumbai.

Workshop/Trainings Attended

1. Azadi Ka Amrit Mahotsav, Webinar on "Recent Advances in Cancer Research and Treatment: Conventional and Herbal Methods", 27^{th} September 2021, Jointly Organized by Department of Botany, Rajiv Gandhi University, Arunachal Pradesh, Tata Memorial Centre ACTREC, Kharghar, Navi Mumbai and Mahatma Gandhi Institute of Medical Sciences, Sevagram
2. "Two days National Workshop (Online) on Plant Identification and Herbarium Methodology", 29^{th} October and 1^{st} November 2021, Jointly organized by Botanical Survey of India, Central National Herbarium (Howrah) and Bareilly College, Bareilly (UP), NSS Unit-I(Boys)

NATIONAL CONFERENCE
OF
STAKEHOLDERS ON CONSERVATION, CULTIVATION, RESOURCE DEVELOPMENT AND SUSTAINABLE UTILIZATION OF MEDICINAL PLANTS OF NORTH-EASTERN INDIA

March 6 – 7, 2019

Venue: Nagaland University Lumami

Jointly Organized
by
Department of Botany, Nagaland University, Lumami Nagaland
&
Society for Conservation And Resource Development of Medicinal Plants (SMP), New Delhi

Certified that Prof/Dr/Mr/Ms Pangwan M. Konyak
Participated/Presented a paper (Plenary/Invited/Oral/Poster) in the National Conference of 'Stakeholders on Conservation, Cultivation, Resource Development and Sustainable Utilization of Medicinal Plants of North-Eastern India' held on 6 to 7 March 2019 at Nagaland University Lumami, Nagaland.

(Prof. A.K. Bhatnagar)
President
S.M.P. New Delhi

07/03/19
(Prof. S.K. Chaturvedi)
Organizing Secretary & Head
Dept. of Botany, N.U. Lumami

1st Virtual International Conference on Biotechnology and Bioinformatics (ICBB-2022)
On 26th-27th February 2022
Organized by
Insight BioSolutions, France
In technical collaboration with
Indian Science and Technology Foundation, India

CONFERENCE CERTIFICATE

This is to certify that Mr. Pangwan M Konyak has participated in the ICBB 2022 conference and presented an Oral presentation title *"Phytochemical Screening and Estimation of Total Phenolic, Flavonoid and Triterpenoid Content in Clematis napaulensis DC"* in the conference.

Organizer, IBS

President
National Head Quarter
Indian Science and Technology Foundation
C-1/31, Yamuna Vihar, New Delhi- 110053

President, ISTF

SHIROMANI GURDWARA PARBANDHAK COMMITTEE's
Guru Nanak Khalsa College of Arts, Science & Commerce (Autonomous)
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Department of Botany & IQAC Organizes [RUSA Sponsored]
Online International Conference on
"NOVEL APPROACHES IN LIFE SCIENCES"

Certificate

This is to certify that Mr. Pangwan M Konyak from Nagaland University, Lumami- 798627, Zunheboto Nagaland has presented a paper titled Anti-Inflammatory and FRAP Assay of Clematis napaulensis DC. used by Chakesang tribe of Nagaland and participated in the International Conference on "Novel Approaches in Life Sciences" held on 8th and 9th April, 2022 at Guru Nanak Khalsa College of Arts, Science & Commerce, Matunga, Mumbai.

Dr. Gopa Sarma
Convenor, HOD-Botany Department

Dr. Dinesh Kala
Co-ordinator, IQAC

Dr. Kiran Mangaonkar
Principal

CERTIFICATE ID: NALS94202215

TWO DAYS NATIONAL WORKSHOP (ONLINE) ON
PLANT IDENTIFICATION AND HERBARIUM METHODOLOGY

Jointly Oraganized By
Botanical Survey Of India, Central National Herbarium, Howrah
and
Bareilly College, Bareilly (U.P.) NSS Unit-I (Boys)

On 29th October And 01st November 2021

Certificate of Participation

Presented to
Pangwan M Konyak
for his/her active participation in the event.

Dr. R.K. Gupta
Head, CNH
BSI, Howrah

Dr. Rajeev Kumar Yadav
Organizing Secretary
Asstt. Professor (Botany)
P.O. NSS
Bareilly College, Bareilly (U.P.)

Dr. Nisha Verma
Convenor
Asstt. Professor (Botany)
VRAL Govt. Girls Degree
College, Bareilly (U.P.)

Made for free with Certify'em

Azadi ka Amrit Mahotsav

Webinar
on
"Recent Advances in Cancer Research and Treatment : Conventional and Herbal Methods"

September 27th, 2021

This is to certify that

PANGWAN M KONYAK

participated in the webinar
jointly organized by

Department of Botany,
Rajiv Gandhi University,
Arunachal Pradesh

Dr. Hui Tag
Webinar Convener
Professor, Dept. of Botany
RGU, Arunachal Pradesh

Tata Memorial Centre,
ACTREC,
Kharghar, Navi Mumbai

Dr. Ashok Varma
Webinar Convener
Co-ordinator, Bioinformatics Centre, ACTREC

Mahatma Gandhi Institute
of Medical Sciences,
Sevagram

Dr. Satish Kumar
Webinar Convener
Director, Professor & Head,
Dept. of Biochemistry & JBTDRC, MGIMS, Sevagram

List of Publications

1. **P.M. Konyak, Moaakum, Z. Hiese, S. Lakshmana Prabu, K. Ruckmani, V. Hiese, T. Ajungla and H.S. Rathore**. 2022. Persuasive and Strategic Approaches for Preliminary Phytochemical Screening, Quantitative Analysis and Antioxidant Activity of Crude Plant Extracts from Clematis napaulensis DC Indigenous to Nagaland, India. *International Journal of Plant Science Research*. 38(2): 739-751.
2. **P.M. Konyak, Moaakum, Z. Hiese, S. Lakshmana Prabu, K. Ruckmani, V. Hiese, T. Ajungla and H.S. Rathore**. 2024. Efficient Extraction of Triterpenoid Saponins from Leaves of *Clematis napaulensis* DC. – An Indigenous Medicinal Plant used in Treatment of Rheumatoid Arthritis. 15(1): 6-15.