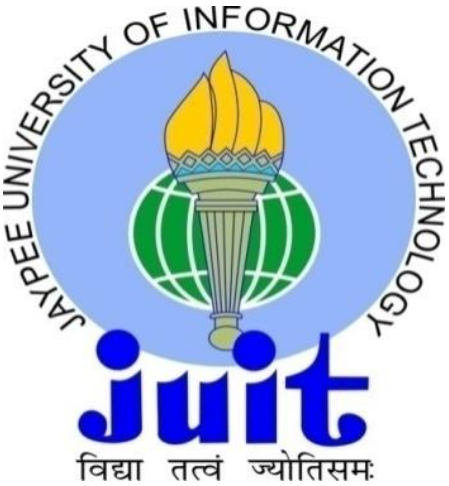
ANTI-ARTHRITIC POTENTIAL OF STANDARDIZED EXTRACT OF *CLERODENDRUM SERRATUM* (L.): A TRADITIONAL MEDICINAL PLANT

Thesis submitted in fulfillment of the requirement for the Degree of

DOCTOR OF PHILOSOPHY

by

RAJ KUMAR TIWARI



Department of Pharmacy

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY

WAKNAGHAT, SOLAN-173234, HIMACHAL PRADESH, INDIA

January 2018

Devoted

• • • *To My Family*

TABLE OF CONTENTS

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DECLARATION BY THE SCHOLAR

I hereby declare that the work reported in the Ph.D. thesis entitled **"Anti-Arthritic Potential of Standardized Extract of *CLERODENDRUM SERRATUM* (L.): A Traditional Medicinal Plant"** submitted at **Jaypee University of Information Technology, Waknaghat, India,** is an authentic record of my work carried out under the supervision of **Dr. Udayabanu M**. I have not submitted this work elsewhere for any other degree or diploma. I am fully responsible for the contents of my Ph.D. thesis.

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SUPERVISOR'S CERTIFICATE

This is to certify that the work reported in the Ph.D. thesis entitled **"Anti-Arthritic Potential of Standardized Extract Of *CLERODENDRUM SERRATUM* (L.): A Traditional Medicinal Plant"** submitted by **Raj Kumar Tiwari** at **Jaypee University of Information Technology, Waknaghat, India,** is a bonafide record of his original work carried out under my supervision. This work has not been submitted elsewhere for any other degree or diploma.

(Signature of Supervisor)

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Acknowledgement

First with my folded handed to almighty God, I would like to express his enclosing presence to point the life and displaying me the direction of life.

I whim, my heartiest indebtedness to my beloved guide Dr.Udayabanu M for his constant encouragement and all the invaluable and timely suggestions and help during the entire course of the project.

I express my deep sense of gratitude, immense respect to Dr. Sayeed Ahmad, Assistant Professor, Dept. of Pharmacognosy and Phytochemistry, Jamia Hamdard, New Delhi for allowing and providing me all the necessary facilities required to conduct experiments during the project.

I sincerely thank Dr. Sudhir Kumar Syal, Head BT, BI & Pharmacy, JUIT for providing me the required facilities and the suggestions for my work.

I am very grateful and thankful to all my doctoral committee members Prof. (Dr.) Ghanshyam Singh, Dr. Jitendraa Vashistt, Dr. Gopal Singh Bisht for their advice and warm inspiration throughout the advancement of my Ph.D. program.

It is a pleasure to acknowledge the helpful instruction, I received from Vice Chancellor Prof. (Dr.) Vinod Kumar, Prof. Samir Dev Gupta, Dean (A&R), Registrar and Dean of students, Maj Gen. Rakesh Bassi, Jaypee University of Information Technology, for their important remarks during the research and providing all the requisite for carrying my research work.

I thank librarian Dr. Suresh Kumar Chauhan along with all library staff of JUIT for their help throughout the study for my research work.

I am thankful to Sonika Gupta, Somlata Sharma, Ismail Siddiqui, Ravikant, Baleshwar and Kamlesh, for their support and helps with the regular function of the lab.

I am thankful to JUIT, Waknaghat, for furnishing the facility, support and opportunity to work overall and also for providing the fiscal abutment. I also praise complete JUIT members who have directly or indirectly helped me in the completion of my dissertation.

I would like to thank Executive director, Dr. Anju Saxena, Sunder Deep Group of Institutions for always encouraging and motivating throughout my work.

It is a pleasure to acknowledge my Director, S.K. Sharma, Sunder Deep Pharmacy College for always allowing and supporting me during my PhD work.

With all my love and affection, I am thankful to god for my beloved parents, especially my father Shri. A. K. Tiwari who is really, the most important person in my life as a teacher, motivator for all problem elucidator and my mother Smt. Rama Tiwari who guided the learning of being patience.

I am blessed with caring elder my brother-Pawan Tiwari and sister in law-Nutan Tiwari along with my maternal father Shri Shiv Kumar Tiwari for their support and love. Lastly, I am thankful to my wife Silpi Chanda for all the help and co-operation during the course of Project work.

Raj Kumar Tiwari

ABSTRACT

Arthritis is a chronic inflammatory disease with restricted joint movement. The pathophysiology of arthritis is not clearly illustrated. An external factor like (e.g., smoking, infection, or trauma) that give rise to an autoimmune reaction, enduring inflammation of joints associated with hidden extra-articular manifestations. The synergy of genetic and environmental factors rises various types of complex immune response, which finally damages joint and bone. Epidemiologic data indicate that globally about 1% of people suffer from arthritis.

Management strategy includes nonsteroidal anti-inflammatory drugs (NSAIDs), use of disease-modifying anti-rheumatic drugs (DMARDs) and corticosteroids (steroids). Above mention existing synthetic drugs have several drawbacks due to extra-articular manifestations or co-morbidities. Major side effect includes gastrointestinal bleeding, cardiovascular problems associated with chronic arthritis conditions.

Natural products play an important role for new pharmaceuticals and chemical entities. WHO guidelines also rigorously commend the standardization of medicinal plants for their safety and efficacy. With this background, current work is invented to standardize the *Clerodendrum serratum* extract and evaluate biological activity as anti-arthritic and for scientific validation of traditional claim as an anti-arthritic agent. The objective was to assess the richness of ethno-medicinal plant species with an appropriate scientific approach, practiced by the different communities for arthritis disorders.

Roots of *Clerodendrum serratum* L. (Verbenaceae) was procured from the local market of Faizabad, Uttar Pradesh, India. The taxonomical recognition was done from Department of Agronomy, Aacharya Narendra Dev Agriculture Technical University, Dist. Faizabad, Uttar Pradesh.

The collected brown colored roots (100gm) were subjected to extraction (decoction) with water (500ml) maintaining temperature around 110˚C for 2hrs. The aqueous extract obtained, was allowed to cool, filtered and lyophilized. The yield of the dried extract was 10%w/w.

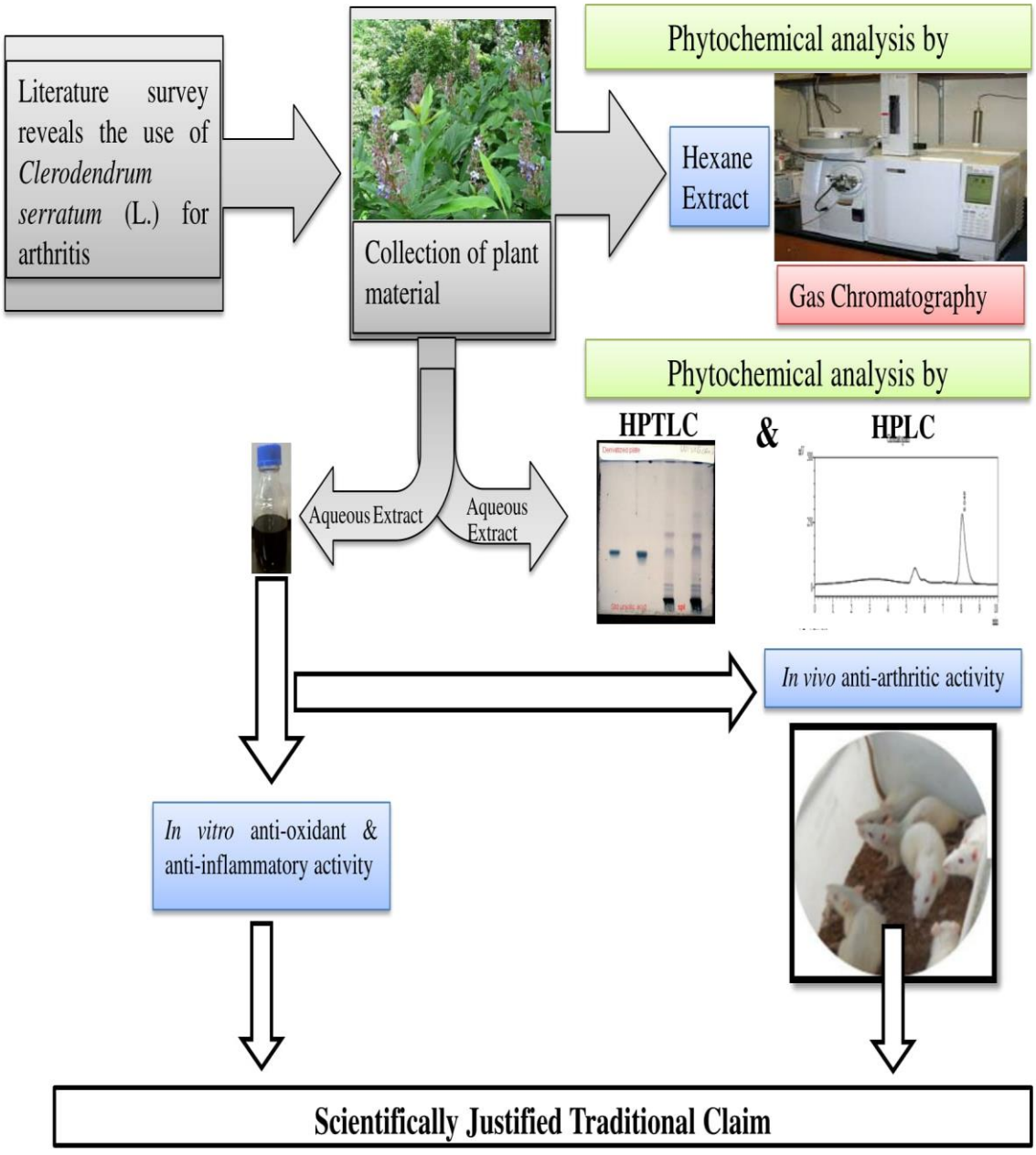
Phytochemical analysis of aqueous root extract marked the existence of secondary metabolites like triterpenoids, volatile oil, sterols, alkaloid and glycoside. Both the HPTLC and HPLC

analysis reveals the presence of ursolic acid. Moreover GC-MS investigation of hexane root extract conceded the presence of 15 phytoconstituents. HPLC method validation was done for plant extract according to ICH guidelines. The validated HPLC method is well-established for ursolic acid. Ursolic acid 0.059% was quantified in the extract was determined from the regression equation and HPLC method was validated for the quantitative assessment of ursolic acid from *Clerodendrum serratum* extract. The method was found to be accurate, precise, within the range according to ICH parameter. Moreover, evalution of *Clerodendrum serratum* for its quality control and adulteration in the raw materials, extract can be done by HPLC.

Antioxidant potential of the plant extract was assessed by DPPH radical scavenging and Superoxide scavenging assay showing IC_{50} values around 85.43 µg/ml and 107.59 µg/ml respectively, while FRAP assay showed significant reducing power activity with increased absorbance.

Further, *In-vitro* anti-inflammatory evaluation of the extract was observed around 8% and 40% inhibition for membrane stabilizing and protein denaturation with 100 µg/ml concentration. The anti- arthritic activity of aqueous extract of *Clerodendrum serratum* exhibited COX-2 and TNF-α inhibition as observed in various parameters like paw edema, arthritic index and joint diameter. Plant extract showed reclamation of arthritis in regard to body weight, arthritic score, paw edema, joint diameter. The extract showed significant results for TNF-α and COX-2 (***P<0.001).

In summary, the current study established the scientific basis of ethnomedicinal use of *Clerodendrum serratum* for arthritis. The study also reveals the medicinal importance of the plant as an anti-inflammatory and free radical scavenger. The plant has potential to be commercialized in the future. The study also documents the use and significance of *Clerodendrum serratum* as ethnomedicinal plant and also suggest a possible alternate solution for arthritis to overcome limitation and drawbacks of current medicines.



Overview of the study

LIST OF ABBREVIATIONS

$AlCl_3$	Aluminium chloride
APC	Antigen presenting cell
BCG	Bromocresol green
COX	Cyclo-oxygenase
CFA	Complete Freund's adjuvant
DPPH	1,1-Diphenyl-picryl-hydrazyl
DMARDs	Disease-modifying antirheumatic drugs
DTH	Delayed-type hypersensitivity
$FeCl_3$	Ferric chloride
FLS	Fibroblast like synoviocytes
GAE	Gallic acid equivalent
GCMS	Gas chromatography and mass spectrometry
HLA-DR	Human Leukocyte Antigen – antigen D Related
HCL	Hydrochloric acid
HPTLC	High performance thin layer chromatography
HPLC	High performance liquid chromatography
H_2O	Water
Hr	Hour
H_2SO_4	Sulfuric acid
NBT	Nitroblue Tetrazolium
IL	Interleukin
ICH	International conference on harmonization
IC_{50}	Half maximal (50%) inhibitory concentration
LOD	Limit of detection
LOQ	Limit of quantification
MeoH	Methanol
M-CSF	Monocyte-colony stimulating factor

$NaNO_2$	Sodium nitrite
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NIST	National institute of standards and technology
FRAP	Ferric reducing antioxidant power
NSAIDs	Nonsteroidal anti-inflammatory drugs
OPG	Osteoprotegerin
O_2	Oxygen
OD	Optical density
OTC	Over the counter
PTH	Parathyroid hormone
p.o	Per oral
R_f	Retention factor
RANKL	Receptor activator of nuclear factor ligand
RSD	Relative standard deviation
SD	Standard deviation
SEM	Standard error mean
TPTZ	Tripyridyl triazine
TLC	Thin layer chromatography
TNF-α	Tumor necrosis factor
TMB	Tetramethylbenzidine
USP	United states of pharmacopoeia
UV	Ultraviolet
WHO	World health organization

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION TO ARTHRITIS

Arthritis is a chronic disorder, mainly causing inflammation, in particular or extended joints, in the human body comprising of more than 360 joints. Most of the arthritic, cases are chronic and continuous, accounts for muscular stiffness. If arthritis left untreated, can further lead to joint torpidity and muscle atrophy. In the pre-arthritic or acute stage, the small joints of the hands and feet are usually affected. Around 100 types of arthritis are reported in all the age groups and various drugs are available for its treatment. Juvenile arthritis, rheumatoid arthritis, and gout are inflammatory in nature, while, osteoarthritis is regressive. Arthritis is a condition of mainly inflamed joints affecting single or in group. Arthritis is associated with disruption of cartilage. The main function of cartilage is to protect and behave as a shield just like the brake shoe of the cycle or motor car for joints, to support smooth movement and locomotion activity. Cartilage has a decisive role in balancing the external shock and pressure on the joint [1-4].

When we step, with a deficient load of cartilage, rubbing of the bones together, results in pain, swelling (inflammation) and rigidity. Almost all ages of people are affected by arthritis but, outset is at intervals of 25-50 with spiked mainly between 40-50 [1, 5].

As we know 100 different forms of arthritis are classified accounting for major and minor arthritic condition. Broadly prevalence is incorporated with osteoarthritis (OA), rheumatoid arthritis (RA), psoriatic arthritis (PsA), fibromyalgia and gout. Response to pain is different in the above mentioned conditioned.

Arthritis and related diseases can cause crippling, chronic pain throughout the life span. Reports tender by Centers for Disease Control and Prevention states that, one-third of the adults, accounting for limiting factor in their office work and daily routine activity. Rheumatoid Arthritis is the one mainly, autoimmune disorder attacked by the body's own immune system may be, from viruses, bacteria and other invaders. In case of Osteoarthritis mostly the cartilage is damaged due to physical and mechanical reasons. RA is commonly an auto-immune disorder

where the lining of the joints called synovium is mainly affected. Over a period of time, the repeated inflammation breaks down the joint and damage it permanently [6].

Psoriatic Arthritis(auto-immune disorder) is condition in which the immune system attacks body itself, inducing inflammation and pain. Mainly affecting connective tissues are tendons or ligaments attach to bones, causing enthesitis and the skin causing psoriasis [7].

Fibromyalgia is considered as central pain syndrome. The different behavior of the brain and spinal cord to process pain signals. Fibromyalgia is characterized by widespread pain across the musculoskeletal structure and intense pain is felt [8-10]**.** Gout is a form of inflammatory arthritis, in which deposition of excess uric acid can form crystals in your joints, resulting in extremely painful joint inflammation [11]**.**

Rheumatoid arthritis (RA) is a long term (chronic), systemic condition damaging joints and tissue. RA still remains a dreadful disease, being capable of producing severe sideline deformities, functional disabilities, cartilage destruction and leads to significant disability. Numerous proinflammatory molecules are released by macrophages mainly free radicals, in the form of reactive oxygen species, eicosanoids like prostaglandins, leukotrienes and cytokines is mainly due to mechanical, genetical and hormonal factors. The mechanism involve in maintaining different types of these mediators stashed by macrophages and other different types of immune cells like T-cells and B- cells and pitching of metabolism involving arachidonic acid mainly by inhibition of enzymes like cyclooxygenase (COX) and lipooxygenase (LOX) are the specific target for various treatments available currently in chronic condition [12].

1.2 NATURAL APPROACH

Natural products have been a major source of therapeutic agents for alleviation or cure human diseases since beginning of civilization and is the core source for development of various drugs in modern era irrespective of its easy availability and complexity of compound reservoir [13]. Most of the FDA approved drugs in last decade for drug development has been from natural product resource in various disease segment like infectious, respiratory, cardiovascular, oncology etc. [14].

The efficacy of some commercialized herbs like Guggul, Ginger, Ashwagandha etc. that has a history of human use since ancient period having anti-inflammatory or anti-arthritic properties due to their scientific approach either pre-clinically and in clinical trials.

Preliminary research has stripped the mechanism by which some plants possess their therapeutic effects. Studies suggest that the extracts or isolated compounds obtained from these herbs are useful for treatment of arthritis in a scientific way with dose dependent treatment. Most of the synthetic treatments are targeting the symptoms, but not the etiology of the disease, whereas natural product has significant impact with less side effects and co-morbidities.

1.3 INTRODUCTION TO PLANT

1.3.1 Phytochemistry

Root part of the plant is mainly traditionally claimed, for various ailments. The phytoconstituents exist in the roots of *Clerodendrum serratum* are mainly carbohydrates, phenolics, flavonoids, and terpenoids. Sachdeva et.al(1965) reported various types of metabolites like primary and secondary in *Clerodendrum serratum* [15]. Zhu et.al(2001) characterize two novel triterpenoid glucoside in *Clerodendrum serratum* [16]. Vasavada et.al(1967) isolated and characterize the presence of γ-Sitosterol from *Clerodendrum serratum* [17]. Garg et.al(1967) isolated and characterize presence of D-mannitol from roots of *Clerodendrum serratum* [18]. Ravi Kumar et.al(2008) isolated and characterize serratin from essential oil of *Clerodendrum serratum* along with lupeol [19]. Ganapty et.al(1997) and Vidya et.al(2007) reported ursolic acid from the stem and root of *Clerodendrum serratum* [20]. Bhujbal et.al(2010) reported presence of Apigenin-7-glucoside from root of *Clerodendrum serratum* whereas Nair et.al,(1976) revealed various types of flavonoids in the leaves of *Clerodendrum serratum* like luteoline 7–0-β-D-glucuronide, Luteolin, scutellarein, Apigenin , 6-hydroxy luteolin, (An uncommon 6-oxygenated flavones) baicalein and 5-hydroxy-7,4-dimethoxy flavones [21]. Banerjee et.al(1969) characterize various terpenoids like Oleanolic acid, Queretaroic acid and Serratagenic acid from bark of *Clerodendrum serratum* [21].Yang et.al(2000) isolated and characterize 7-β-coumaroyl-oxyugandoside and 7-β-cinnamoyl-oxyugandoside from aerial part whereas Wei et.al (2000) isolated Serratoside A and Serratoside B from *Clerodendrum serratum* [21].

1.3.2 Therapeutic Properties and Mechanism of Action

Vazhayil et.al(2017) reported flavonoid content apigenin (API) and luteolin in *Clerodendrum serratum* (LUT) significantly showed neuroprotective action for depressive-like behavioral symptoms in adult mice [22]. Narayanan, et.al(2011) reported anti-inflamatory and antipyretic activity animal model with ethanolic extract of *Clerodendrum serratum* root in hot plate method at dose of 50,100 and 150 mg/kg [23]. Gupta, et.al(1971) reported the presence of saponins having influence on mast cells of rat mesentery in animal model [24]. Gupta et.al(1968) reported antihistamine and anti-allergic activity with long term uses of saponins from *Clerodendrum serratum* [25]. Gupta et.al(1967) successfully evaluated inhibition of *Solanum xanthocarpum* and *Clerodendrum serratum* on histamine release from tissues [25]. Vidya et.al(2005) revealed hepatoprotective activity of *Clerodendrum serratum* 20 mg/kg orally of ethanol extract and 10 mg/kg orally of ursolic acid against carbon tetrachloride induced toxicity in male wistar strain rats [20]. Moreover, further wound healing using 5% w/w ointment gel, from ethanolic extract of leaves and roots part was demonstrated in excision and incision model [21]. Bhangare et.al(2012) reported anti-inflamatory activity of aqueous extract from root of *Clerodendrum serratum* at low dose of (90 mg/kg) and highest dose at (180 mg/kg) in Rats granuloma pouch method [21]. Delayed type hypersensitivity (DTH) reactions along with humoral antibody in mice was reported by Juvekar et.al(2006) for phagocytic and proliferation assays for doses 100 and 200 mg/kg [21].

1.3.3 Traditional / folkoric uses in medicine

Korpenwar et.al(2012) reported uses of *Clerodendrum serratum* root as blood purifier used as decoction in Bhilala tribals of Buldhana district, Maharashtra [21]. Manjula et.al(2013) reported uses of *Clerodendrum serratum* root as rheumatism in Khammam district of Andhra Pradesh with dose of one glass of water twice a day weekly [21]. Padal et.al(2010) summarized *Clerodendrum serratum* root in tribals of Visakhapatnam district of Andhra Pradesh for pain [21]. Devi Prasad and Shyma et.al(2010) stated *Clerodendrum serratum* root in Wayanad district of Kerala state for wound healing in powder form [21].

1.3.4 Ayurvedic formulations of *Clerodendrum serratum* L.

Clerodendrum serratum root are used in Mahavishgarba taila, Mahayogaraja guggulu and Madhusnuhi rasayana commonly for joint diseases, wound healing, stiffness and tightness in all limbs [21].

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CHAPTER 2

REVIEW OF LITERATURE

2.1 REVIEWS ON ARTHRITIS

2.1.1 Epidemiology of Arthritis

The prevalence of rheumatoid arthritis (RA) is comparatively constant in many populations across the globe ranging, at 0.5-1.0%[1]. Epidemiologic data indicates that the development of arthritis disorder and related diseases to the human body will have a significant bounce upon the practice of pharmacy[2-11]. Explanatory style was correlated with an elevated venture of expanding RA conditions and transformed mortality rate in patients with RA[12]. Increased life expectancy had a great impact and burden on knees, leading to increased number of RA patients [13]. The risk of arthritis increases as the age progresses and arthritis is more prevalance in female as compared to males. From 2013- 2015, an estimated 54.4 million US adults (22.7%) annually had ever been told by a doctor that they had some particular type of arthritis. Persons ages 65 or older were diagnosed with arthritis around 49.6% by doctor of the total population [14]. Arthritis cases, accounts for 15% of the total population and around 180 million people in India are suffering from the disorder. This prevalence is larger than other diseases such as diabetes, AIDS and cancer. Rheumatology is a branch of medicine that accord for various types of arthritis and rheumatism. Rheumatism illustrate study of joints pain related to various part of musculoskeletal system. More than 100 types of rheumatological anarchy are classified by the World Health Organization. Among the chronic rheumatic diseases, hip and knee osteoarthritis (OA) are the most prevalent and is a dominant root of pain and disability in most countries worldwide [15].

Rheumatoid arthritis (RA) is a prevailing autoimmune disorder stirring approximately 1% of the worldwide community. The synergistic connection of

eugenic and environmental aspect results in a fountain of immune reactions, which ultimately spark to breakthrough of cartilage, affecting joint and causing structural bone damage. These, will have overall affect both on social and economical part of the society and is challenging for the pain management people in regard to treatment. Extra-articular manifestations and comorbidities are commonly co-orrelated with RA pateients, resulting in more and more mortality rate, so it is important to have effective treatment which can be occupied to prevent established changes permanently in early stage [16].

Various epidemiologic data indicate that the existence of arthritis and its form of related diseases will have a significant crunch upon the pharmacy practice. Osteoarthritis and rheumatoid arthritis are the highest trending diseases. These two diseases currently affect millions of Americans and also peoples across the world widely. Data is presented that facilitate awareness of the need to develop ways to economically provide pharmaceutical care with the expanding epidemic of rheumatic diseases [17]. Internationally, the prevalence of arthritis is believed to be 1% of global population. Centers for Disease Control (CDC) mentioned that adults suffer from arthritis around 67 million by 2030 in USA. An estimated 35 million adults (almost 44% arthritis patient) will report arthritis-traceable movement limitations by the year 2040. The arthritis prevalence rate is around 15% people affecting 145 million of the Indian sub-continent. Moreover prevalence rate is much higher than diseases such as diabetes, AIDS and cancer [18].

2.1.2 Pathophysiology

Several clinical features are consistent with nervous system involvement in the pathogenesis of rheumatoid arthritis [19]. Rheumatologists practitioner in clinical practice has increased in last 15 years, though the statistic of patients with (RA) has increased in different region of the world.

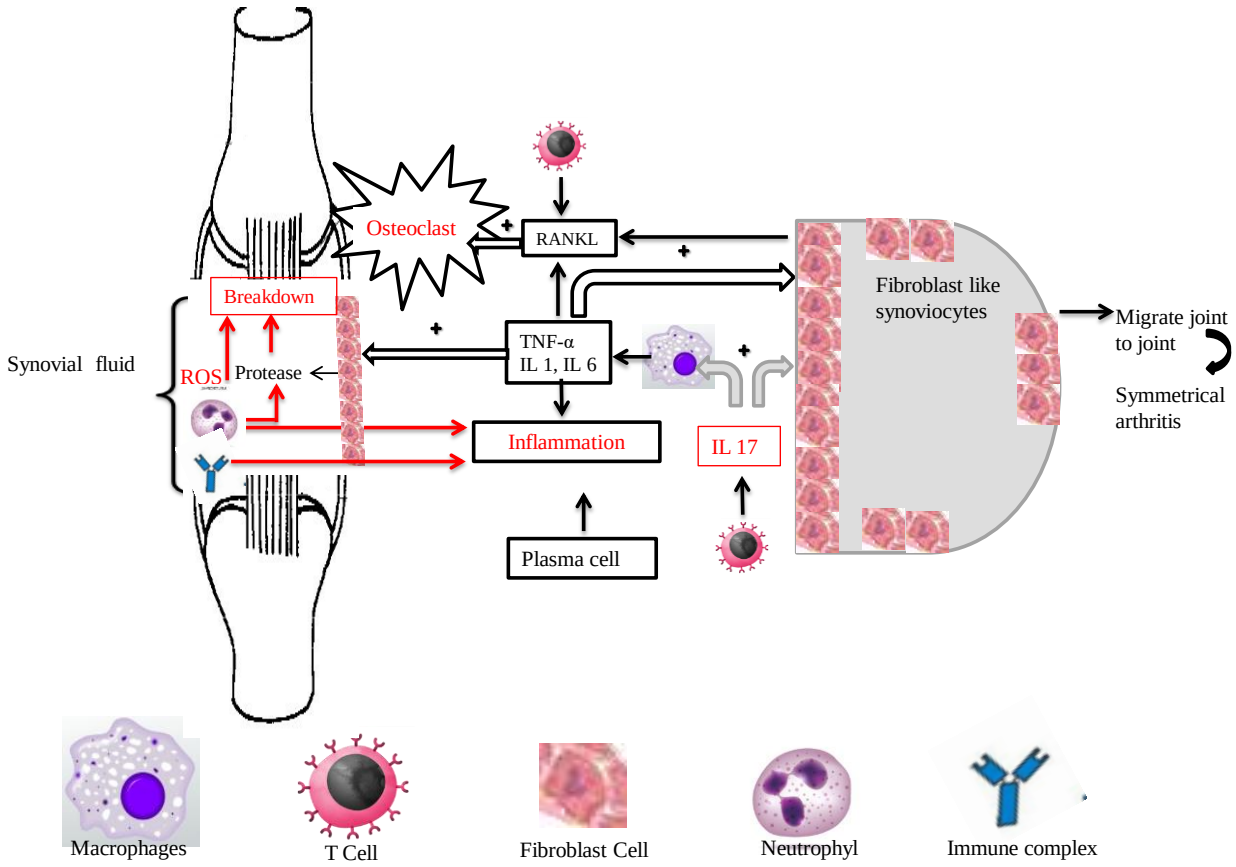


Figure 2.1: Pathogenesis of arthritis

The poor projection for patients is observed with this disease due to a lack of shortcoming therapies. Although the specific cause is not known till now, a variety of reports reveal that RA results from the appearance of a relevant antigen to an immunogenetically susceptible host forming a immune complex and targeting its own tissue. Study highlight vastly understanding the biology of arthritic disorder especially its pathophysiology predicting that the host antigen interaction are mainly cause at cellular level forming a immune complex [20]. Role of endothelial cells is very vital in the pathogenesis of rheumatoid arthritis (RA) [21]. Moreover both, TNF-α along with IL-17 are supreme or uppermost inflammatory cytokines convoluted in the etiology of RA. The contribution of TNF-α for arthritic condition may be due to an imbalance in OPG expression

and osteoclastogenesis resulting in a systemic increase of osteoclast precursor and their movement in between joints, causing cartilage damage and destruction of bone [22].

As we know RA is an autoimmune systemic endemic of joints that principally affects joints. Various types of cells are involved like macrophages, T and B cells, fibroblasts, chondrocytes and dendritic cells in the etiology of the disease. Despite lots of research, provide information that many genes and epigenetic modifications in the development and evolution of the disease, in most RA patients there is no clear preliminary factors present. Environmental factors involved in RA pathogenesis are smoking, industrial outcomes like silica crystals, GIT disturbances, lung, and oral microflora and some specific bacterial and viral infectious agents and their components. At the early stage of disease qualitative and quantitative imbalance of peptide citrullination as well as other protein modifications, followed by antigen presenting cell (APC) (macrophages and dendritic cells) and fibroblast like synoviocytes (FLS) activation is seen. It has also seen in studies that some of the microbes stimulate this activity by APC and FLS direct and indirect activation. In the next stage APC's elicit specific humoral B cell response resulting in specific antibodies production and T cell autoreactivity. Acquired and transferred defects in T and B cell responses from one generation to successive generation caused by the repeated renewal of innate immunity as well as loss of resistance, bring out chronic autoimmune inflammation, primarily of synovial membranes, and development of cellular panus. Pathologic stimulation of the osteoclasts affects liberation of the immune system effector molecules, proteolytic enzyme destruction of the cartilage, bone and tendons composition and structure. Continual inflammation through its complex mechanisms results in many systemic and extra-articular RA manifestations of almost all vital organ systems, resulting in severe obstacle and comorbidities such as rheumatoid lung, carditis, vasculitis, cahexia, anemia, accelerated atherosclerosis, myocardial and cerebrovascular disease, lymphoma, osteoporosis, depression in the human body etc. Accumulated complications and

comorbidities finally result in handicap, social dysfunction and premature death [23].

Osteoporosis is one of the common complexity observed in rheumatoid arthritis RA surrounding. Threatening part is abrupt loss of bone in RA patients, which is a serious concern especially in females. The RA pathogenesis is crucial may for better accepting the reason of bone loss. The contrivance behind the bone loss is not well elucidated in RA, despite cytokines like interleukin-1 and tumor necrosis factor alpha (TNF-α) have been firmly questioned. In recent years drugs for TNF-α had been targeted, which have revolutionized the cure of RA. Various reports, illustrate link between antagonists for TNF-α and abolishment of bone resorption [24].

RA is common, but composite, disorder and RA, physician face a multiple grid of cytokines. Exact trigger of the disease is yet to be concluded and specialists prescribing numerous, modern anti-rheumatic medications are having their own choices of treatment [25].

Despite various targets, medicaments counter TNF and IL-6, apart from this inhibition and reduction of T-cell and B-cell, both have analogous effects, causing rheumatoid arthritis. All these assessment hold-up queries whether all the potential targets of the possible treatment might target for all the pathogenic targets. On the other hand, cytokinins mentioned above and its inhibition has a analogous effect in damaging joints, whether in initial or later stage of disease. Further, it was also observed that reduction in the amount of B-cell and T-cell has a long term effect on joint conditions, which concede that these accession involve obstinate pathogenetic changes. Various studies have been reported regarding imbalance in the immune system and the current expectation is to investigate, even if clinical trials are appropriate way to access the definite target for obstructing pathogenetic complexes in RA [26].

Rheumatoid arthritis are governed by various inclusive pre-arthritic factors regulated by Cytokines. In rheumatoid joints, most of the reports highlight that

broadly imbalance between pro- and anti-inflammatory cytokine activities, leads to immune complex reactions resulting in auto-immune disorder damaging joint. Cytokines are classified within a laminated governing grid,however its not cleared till now, which is the exact channel to be targeted and is a matter of clinical intervention at priority [27].

Receptor activator of nuclear factor ligand (RANKL) is protein, mainly responsible for the osteoclasts mechanism for bone development, belonging to the class of TNF cytokines family. The Major function of RANKL is its involvement in osteoclastogenesis, activation, and endurance of osteoclasts. The attachment between monocyte-colony stimulating factor (M-CSF) and RANKL mainly lead to differentiation of macrophage/monocyte-like cells into osteoclasts by their respective plasma-membrane receptors, and RANK present. Osteoprotegerin (OPG), a soluble protein from the class of TNF receptor is synthesized chiefly by osteoblasts, is a promoter receptor that gets attached to RANKL. By this approach OPG match with RANK leading to inhibition of osteoclastogenesis and bone absorption. Moreover, it is reported that the RANKL/OPG ratio balances mainly bone resorption and synthesis for growth and development maintaining osteoclast-osteoblast mechanism. Production of RANKL is mainly done by osteoblasts and bone marrow stromal cells during normal conditions. During arthritic conditions or pathological conditions such as rheumatoid arthritis (RA)RANKL is also produced by various types of immune cells like T and B lymphocytes, and other cells of body as monocytes, fibroblasts, synoviocytes etc. Apart from M-CSF and RANKL causing osteolysis in RA, other class of cytokines like (TNF, IL-1beta, IL-6, IL-7, IL-11, IL-15, IL-17, IL-18), hormones (PTH, PTH-rP, corticosteroids), and prostaglandin E2 also causes osteolysis. On the other sides, OPG, interferon gamma, IL-4, TGF-beta, bifosfonians and estrogens constrict RA-related osteoclastogenesis. Recently, therapeutics targeting RANKL pathways have been used for the regimen of postmenopausal osteoporosis [28].

Treatment with (DMARDs) for rheumatic disorder is arduous and challenging due to its extra-articular manifestations. Currently advancement in anti-cytokine therapies targeting TNF-α, IL-1, IL-6, and IL-15, for combatting new responses, has led therapies as biological. The clinical trials highlight of arresting TNF along with, IL-1 to some degree, has been established in DMARD-resistant RA patients. Recently, clinical trials revealed inhibition of IL-6 and IL-15 expressing efficacy equivalent to TNF blockers. Combinations of these targets with drugs like methotrexate showed fruitful results. Inspite of various clinical trials for anti-cytokine treatment, these drugs decreases level of disorder, in combination and increases quality of life in RA patients [29].

The pathogenesis of RA have not been understood in extensive pattern. Various types of cells involved in pathogenesis are characterized as: monocyte and macrophages, T and B cells, synoviocytes and endothelial cells. Various types of functions include cell bonding and movement, activation of T and B cell, release of cytokine and destruction of joints. Involvement of molecules that contend in various types of complex interactions have, broadly, been cloned and sequenced including the function of their products conclusively. However, despite the knowledge gathered in regard to pathogenesis of arthritis is limited and today also we still do not perceive fully the pathogenesis of the disease. The problem is complex and we can understand the fact that the synovitis is taking place within a patient comprising of various types of reactions competing with immune systems. Further the neuroendocrine system is apparently an important factor because of its capacity to deregulate inflammation through the secretion of cortisol. The study also revealed that hypothalamic-pituitary-adrenal axis in patients with RA has shown that it reaction indisposed to inflammatory stimuli. This drawback is one of the important factor in concluding the severity of joint inflammation, but keeping an important early episode in the transformation from acute to joint inflammation. These findings fortify that high quality clinical research is still required for concluding complex biological processes such as RA [30].

Genetic, environmental, and hormonal factors and their interactions are considered to play an important role on disease development[31]. There has been significant correlation between inborn resistance and immunological biomarkers, considered to be corresponding to sex hormones and supplementary neuroendocrine aspect [32-36]. Numerous reasons for the disease is composed of factors like genetic sensitivity, gender and age, smoking, infectious agents, hormonal, diet, socioeconomic, and ethnic factors throughout the globe. Most of these factors are prone to be linked with both disease occurrence and acerbity [37]. Dominant trigger for RA patients is TNF-α an leading cytokine in the musculoskeletal system [38]. Proinflammatory cytokines, IL-1alpha/beta and TNF-alpha trigger the intracellular molecular signalling pathway responsible for the pathogenesis of RA [39]. Porphyromonas gingivalis can also raise autoimmune conditions resulting in arthritic condition [40]. Mostly Smoking and age were correlated and both these factors has been observed for prevalent and inflammatory joint signs for RA patients [41]. Immunological and genetic studies are causes of RA [42]. Both smoking and Porphyromonas gingivalis are reasons for RA [43]. Production of Inflammatory cytokines in the myocardium can account for congestive heart failure in synovium [44]. Smoking >5 pack-years after RA diagnosis significantly increased mortality beyond the risk of non-RA comparators [45]. Smoking is a vigorous able dangerous thing for radiographic advancement in early RA [46]. Moreover smoking is an active forecaster in pre-rheumatoid arthritis patients when treated with combination therapy of methotrexate and sulfasalazine [47-48]. Moreover, it is revealed that smoking and HLA-DR shared epitope (SE) type of genes which may interact in stimulating immune reactions forming immune complexes to citrulline-modified proteins [49]. Stress initiation can also lead to affliction through endocrine and nervous system in initiation of this diseases [50]. Psychological stress in relation through hormones and nerve tissue may heighten rheumatoid arthritis [51]. Plasma levels get elevated for leptin and visfatin was big and marked up with patients of rheumatoid arthritis [52].

2.1.3 Treatment

NSAIDs are repeatedly prescribed to patients with rheumatoid arthritis (RA), as the early figure of regimen as drugs, and are directed over a long period of time. NSAIDs are classified principally into acidic and basic qualification, and the former is more universally used for the treatment, since the terminal possesses no anti-rheumatic effect. Moreover, the acidic NSAIDs are restricted into salicylates, arylacetic acid, pyrazolone, fenamates and oxicams. Arylacetic acid has a strong analgesic effect. Pyrazolone is well stabilized between analgesic, anti-inflammatory and antifebrile effects. The fenamates and oxicams are long acting form of drugs. [53].

Cardiovascular risk are associated with prescribed drugs for RA patients. corticosteroids, NSAIDs and COX-2 inhibitors could influence cardiovascular risk negatively, though confined report is available for cardiovascular risk about different drugs in RA patients[54].

Currently about 30 million people throughout the world use prescription related to (NSAIDs) everyday. Dominant drawbacks of NSAIDs administration to patients is gastrointestinal (GI) toxicity which is revealed as known clinical complication, accounting for around 25% of all characterize side effects correlated with NSAIDs. Over-the-counter (OTC) formulations are the major set back along with prescription of NSAIDs throughout the world. Various clinical reports in centers of North America clearly indicate GI risks with the usage of over the counter (OTC) NSAIDs. Uprightly GI cases lead to bleeding in stomach, forcing ulceration and even hospitalization of the patient. COX was found to be a major threat in various demographic region. Paracetamol is not associated with GI problem and can be prescribe for treatment as first line targets [55]. (NSAIDs) might cause hepatic damage, affecting liver associated with serious events and death of patients [56].

Currently new pharmacological treatment options for rheumatoid arthritis (RA) are available, though major limitations of (NSAIDs) are its GI and renal toxicity along with hepatic damage.[57-58].

2.1.4 Challenges to treatments

NSAIDs are effective in treatment of nociceptive arthritis. However, safety concerns of NSAIDs is a major challenge for all the clinicians [59]. Currently aspirin, nonsteroidal antiinflammatory drugs (NSAIDs) are the first choice of treatment along with cyclooxygenase-2 (COX-2) inhibitors for RA patients [60]. On the other hand, NSAIDs sometimes constrain adverse reactions, such as gastroduodenal diseases and renal insufficiency, which are probably convinced by the inhibition of cyclo-oxygenase to reduce prostaglandin content. Therefore, appropriate NSAIDs must be tabbed after consideration on the character, property and adverse reaction of each drug [53]. NSAIDs in rheumatoid arthritis should be limited due to side effects like hepatic and renal failure [61]. Anti-TNF-α treatment has lead to decline of body mass in RA patients [62]. Prednisone use was related with a significantly expanded danger of mortality in patients with RA [63]. 7-coumarine hybrid with ibuprofen showed potent and persistent anti-hyperalgesic effect up to 60 min after administration [64]. Tofacitinib was further discontinued due to various adverse events, as compared to biological DMARDs [65].

NSAIDs are one of the ultimate adopted treatment in the world. Most of the NSAIDs available have severe side effects. With increased awareness about its side effects, safety has become a priority in treatment of arthritis and other inflammatory diseases with NSAIDs [66]. NSAIDs effects describe various types of adverse effect on the body viz. hip fracture, acute interstitial nephritis, keen renal failure, osteoporosis, hepatitis, along with visual disturbance [67-68]. Traditional drugs generally had fewer side effects, comparative to NSAIDs should be better alternative for the therapy of arthritis condition. Traditionally,

medicinal plants of various families are used in the treatment of arthritis. They exhibit individual and multiple mechanism of action like antioxidant, anti-inflammatory, protective action, inhibition of cytokine production, TNF-α etc. in the management of arthritis. Table 2.1 listed the names of the plant showing anti-arthritic activity with their mechanism. There are several reports for the drug/chemicals derived from natural product possess anti arthritic activity [49-72].

2.2 REVIEWS ON NATURAL APPROACHES

Table 2.1: Plants used In Arthritis Disease

Plant name	Plant part / Formulation	Extract/Isolated component	Mechanism involved	References
Campomanesia lineatifolia	Leaves	Dichloromethan, ethyl acetate, and water	anti-TNF-α	[69]
Stryphnodendron adstringens	Leaves	Dichloromethan, ethyl acetate, and water	anti-TNF-α	[69]
Terminalia glabrescens	Bark	Dichloromethan, ethyl acetate, and water	anti-TNF-α	[69]
Morus mesozygia	Leaves	Acetone	Protein denaturation assay	[70]
Heteromorpha arborescens	Leaves	Acetone	Protein denaturation assay	[70]
Terminalia lanceolata	Fruit	Methanolic	Inhibition of cytokine production	[71]
Terminalia pruinoides	Leaves	Aqueous	Anti-Proteus activity	[72]
Terminalia. Sericea	Leaves	Aqueous	Anti-Proteus activity	[72]

Oroxylum indicum	stembark	Aqueous	Reduced myeloperoxide release	[73]
Derris scandens	stem	Aqueous	Reduced myeloperoxide release	[73]
Claoxylon indicum	Root	Ethanolic	TNF-alpha	[74]
Pterocephalus hookeri	Whole plant	Ethanolic	Inhibiting the expression of NF-κB p65 as well its antioxidant activity	[75]
Vatari Guggulu	seed	Alcoholic	TNF- α	[76]
Dashanga Ghana	Ayurvedic Formulation	-	Significant anti-arthritic activity, Mechanism not reported	[77]
Alchornea cordifolia	Leaf	Hydroethanolic	Antioxidant mechanism	[78]
Vitellaria paradoxa	Stem bark	Methanol	Inhibition of production of release of TNF-α	[79]
Xanthium strumarium	Fruit	Aqueous	Decreased level of COX-2 and 5-LOX	[80]
Oroxylum indicum	Root bark	Chloroform, ethyl acetate and n-butanol extracts	Increased catalase, glutathione content	[81]
Tridax procumbens	Whole plant	Ethanol	Increased RBCs and Hb content	[82]
Fagopyrum cymosum	Rhizomes	Ethanol	Decreased TNF-α production	[83]
Cardiospermum halicacabum	Leaf	Ethanol	Free radical scavenging	[84]
Vitex negundo	Leaf	Agnuside	Inhibition of (Th1/Th2) cytokines along with T-	[85]

			cell mediated inflammatory suppression	
Terminalia chebula	Fruit	Hydroalcoholic	Reduced serum TNF- α level	[86]
Premna serratifolia	Wood	Ethanol	Not reported	[87]
Sophora flavescens	Root	Alkaloid-free prenylated flavonoid-enriched fraction	Inhibited cyclooxygenase-2	[88]
Barringtonia racemosa	Fruits	Bartogenic Acid	Decreased in ESR and increased Hb level	[89]
Sida rhombifolia	Aerial parts	Ethanol and aqueous extract	Not Reported	[90]
Euphorbia tirucalli	Not known	Biopolymeric fraction	Abolishment of CD4(+) and CD8(+) T cells along with inhibition of Interleukin-2 (IL-2) and Interferon-γ inhibited vascular permeability	[91]
Cleome gynandra	Leaves	Methanol	Not reported	[92]

2.2.1 *CLERODENDRUM SERRATUM* (L.)

Clerodendrum serratum (L.) belonging to the family Verbenaceae, commonly known as Bharangi is a popular indigenous plant in India. The Plant is widely distributed across the globe ranging through various continents from Asia to Africa.

Figure 2.2: *Clerodendrum serratum* whole plant

Figure 2.3: *Clerodendrum serratum* root

2.2.2.1 Synonyms

Rotheca serrata (L.) Steane & Mabb

2.2.2.2 Plant Part used

Root

2.2.2.3 Ayurvedic Properties and Action:

Rasa : Katu, Tikta, Kasaya

Guna : Laghu, Ruksa

Virya : Usna

Vipaka : Katu

Karma : Dipana, Kaphahara, pacana, Rucya, Vatahara, Swasahara

2.2.2.4 Vernacular Names [93]

Taxonomical identification

Kingdom: Plantae

Phylum: Tracheophyta

Sub-phylum: Euphyllophytina

Division: Angiospermae

Class: Magnoliopsida

Subclass: Lamiidae

Order: Lamiales

Family: Lamiaceae/ Verbenaceae

Genus: Clerodendrum

Species: serratum

2.2.2.5 Macroscopical and Microscopical Character

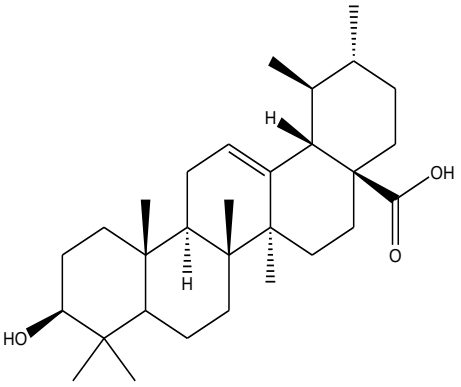
Macroscopy: Fully mature root is hard, woody in appearance, cylindrical in shape, 5 cm in thickness, with appearance of lenticels at the external surface with light brown color; bark appears very fragile and readily distinct from a broad wood showing striking medullary rays. Parallel growth rings are commonly observed in transversely cut section; having short fracture and acrid taste [94].

Microscopic: Riped root was found to have stratified cork holding numerous 14-20 layers of thin-walled, tangentially separated strechted cells; each stretched cells comprises of around 3-5 layers of cells appearing; secondary cortex wide, exterior 2 or 3 layers radially formed and tangentially extended, whereas innermost part of the cells are polyhedral or circular to egg-shaped with intercellular spaces; some of these cells get converted into stone cells with highly thickened wall having aligned striations and expanding channels with narrow lumen; some of the cells are fused with acicular crystals of calcium oxalate and a very less quantity of brown colouring matter; secondary phloem lasting to sieve elements and parenchyma largely fall in outer region [94].

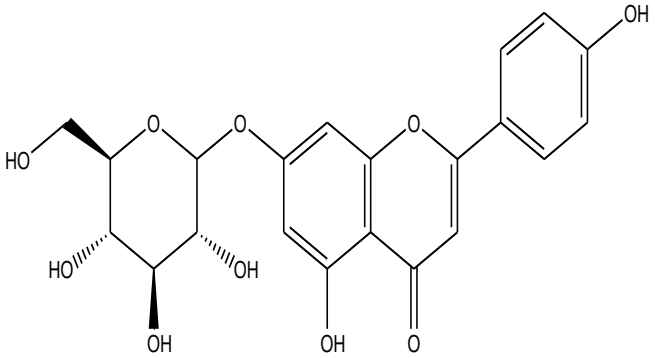
2.2.2.6 Chemical constituents

Roots has been found to report various chemical constituents as terpenoids like Ursolic acid, sterols like γ sitosterol, flavonoids like Apigenin-7-glucoside and carbohydrate like D- mannitol.

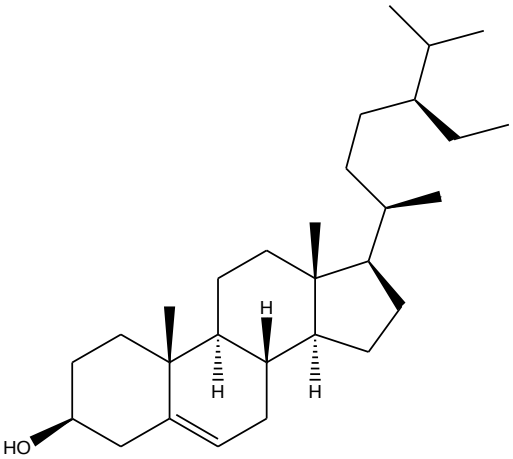
1. Ursolic acid
2. Apigenin-7-glucoside
3. γ sitosterol
4. d-mannitol



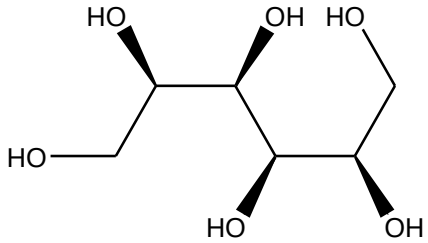
ursolic acid



apigenin-7-glucoside



(3b,24S)-Stigmast-5-en-3-ol



d-mannitol

Figure 2.5: (a) Chemical Structure of Ursolic acid, (b) Chemical Structure of apigenin-7-glucoside(c) Chemical structure of γ sitosterol (d) d-mannitol

2.2.2.7 Pharmacological activities

Anti-inflammatory, antispasmodic, anticancer, anthelmintic, hepatoprotective.

2.2.2.8 Formulations and preparations

Ayaskrti, Kanakasava, Dasamularitra, Rasnadi, Kvatha churna

2.2..2.9 Safety aspects

Considered as safe due to its traditional use.

2.3 STANDARDIZATION AND VALIDATION

Medicinal plants play a major origin of remedial steward for the alleviation of human diseases since our ancestor times. India is considered as "Botanical Garden of the world." Indian flora and fauna supplemented with large varieties of medicinal plants approximately exceeding 2200 different types of species. Due to the increase in the interest of use of medicinal plants throughout the world, there is a manifold increase of medicinal plants based industries, which are growing at a rate of 7-15% annually. Though, India being the largest number of medicinal and aromatic plant, the contribution in herbal Industry is less than 12%. In India the traditional

knowledge and claims from various ailments about medicinal plants were disappearing due to continuous intrusion and various civilized adaptations over a period of time.

2.3.1 Characteristics that are restricting the development of herbal medicine in India

- Lack of standardization
- Lack of validation of standardized method
- Deficient of market contacts
- Marketing is inefficient, imperfect, selective and opportunistic

Due to these reasons, the number of plants in the official monograph has gone down, either in crude drugs or plant derived form as observed in Indian Pharmacopoeia. There is a practice of using plants and their products in an assorted present day and traditional systems of medicine, without any documented form or regulations. To meet the demand of international market it is mandatory to standardize the product or method and validate written document form. Now days R&D thrust in the pharmaceutical sector focuses on the standardization and validation of the developed standardize technique.

2.3.2 Standardization

Standardization is a system that ensures a predefined amount of quality, quantity and therapeutic effect of the ingredients present in each dose [95]. It is an important aspect of maintaining and assessing the quality, purity and safety of the herbal product/extract/raw material/ formulation to attain the desired therapeutic effect [96]. A standardized herbal extract means a measurable marker substance or substances present, which is extracted from the herb. The markers present in an extract may be active or inactive.The first Indian National Health Policy 1983 claims that India is the richest source of herbs and the drugs should be standardized [97].

2.3.3 Need of Standardization

The resurgence of interest in natural products and various phytochemicals isolated from plants in the last decade, mainly because of the its demographic distribution and acceptance due to its traditional claim having less side effects than synthetic one. This lead to the rapid spurt of demand for health products of traditional medicine, which leads to the market of medicinal

plants for commercialization. To fulfill this need there is a practice of indiscriminate and unscientific collection practices, without any consideration for the quality control of the material. This decreases the therapeutic efficacy and quality of the final product. Hence standardization plays a decisive semblance in the quality assurance of herbal drugs and their products. Raw plant material contains various chemical constituents, thus standardizing the herbal drug require more than one analytical technique [98].

2.3.4 Validation

Validation is an apprehension well documented and accepted, since its first draft in the year 1978 in between various countries, for regulation of various procedures in the area of pharmaceuticals [99]. The analytical procedure validation confirms that the procedure adopted is relevant for the intended and drafted used in variable conditions to maintain high degree of quality and efficacy. Moreover, analytical procedure validation requires to establish scientifically that the risk of errors in different analytical steps is acceptably small [100]. Validation is a basic and essential specification to ensure quality control, quality assurance and maintaining total quality management(TQM) of the industrial applications. The objective of validation procedure is to determine the analytical procedure is suitable for its planned purpose, determine by medium of well-reported documents supported by experimental studies [101].

2.3.5 Reasons for validation

There are two chief paramount reasons for validating assays in the pharmaceutical industry. The first, and by to the most important, is that assay validation is an integral part of the quality-control system. The second is that current good manufacturing practice regulation requires assay validation [102]. In industry, it would be difficult to confirm that the product being manufactured is uniform and that meet the standards set to assure fitness for use. The varying nature of the differences between the analytical development laboratory and quality control laboratory is a good reason for the validation program in pharmaceutical analysis.

2.3.6 Benefits of method validation [95]

- An absolutely validated process may have need for less in-process control and end–product testing.

- It expands the understanding of processes, decrease the risks of processing problems, and thus assure the smooth functioning of the process**.**

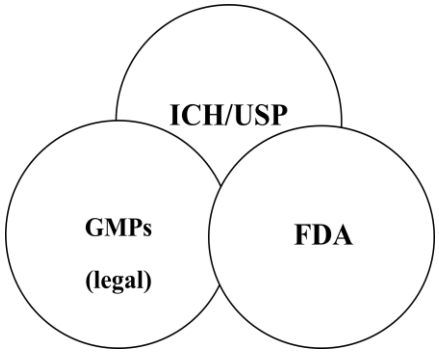
2.3.7 Steps in method validation

- Development of validation protocol, or operation procedure for the validation.
- Programming of application, aspiration and extension of the method.
- Achievement parameter and acceptance criteria have to be reported.
- Validation experiments have to be defined.
- Performance and characteristic of equipment have to be verified.
- Quality materials should be controlled (for e.g. standard and reagents).
- Pre-validation experiments have to be performed.
- Adjustment of method parameter or/and acceptance criteria is necessary.
- Internal and external validation of equipments should be performed.
- Development of SOP for purging the method during analysis.
- Re-validation criteria have to be defined.
- System suitability test and analytical quality control (AQC) have to be recorded.
- Documentation of validation operation and outcome of the result in the validation report.

2.3.8 Today's validation requirements

The objectives of ICH as laid down in their terms of attributing in their early years were:

- To provide a platform for constructive dialog between regulatory authorities and the pharma company by acting as a bridge on the actual and observed differences in the technical claim for product registration in the countries like the Europe, United States, and Japan.



- Identification of various areas in which adjustment in technical requirements or greater mutual acceptance of R&D procedure could lead to a more cost-effective use of animal and human being without composing safety.
- To make suggestion on practical guidance to accomplish greater harmonization in the analysis and operation of technical guidelines and requirements for registration [101].

There are two guidelines of validation issued by the US (FDA), one is accessing applicant and the other is focused on inspection and reviewing the data. The major objective of first guidelines is that confirmation of experiments, that requires a disclosed depiction of finished product whether raw material or reference standard, along with potential impurities and defined procedure subjected to analysis. The second guidelines targets RP-chromatography and had an eyesight on details in regard to implication of results for the procedures adopted[101].

There are specific guidelines given by ICH, FDA, and USP. There are different validation characteristics normally classified in the distant types of test procedure: -

- Specificity
- Linearity
- Range
- Accuracy
- Precision (Repeatability, Intermediate precision, Reproducibility)
- Limit of detection
- Limit of quantification

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CHAPTER 3

RATIONALE OF THE STUDY AND OBJECTIVES

3.1 Rationale of the study

Arthritis has been a major challenge in regard to its treatment as the life expectancy of the people has increased with advances in medical sciences, leading to increased burden on joints. Various causes of arthritis include immune response, mechanical, genetical, ageing etc. Choice of drugs in modern days are mainly pain killers like non-steroidal anti-inflammatory drugs (NSAIDs), disease-modifying anti-rheumatic drugs (DMARDs) and corticosteroids (steroids) for the treatment of arthritis [1-3]. Opioids, non-opioids, NSAIDs and corticosteroids are commonly recommended for pain management. All these medications are associated with possible side effects [4]. Various studies are reported for gastrointestinal bleeding, with the short term adoption of (NSAIDs) like aspirin, ketorolac, diclofenac, and nimesulide. Moreover, literature also reveals that piroxicam in short-term and chronic conditions was found to marked up the prospect of bleeding [5-6]. Opioids are the frequently used administered drugs during post-surgical pain [7]. Currently, a much more combative approach is recommended for people with arthritis, with prescription of non-biologic DMARDs in early stage to prevent joint deformity [8-10].

Physicians frequently prescribe anti-inflammatory, analgesic, and anti-pyretics for the treatment of all rheumatic conditions [11-12]. GIT pain and disturbance, heartburn, nausea, diarrhea, vomiting, cardiovascular problems are major adverse effect in case of chronic arthritis [4, 13-16]. Existing synthetic drugs have several limitations due to extra-articular manifestations or co-morbidities. The modern medicine confesses that ayurveda and herbal medicine, has a lot of

positive influence on the treatment of arthritis. Herbal drugs either in crude form or isolated component have been evaluated for arthritis [17-19].

Phytopharmaceutical Approaches

Plants contain various types of secondary metabolites like phenols, coumarins, essential oils, monoterpenes, catechins, quinones, carotenoids, flavonoids, alkaloids, anthocyanins and xanthenes which are useful for various therapeutic use [20-21]. Herbal drugs are commonly used in the treatment of arthritis like Guggul, ginger, Ashwagandha etc.,. Basic scientific research has uncovered the mechanism by which some plants afford their therapeutic effects. Various studies report that most of the herbs with the systemic and scientific approach play an important role in the treatment of joint disorder with safety aspects.It has been observed that from North to South and east to west part of India, different community of people uses *Clerodendrum serratum* in different ailment.

Natural products have been an important basis for the development of new pharmaceuticals and has a reservoir of lead structure. WHO guidelines strictly advise the standardization of medicinal plants for their safety and efficacy. The first Indian National Health Policy 1983 claims that India is the richest source of herbs and the drugs should be standardized. Standardization is a system that ensures a predefined amount of quality, quantity and therapeutic effect of the ingredients present in each dose. We aim to standardize and evaluate *Clerodendrum serratum* not only having a unique benefit to medicinal property, but also evaluation and analysis of plant, therefore, offer a new platform for the development of herbal drug and can serve as a key role in the safety and cost effective especially in the area of arthritis management.

3.2 Objectives

The current study has the following objectives:-

3.2.1 Phytochemical analysis and standardization of *Clerodendrum serratum* extract

3.2.1.1 Qualitative phytochemical analysis

3.2.1.2 Quantitative analysis of phytochemicals by spectrophotometer

3.2.1.3 Qualitative HPTLC study of Ursolic acid

3.2.1.4 Phytochemical analysis of volatile principles

3.2.2 HPLC method validation of plant extract, according to ICH Guidelines of extract

3.2.3 Evaluation of Anti-arthritic activity

3.2.3.1 Antioxidant activity (DPPH, Superoxide scavenging assay and Ferric reducing antioxidant power (FRAP) assay.

3.2.3.2 *In-vitro* Anti inflammatory Activity: Hypotonic solution –Induced haemolysis (membrane stabilizing activity) and Effect of Protein Denaturation.

3.2.3.3 *In- vivo* anti arthritic study

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CHAPTER 4

MATERIALS AND METHODS

4.1 Selection and Collection of plant materials

Depending upon the Literature survey report and traditional claim in various communities, the plant *Clerodendrum serratum* L. was chosen for current work. Plant materials (root parts) were collected from the surrounding market of Faizabad, where the people come to commercialize their raw material for financial benefit.

4.2 Collection and authentication of plant material

Roots of *Clerodendrum serratum* L. (Verbenaceae) was purchased from the local market of Faizabad Uttar Pradesh, India. The taxonomical recognition was done from Department of Agronomy, Aacharya Narendra Dev Agriculture Technical University, Dist. Faizabad, Uttar Pradesh.

4.3 Preparation of Extract

The collected brown colored roots (100gm) were extracted (decoction) with water (500ml) at a temperature not exceeding 110^0C for 2hrs. The extract was allowed to cool, filtered and lyophilized. The yield of obtained dried extract was 10%w/w. The solvents and other chemicals used were of the analytical grade.

4.4 Qualitative phytochemical analysis

The phytochemical screening of aqueous extract was done to identify the secondary metabolites present in the aqueous extract of *Clerodendrum serratum* by different chemical reactions, including tests for alkaloids, glycosides, tannins, carbohydrates, saponins, proteins and amino acids, phenolic compounds and flavonoids in the root extract of *Clerodendrum Serratum* [1-2].

4.4.1 Test for tannins

Quantity sufficient of *Clerodendrum serratum* aqueous extract was dissolved in distilled water and filtered. 2-3 drops of 10% of $FeCl_3$ were added to 2 ml of the filtrate obtained, for further

testing. The evolvement of black-blue or blackish-green coloration illustrate the presence of tannins. Further tannins were confirmed by adding 1 ml of bromine water to 2 ml of the filtrate due to decoloration of bromine water.

4.4.2 Test for flavonoids

Shinoda test : Sufficient quantity aqueous extract was dissolved in methanol and subjected for heating. The Magnesium metal chip was combined into the mixture, followed by the inclusion of a slight drops of concentrated HCl. The appearance of a red or orange coloration indicate the presence of flavonoids.

4.4.3 Test for saponin glycosides

Hemolytic test: Agar medium about 7% fresh preparation was used and wells were contrived in it. Distilled water was applied to aqueous extract, whereas methanol was used as negative control. Commercial saponin (BDH) solution was used as positive control for testing. The incubation of the plates was done at 35°C for 6 hr. Presence of saponin glycoside was confirmed by complete haemolysis of the blood around the extract.

4.4.4 Test for anthraquinone glycosides

Borntrager`s test: Extract was moistened with, dilute H_2SO_4 boiled and filtered, followed by the addition of equal proportions of benzene or chloroform. The organic layer was collected and ammonia was added. Pinkish red color of ammoniacal layer indicates the presence of anthraquinone glycoside.

4.4.5 Test for cyanogenetic glycosides

The powdered drug was moistened with water and taken in a conical flask and corked it. 10% picric acid followed by 10% sodium carbonate was moistened to filter paper. The moistened filter paper was placed above the conical flask. Appearance of brick red or maroon coloration confirms the presence of cyanogenetic glycosides.

4.4.6 Test for cardiac glycosides

Aqueous extract was fused with glacial acetic acid comprising 1 drop of 1% $FeCl_3$. This was placed underneath with concentrated H_2SO_4. A circular brown ring was observed at the intersection indicating the existence of deoxy sugar, symbolic for cardiac glycosides.

4.4.7 Test for alkaloid

To the alcoholic, aqueous and chloroform extract, dilute hydrochloric acid was added and filtered.To the 2 ml of filtrate, Dragendorff`s reagent was added. The development of orange, brown precipitate indicates the presence of alkaloids.To the 2 ml of above filtrate, Hager`s reagent was added. Observation of yellow precipitate confirmed alkaloid.

4.4.8 Test for steroidal moiety

Salkowski method was adopted to test steroids. Accurately weighed extract was fluidified in chloroform and strained. Filtrate obtained was combined with a concentrated H_2SO_4 form a secondary layer at the bottom. Appearance of reddish brown color was considered as affirmative for steroid.

4.4.9 Test for reducing sugars

One ml of both Fehling's solutions I and II was supplemented to 2 ml aqueous extract. The obtained mixture was further subjected to heat in a boiling water bath for about 2-5 min. The intense brick red precipitate certified the subsistence of reducing sugars.

4.4.10 Test for amino acids

To the 3 ml of test sample, 4% of sodium hydroxide and a few drops of 1% copper sulfate was added. Formation of violet or pink color illustrated the existence of amino acids.

4.5 Quantitative analysis of phytochemicals by spectrophotometer

4.5.1 Spectrophotometer Quantification of Total Phenolic Content

Folin Ciocalteu reagent was intended for estimation of total phenolic content. Gallic acid was taken as standard for the determination of phenolic content and it was asserted as mg/g Gallic acid equivalent (GAE). Different combination ranging from about 0.01-.05 mg/ml of Gallic acid was processed in methanol. Concentration of plant extract was 1mg/ml using methanol as diluent. About 0.5ml of test sample was placed in a test-tube and admixed with 2.5ml of Folin Ciocalteu reagent. Sodium carbonate 2 ml of (7.5%) was added. The tubes were granted to standpoint for 30 minutes at room temperature, covered with paraffin, followed by taking the absorbance at 760 nm. Reading was taken in triplicate. Blue coloration was observed and measured spectrophotometrically [3].

4.5.2 Spectrophotometric Quantification of Total Flavonoid Content

Total flavonoids were measured by a colorimetric assay [4]. An aliquot of sample (1mg/ml) or standard solutions (10-50µg/ml) of rutin was added to a 75 µl of sodium nitrite ($NaNO_2$, 5%) solution, and mixed properly. After 5-6 minute, 0.15 ml aluminium chloride ($AlCl_3$, 100 g/L) was added, followed by addition of sodium hydroxide 0.5 ml (NaOH, 4%) after 5 min. Volume adjustment was done upto 2.5 ml with distilled water and mixed thoroughly. Optical density was measured at 510 nm for sample, as well as blank. All samples were analyzed in three replications for sample and standard [5].

4.5.3 Spectrophotometric Quantification of Total Saponin Content

Standard curve 50, 62.5, 75, 87.5, 100, 112.5 and 125.5 µg/ml of the standard saponin solutions was made with aqueous methanol (80%). Diosgenin was taken as standard prepared by dissolving 10 mg(diosgenin) in 16 ml methanol and 4 ml of distilled water. Vanillin reagent (8%, 0.25 ml) and sulphuric acid (72% v/v, 2.5 ml) was combined. After proper mixing the tubes were placed in water bath at 60°C for 10 minutes and cooled in ice bath for 3-4 mins. The absorbance was done at 544 nm against the reagent blank. 0.1gm of sample was prepared by dissolving in aqueous methanol (80%) [6].

4.5.4 Spectrophotometric Quantification of Total Alkaloid Content

For the standard curve of atropine, total 5 different concentrations were used. 100 µg/ml solution was initially prepared from atropine (1 mg in 10 ml of distilled water). From this stock solution, exactly 0.5, 1, 1.5, 2 and 2.5 ml of atropine solutions was transferred to five different separating funnel. To each and individual funnels, 5 ml of phosphate buffer (pH4.7) and 5ml of Bromocresol green (BCG) solution was added and mixed vigorously. The formed complex mixture is extracted with chloroform. Chloroform fraction was collected and final volume was adjusted in 10 ml volumetric flask with chloroform. Absorption at a wavelength of 470 nm of each flask was measured and calibration graph was drawn.

Plant extract (1mg/ml) was admixed in HCl(2N) and filtered. The pH of the plant extract was fixed to neutral with 0.1 N NaOH. Solution(1ml) was transferred to a separating funnel and around bromocresol green(5ml) and phosphate buffer(5ml) was combined and mixed properly.

The mixture was further extracted with chloroform (5ml) and transferred to 10 ml of volumetric flask and final volume was adjusted. The estimation was done by taking absorbance at 470 nm [7-8].

4.6 Qualitative HPTLC study of Ursolic acid

The presence of ursolic acid was confirmed by performing the High performance thin layer chromatography (HPTLC) [9]. Aqueous extract prepared in section 4.3 was used for qualitative estimation of ursolic acid. The solvent system was used toluene: ethyl acetate (8:2 v/v). Anisaldehyde sulfuric acid was used as spraying reagent to detect the spots. Standard ursolic acid was used as reference standard. The R_f value was calculated by using the formula as mentioned below:

$$\mathrm{Rf} = \frac{D\text{istance travel by solute}}{\text{Distance travel by solvent}}$$

4.7 Phytochemical analysis of volatile principles

4.7.1 Extraction and isolation of essential oil

Root part of *C. Serratum* L. was size reduced, dried (shade) at room temperature for 15 days and subjected to grinding mill. Coarse powder obtained was further passed through sieve. No. 44. 10 gm of root powder was macerated with 30 ml of hexane overnight followed by sonication for 15 minutes. Further, hexane extract was subjected to reflux for 2 hours. Centrifugation was done for the refluxed sample for 10 minutes and kept in a sealed vial in the refrigerator (4°C) until further use [10-11].

4.7.2 Preparation of sample for GC/MS analysis: 5 ml of n-hexane plant extract was vortexed properly, centrifuged for 5 minutes and filtered through 0.22 mm syringe filter (Millipore Corp., Bedford, MA, USA). One µl aliquot of each sample solution was injected into the GC/MS system for the requisite analysis.

4.7.3 Instrumentation and chromatographic conditions: The phytoconstituents present in the n-hexane root extract was evaluated by gas chromatography–mass spectrometry using Clarus 500 Mass Spectrometer. Temperature programmed with a ramp 8°C /Min upto 240°C and ramp 6°C /Min upto 280°C hold for 15 min while the injector temperature kept at 280°C at injection

volume of 1µL with a syringe size of 10µL. Flow rate was maintained at 1.2ml/min. Oven was maintained for 70 min having transfer line temperature for about 280˚C. Scanning was done in the range of 35-500 amu. Identification of phytoconstituents was made by comparison of their mass spectra with NIST libraries mass spectra[12].

4.8 HPLC method validation of plant extract, according to ICH Guidelines of extract

4.8.1 Optimization of HPLC chromatography solvent system

Different solvent systems were tried for developing an HPLC system for identification of constituents in the extract based on the literature survey and keeping in mind the chemical nature of the constituents and the one showing maximum separation was selected as mobile phase for the study [13-14].

The following solvents were used for the development of the HPLC system*:*

1. Methanol and Acetonitrile in the ratio (30:70, v/v)
2. Methanol : Acetonitrile (5:95, v/v)
3. Methanol: Acetonitrile (10:90, v/v)
4. Methanol: Acetonitrile (15:85, v/v)
5. Methanol: Acetonitrile (20:80, v/v)
6. Methanol: Acetonitrile (25:75, v/v)

4.8.1.1 Mobile phase: Mobile phase mixture was prepared, filtered and degassed using methanol and Acetonitrile in the ratio(30:70, v/v)

4.8.1.2 Preparation of Reference Solution: 10 mg of ursolic acid working standard was taken into volumetric flask. Methanol (5 ml) was added and subjected to sonication. The solution was settled to room temperature, dilution was done up to the mark with diluent and mixed. 3 ml of the solution was taken in a volumetric flask diluted with diluent and mixed.

4.8.1.3 Preparation of Sample Solution: 1.5 gm of test sample was taken in Iodine flask. 25 ml of water was added and sonicated for proper mixing. Reflux was done for about 30 min in

reflux assembly and filtered. Process was repeated twice and filtrate was evaporate to dryness. Residue was dissolved in methanol volume was make up with 10 ml methanol. Further 0.5 ml of this solution was taken into 10 ml volumetric flask diluted with methanol.

4.8.2 Development of HPLC method and validation for Ursolic acid.

4.8.2.1 Chromatographic condition

Chromatographic System:

HPLC Method : Isocratic condition

Equipment : HPLC

Column : C18 (25 cm X 4.6 mm) 5 µm

Column Temperature : Ambient

Detector : UV

Wavelength : 210 nm

Flow rate : 0.6 ml/min

Injection volume : 20 µl

Blank : Diluent

4.8.2.2 Procedure: Injection of equal volumes of the reference solution was done, chromatograms were recorded along with measurement of peak area. Solutions number of injections 1, Diluent 1 and Reference Solution 5 were run in HPLC system.

Calculation: Percentage of ursolic acid was calculated by the formula:

$$\% \text{ of Assay} = \frac{\text{At X Cs X P}}{\text{As X Cu}}$$

At= Peak area of test sample.

As = Peak area of reference standard

C_S = Concentration of reference standard

CU = Concentration of test sample

P = Potency of Ursolic Acid working standard

4.8.3 Method validation

Method validation was done, to confirm that analytical method occupied for the distinct analysis is applicable to its expected use. Above method validation can be used to check its quality, authenticity and consistency [15-16]. The method was developed and validated according to ICH guidelines (ICH, 1997). An analytical method to be validated can be done by any of the categories mentioned below:

a) Identification test

b) Quantitative estimation of actives.

c) Quantitative estimation of impurities

Estimation of marker compounds in *Clerodendrum Serratum* L. extract is classified under category i.e. quantification method. The analytes to be estimated were ursolic acid. The characteristics required for its validation are listed below:

1. Linearity
2. Specificity
3. Accuracy
4. Range
5. Precision
6. Repeatability
7. Intermediate precision
8. Robustness
9. Limit of Detection
10. Limit of Quantification

4.8.3.1 Validation of linearity

Linearity was determined by applying a series of dilutions for standard in triplicate within a span between 50-200% of the expected working range in different concentrations. The Chromatogram was recorded to determine the peak area, % peak area and retention time.

The stock solution of Ursolic acid was prepared in HPLC grade methanol by dissolving 1 mg of individual sample in 1ml of HPLC grade methanol. Further dilution was made to obtain a concentrate of the range of 33 to 1000µg/mL of concentration. The calibration graphs were plotted peak areas vs. concentration. For assessing the linearity, the least square regression equation correlation coefficients were determined.

20 µL of standard ursolic acid of different concentrations (33 -1000 µg /ml) were injected. It was repeated for 3 times for all concentration. The peak area was documented and calibration curve was drawn.

Acceptance criteria**:** Coefficient of correlation < 0.99 r^2

4.8.3.2 Validation of specificity

The specificity was done to evaluate the peak purity of the analyte by observing resolution in comparison to other related components. Spectra were recorded to specify the peak of the analyte in comparison with a standard. The peak area of ursolic acid was confirmed by matching the retention time and the spectra of the test sample with the standard.

Acceptance criteria: - Resolution > 2%.

4.8.3.3 Validation of accuracy

The accuracy was done by spiking the plant extract, with known amount of standard in different concentration. The test sample previously analysed was spiked with standard at four different concentration levels, i.e. 0%, 50%, 100% and 150% using the adopted method of analysis for validation. The sample was analyzed according to the method and the assay value along with % recoveries was reported to check the assay content. The different batches of *Clerodendrum Serratum* extract were analyzed to find out the actual content of ursolic acid.

Acceptance criteria: Assay > 10%, < 95; recovery between 95-110 %

Assay > 0.5%, < 7.5; recovery between 85-120 %

4.8.3.4 Validation of Range

Linearity studies were considered for determining the specific range. The computation was done for the test sample from the linearity graph, within lowest and highest concentration of the given sample interpolated for linear, precise and accurate response.

Acceptance criteria: RSD < 2.5

4.8.3.5 Validation of Precision

The precision is done to check the proximity of results of the same sample by doing series of analysis with the defined procedure and conditions. The precision of the proposed method was obtained by repeatability and intermediate precision. Experiments are done for evaluation of Inter-day and Intra-day precisions for checking whether the results are coming within the range be performing analytical procedures for the analyte on the same day, and on other days by same analyst and different analyst. As we know the procedure should be giving similar results for analysis, precision value should be within the % RSD<2 to confirm the validity.

4.8.3.6 Validation of Robustness

Robustness of the analytical procedure is the evaluation of analytical method under adverse conditions by intensely changing various frameworks and providing an implication that the procedure is acceptable under different conditions for its accurate and precise use as compared to conventional usage. Design and procedural changes are intentionally done within the limit to the analytical procedure in regard to mobile phase and detection wavelength or change of column used for analysis.

4.8.3.7 Limit of detection (LOD) and Limit of quantification (LOQ)

The LOD and LOQ is accounted for the analyte to know its threshold level of quantification and detection within acceptable range supporting other parameters of validation as mentioned above for the analytical procedure. Signal to noise ratio was used as indicator for concentration of the sample for detection of LOD with minimum criteria of three observations, whereas often indication for signal to noise ratio was fixed as LOQ.

4.9 Evaluation of *in-vitro* antioxidant activity

4.9.1 Chemicals

1,1-Diphenyl-picryl-hydrazyl (DPPH) was procured from Sigma Aldrich,USA. Rutin (Ozone, Mumbai) was used as a standard drug. All other chemicals/solvent used during experimental procedure was of analytical grade.

4.9.2 Preparation of Extract

The collected brown colored roots (100gm) were extracted (decoction) with water (500ml) at a maintaining temperature around 110°C for 2hrs. The extract was strained and lyophilized as mentioned in section 4.3.

4.9.3 DPPH* radical scavenging activity

The DPPH assay of extract was determined by using UV-Spectrophotometer [17-18], which involved the preparation of standard, test and control.

4.9.3.1Preparation of Standard Rutin solutions

Different solutions (20 - 100µg/ml) of the rutin were prepared in methanol. 1.5 ml of each solution of rutin were blended with 200µM DPPH (1.5ml) solution and fecundated for 30 min time duration at room temperature. OD was taken at 517 nm from test sample and standard for all the test tubes prepared.

4.9.3.2 Preparation of test solutions

Test samples were prepared similarly in the concentrations range of 20-100µg/ml as mentioned in standard rutin solutions.

4.9.3.3 Preparation of control solution

For control, 1.5 ml of methanol was mixed with 200 µM DPPH taking absorbance at 517 nm. The Percentage antioxidant activity of plant extract and rutin was determined by below mention formula:

$$\% \text{ inhibition} = [(A_0 - A_1/A_0)] \text{ X } 100$$

A_0 was the absorbance of control (blank, without extract) and A_1 was the absorbance of the tests sample i.e. plant extract or standard rutin. The 50% inhibitory dose (IC_{50} value) was found by interpolation by using graph pad (prism 6 software) and compared with standard.

4.9.4 Superoxide scavenging assay

4.9.4.1 Reagents and chemicals

1 ml of alkaline DMSO having a composition of 5mM NaOH(0.1ml) and DMSO(0.9ml). NBT (Nitroblue Tetrazolium) solution was processed by defronting 25 mg of nitroblue tetrazolium diluted to DMSO(25ml) with final concentration of 1 mg/ml [19-22].

4.9.4.2 Preparation of standard and test sample

Different concentration (20-100µg/ml) of rutin and test sample was prepared. Test tubes was composed of 0.1 ml of NBT, 0.3 ml of aqueous extract and 1 ml of alkaline DMSO. Absorbance was fixed at 560 nm. Plain DMSO used as blank as control. Depreciate absorbance concluded for superoxide anion scavenging activity in different concentrations. 50% inhibition of the extract was determined by plotting a graph between absorbance and concentrations.

4.9.5 Ferric reducing antioxidant power (FRAP) assay

The endowment for reduction of ferric ions was deliberated using the method [23].

4.9.5.1 Reagent preparation

FRAP reagent was accomplished by interweaving 300 mM sodium acetate buffer (pH 3.6), 10 mM (tripyridyl triazine) TPTZ solution and 20 mM and $FeCl_3.6H_2O$ solution in a proportion of 10:1:1 in volume.

4.9.5.2 Preparation of standard and test sample

Rutin and Test samples at divergent combinations (100 to 500 µg/ml) was supplemented to 3 ml of FRAP reagent and the counterpoise was nurtured at 37°C for 30 min. Increase in absorbance of test and standard at 593 nm was measured. Reduction of ferric ions to ferrous ions was illustrated for the the antioxidant capacity.

4.10 In-vitro Anti inflammatory Activity

4.10.1 Hypotonic solution –Induced haemolysis or membrane stabilizing activity: This test was done according to the method mentioned. Extract sample subsisted of stock erythrocyte (RBCs) suspension 0.03ml infused with hypotonic solution(5ml) (154 mM NaCl in 10mM

Sodium Phosphate Buffer at pH 7.4) enclosing test sample ranging from concentration 100-500 µg/ml. The blank was performed. The standard drug acetylsalicylic was treated similarly. The experiment was done in triplicate. The mixtures were allowed for incubation at room temperature for around 10 minutes, followed by centrifugation (10 minutes at 3000 rpm). Absorbance of the supernatant was uniformed spectrophotometrically at 540 nm. The percentage inhibition of haemolysis was computed by following equation [24-26].

$$\%\ \text{inhibition of haemolysis} = 100\ \text{X}\ [\text{A1} - \text{A2}\ /\text{A1}$$

Where: A1 = Absorbance of blank; A2 = Absorbance of test and standard sample

4.10.2 Effect of Protein Denaturation: Protein denaturation was executed as mentioned below. Different concentrations ranging from 100-500 µg/ml for both test sample and standard acetylsalicylic was composite with egg albumin solution (1mM,1ml) and incubated at 27˚C for around 15 minutes. Further reaction mixture was maintained at 70˚C in a water bath for 10 minutes for denaturation. Samples were allowed to cool down and od was spectrophotometrically at 660 nm. Percentage inhibition of denaturation was calculated from control without sample and standard. All the experiment was performed in triplicate [27].

The percentage inhibition of denaturation was done by below mentioned formula.

$$\%\ \text{Inhibition of haemolysis} = 100\ \text{x}\ [\text{A 1} - \text{A 2}\ /\text{A 1}]$$

4.11 Evaluation of In-vivo anti arthritic study

4.11.1 Complete Freund's adjuvant (CFA) induced arthritis:

Complete Freund's adjuvant (CFA) model was performed to evaluate the anti Arthritic Activity. Albino Wistar male rats of weight 200±25gm were used for the experiment. Route of administration was P.O. Animals were placed in different cages in group under controlled conditions of temperature (22 ± 2°C). Golden feed diet and water were administered to all the animals regularly. The dose adopted 100 mg/kg and 200 mg/kg b.w. Paw diameter, joint diameter, arthritic score, body weight was used as a parameter for the activity. Estimation of TNF-α and COX-2 was also done. Institutional Animal Ethics Committee (IAEC) approval

was taken (Reg No. 1824/PO/ERe/S/15/CPCSEA). Protocol Approval Reference No.IAEC/PN-16045 before performing the experiments.

Animals were divided into five groups. Vehicle/drug was administered orally and after 30 minutes of waiting period, 0.1 ml of CFA (0.05% Mycobacterium butyricum in mineral oil) was injected into left hind paw(subplantar surface) with a 26 gauge needle. Paw diameter, joint diameter, arthritic score, body weight measurements was carried out on days 3rd, 7th, 14th and 21st. After completion of protocol on the 21stday blood(terminal) was collected. TNF-α and COX-2 levels were estimated of the serum obtained using ELISA kit assay for all the groups. The ankle joints were composed and fixed in formalin for histology. All were compared to standard Indomethacin and evaluated.[28-33]. The overview of all the methods is given in table no. 4.1.

Animal Grouping

Table 4.1: Animal models used to evaluate anti arthritic activity

S. No.	TREATMENT	DOSE	No. Of Animal
1.	Vehicle (Normal Saline)	10 ml/kg	06
2.	CFA + Normal saline	10 ml/kg	06
3.	CFA + Std (Indomethacin)	3 mg/kg	06
4.	CFA + RKT	100 mg/kg	06
5.	CFA + RKT	200 mg/kg	06

4.11.2 Arthritic score

The structural (morphology) features were closely observed like redness, swelling and erythema for arthritis. Visual criteria were observed as mentioned: score of normal paw = 0, whereas mild swelling and redness of digits were = 1, swelling and redness of the digits in rats as = 2, lastly for severe swelling and redness = 3, finally gross deformity and the inability to use the limb was observed = 4.

4.11.3 Estimation of Tumour Necrosis Factor – Alpha (TNF-α)

4.11.3.1 Preparation of working Reagents:

Serum/plasma samples was diluted with (Diluent A) whereas (Diluent B) was 5-fold diluted with deionized or distilled water.

4.11.3.2 Preparation of standard:

Vial of Standard Recombinant Rat/Mice(Item C) TNF-α was spinned to shake the material and 400 µl Assay Diluent A (for serum/plasma samples) was added to produce a 100 ng/ml standard. The Powder was carefully mixed and from this solution 200µl was taken into a tube with 400µl of diluent A to prepare a (20,000 pg/ml) stock standard solution. Afterward 100 µl of this solution was mixed with 400 µl diluent A into each tube. Stock standard solution was used to produce a serial dilution (shown in figure 3.1) as mentioned below. All the tubes were mixed carefully before further dilution. Assay of diluent A served as the zero standards (0 pg/ml).

4.11.3.3 Preparation of wash buffer:

Wash Buffer Concentrate(20ml) was diluted with deionized or distilled water to yield wash Buffer solution.

4.11.3.4 Preparation of Detection Antibody vial (Item F):

The content of the detection antibody vial (item F) was proportionately added with 1x assay diluent A until the Detection Antibody concentration diluted to 80-fold.

4.11.3.5 Preparation of HRP-Streptavidin concentrate vial (Item G):

HRP-Streptavidin concentrate was diluted with diluent A. Dilution has been shown in figure 4.1.

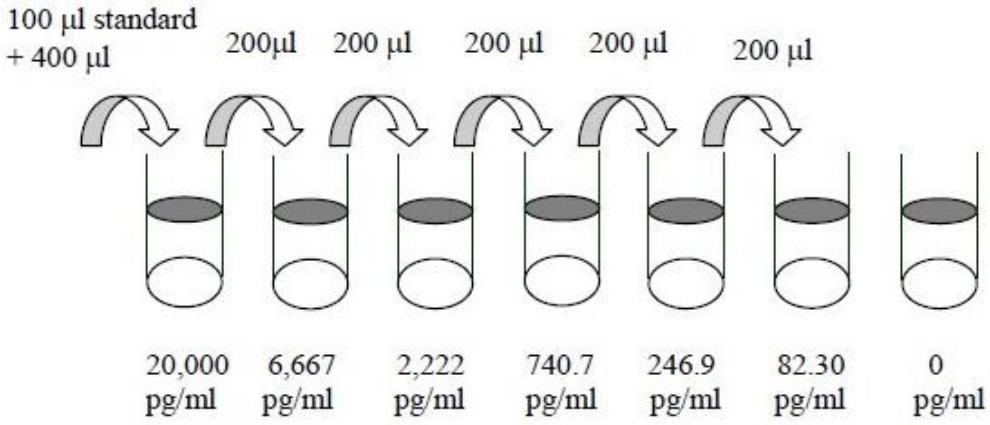

Fig 4.1 : Dilution series for Stock standard solution (TNF- α)

4.11.3.6 Assay procedure:

Duplicate run was done for standard and test sample in 96-well plate. 100 µl solutions of both standard and sample was combined into applicable wells and enclosed with gelatin strip and

incubated (2.5 hours, 4°C) at room temperature or overnight at with affable shaking. Above solution was run-down and spelled 4 times with wash Solution(300 µl). Aspirated for complete removal of wash buffer and plates were upturned and marked.

Biotinylated antibody solution(100 µl) was admixed to each well and incubated for residence time of 1 hour followed by washing and aspiration.

Streptavidin solution(100 µl) was added to individual well and incubated for 45 minutes at room temperature. All the solutions were discarded from, well followed by washing 4 times with wash buffer as mentioned earlier (300 µl). Lastly, all the plates were aspirated and inverted.

Tetramethylbenzidine (TMB) substrate 100 µl (Item H) was incorporated to all the wells, incubated and finally displaced with 50 µl of Stop Solution (Item I) to stop the reaction.

Absorbance was taken at 450 nm using ELISA micro-plate reader. Mean absorbance was calculated for (standards, controls and samples) in duplicate.

4.11.4 Estimation of COX-2

All the test sample, standard and blank was duplicated in 96-well plates for the mentioned quantity.

(A) Standard wells: Standard 50µl, followed by addition of COX-2-antibody 10µl labelled with biotin was added and addition of streptavidin-HRP 50µl was done (since the standard already has mixed biotin antibody, it is not paramount to add the antibody). Subjected to sealing and incubated at 37°C for 60 minutes.

(B) Test wells: Sample(extract) 50µl was added, followed by COX-2-antibody 10µl labelled with biotin and streptavidin-HRP 50µl. The Sample was incubated 60 minutes at 37°C.

Chromogen solution A & B (50µl) was added to each well to stop the reaction. With gentle mixing, subject for incubation for 10 min at 37°C in the dark. Further 50µl chromogen was finally added to have visual observation of color(the blue changes into yellow immediately).

OD was measured at 450 nm after 15 min addition of chromogen taking blank well as zero.

A linear regression equation was applied for calculation of Corresponding sample's concentration from the the standard curve OD values.

4.12 Statistical Analysis

Statistics were asserted as mean ± Standard Error Mean (SEM). Differences were considered significant at ***P<0.001, or **P < 0.01 or * P<0.05 when compared test group vs control (-ve) group. For numerical results, one-way analysis of variance (ANOVA) (compare all vs. control) was performed using Graph Pad InStat Version 3 (GraphPad Software).

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CHAPTER 5

RESULTS AND DISCUSSIONS

5.1 Collection of plant material

The root part of the plant was procured from the local dominated market of Faizabad, Uttar Pradesh.

5.2 Authentication of plant

Roots of *Clerodendrum serratum* L. (Verbenaceae) was purchased from the local market of Faizabad Uttar Pradesh, India. The taxonomical recognition was done from Department of Agronomy, Aacharya Narendra Dev Agriculture Technical University, Dist. Faizabad, Uttar Pradesh.

5.3 Preparation of Extract

Aqueous extract was prepared by decoction for phytochemical analysis and biological evaluation. The percentage yield of the solvent free extract was 10%w/w.

5.4 Qualitative phytochemical analysis

Preliminary phytochemical analysis was done for investigation of different kinds of secondary metabolites. As secondary metabolites of the plants present in nature are directly related to the therapeutic responses of any drug, it is becoming very important to analyze the metabolites present in an extract [1-3]. The chemical test includes tests for tannins, glycosides, alkaloids, steroidal moiety, reducing sugar, amino acids etc. in aqueous extract of the root of *Clerodendrum serratum.* The secondary metabolites present in the aqueous extract are tabulated in table no.5.1.

Table 5.1: Secondary metabolites present in aqueous extract of *Clerodendrum serratum* L.

Sl. No.	Secondary Metabolites	Result
1	Glycoside	Present
2	Alkaloid	Present
3	Sterols	Present
4	Volatile oil	Present
5	Tannins	Present
6	Triterpenoids	Present

5.5 Quantitative analysis of phytochemicals by spectrophotometer

5.5.1 Chemicals and reagents

Total phenolic content, total flavonoids content, saponin content and alkaloid content were determined as mentioned in the appropriate section of materials and methods. All the chemicals were of analytical grade and quantification was done by spectrophotometer.

5.5.2 Phytochemicals quantification:

The activity of any kind of phytomedicine principally depends upon the presence of phytochemicals. In this study, we have quantified the presence of total phenolic, total flavonoid, total saponin and total alkaloid content tabulated in table 5.2. The content of total phenol in the aqueous extract of *C. serratum* was obtained from the gallic acid standard curve. The observed absorbance and calibration curve and of gallic acid is tabulated and figured in table no 5.3 and figure 5.1 respectively. Line of regression from gallic acid graph was used for the estimation of phenol content in the aqueous extract of *C. serratum*. From standard curve of gallic acid line of regression was found to be $y = 0.005x + 0.065$ and $R^2 = 0.976$. The total phenolic content expressed as mg gallic equivalents per gram of sample (mg GAE/g). The total phenolic content in extract was 134.4±0.917 in mg/g equivalent of gallic acid.

Flavonoid is one of the important phenolic compounds existing in nature [4]. The total flavonoid content for aqueous extract of *C. serratum* was measured with the aluminium chloride colorimetric assay where rutin was used as standard. The absorbance and calibration of rutin are tabulated in table 5.4 and figure 5.2. The rutin solution of concentration ranges from 10-50 µg/ml with a regression co-efficient (R^2) = 0.985 (Figure 5.2). The slope (m) and intercept found to be 0.001 and 0.118 respectively. The total flavonoid content (mg of rutin equivalent/ g dry material) of the extract was found to be 67.67±3.512.

In the current study, we also quantified saponin spectrophotometrically at 544 nm by using diosgenin as standard [5]. Table 5.5 and figure 5.3 shows the absorbance and calibration of diosgenin at different concentration. The total saponin content is expressed in mg/g equivalent of diosgenin. Linear regression equation obtained from the calibration curve of diosgenin was used for calculation of total saponin ($y = 0.003x + 0.028$, $R^2 = 0.970$) (Figure 5.3). The total saponin in the extract was found to be 38.2±1.311 in mg/g equivalent of diosgenin.

There are several methods reported for quantification of alkaloid like HPLC [6-7] gas chromatography[8], but in the current work we used the most easy,rapid and accurate spectroscopic method. The alkaloidal content examined from caliberation curve. The absorbance and calibration of atropine at different concentrations is shown in table 5.6 and figure 5.4. The standard curve equation y = 0.006x – 0.003, R2 = 0.997 showing in figure 5.4. The Total Alkaloid content (mg/g equivalent of Atropine) present in the aqueous extract of *C. serratum* was 12.67±3.786. The presence of these phytochemicals indicates its therapeutic importance. The medicinal properties of *C. serratum* might be due to above phytochemicals [9-12].

Table 5.2: Secondary metabolites quantified in aqueous extract of *Clerodendrum serratum*

Flavonoid content in mg/g equivalent of Rutin	
Sample	67.67±3.512
Alkaloid content in mg/g equivalent of Atropine	
Sample	**12.67±3.786**
Saponin content in mg/g equivalent of Diosgenin	
Sample	**38.2±1.311**
Phenolic content in mg/g equivalent of Gallic acid	
Sample	**134.4±0.917**

Table 5.3: Standard Gallic Acid (Concentration Vs Absorbance)

S. No.	Conc. (µg/ml)	Absorbance
		Gallic acid
1.	10	0.110
2.	20	0.176
3.	30	0.247
4.	40	0.298
5.	50	0.326

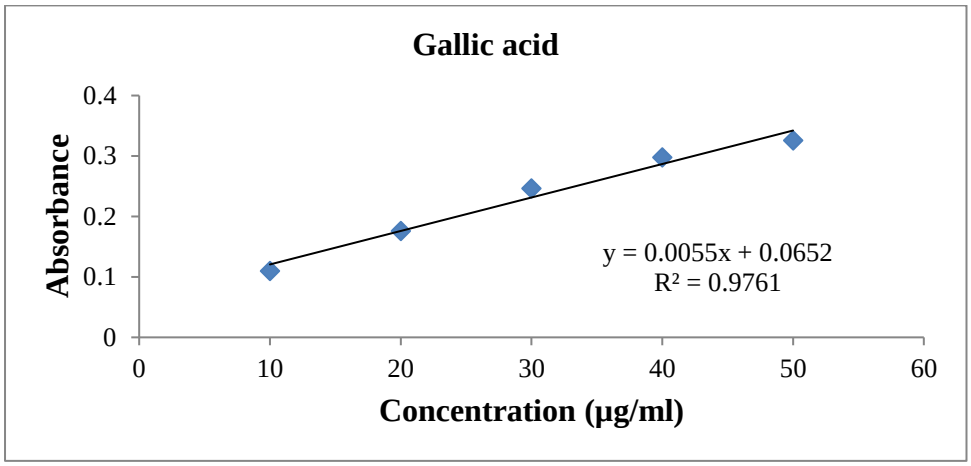


Figure 5.1: Calibration graph for gallic acid standard

Table 5.4: Standard Rutin (Concentration Vs Absorbance)

S. No.	Conc. (µg/ml)	Absorbance
		Rutin
1.	10	0.135
2.	20	0.151
3.	30	0.165
4.	40	0.177
5.	50	0.201

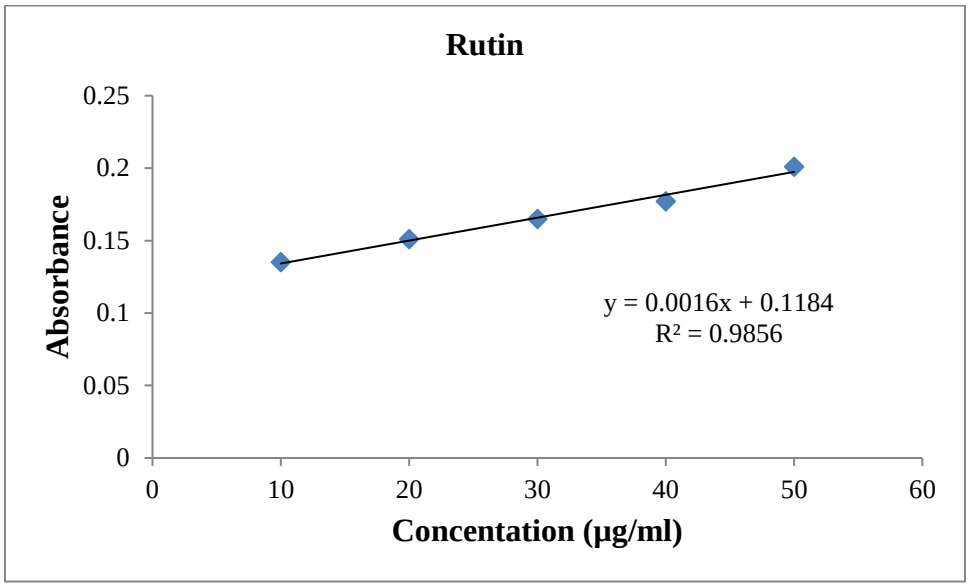


Figure 5.2: Calibration graph for rutin standard

Table 5.5: Standard Diosgenin Concentration Vs Absorbance

S. No.	Conc. (µg/ml)	Absorbance
		Diosgenin
1.	50	0.172
2.	62.5	0.189
3.	75	0.207
4.	87.5	0.279
5.	100	0.321
6.	112.5	0.384
7.	125.5	0.418

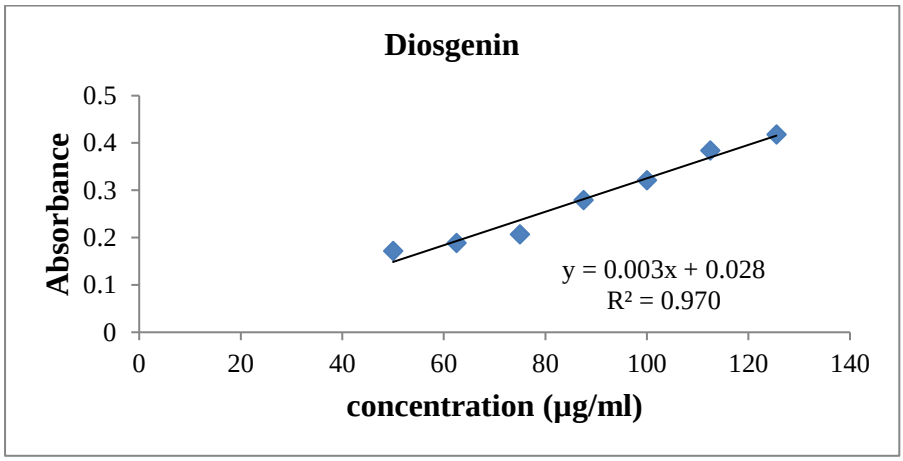


Figure 5.3: Calibration graph for diosgenin standard

Table 5.6: Standard Atropine (Concentration Vs Absorbance)

S. No.	Conc. (µg/ml)	Absorbance
		Atropine
1.	50	0.122
2.	100	0.159
3.	150	0.188
4.	200	0.207
5.	250	0.229

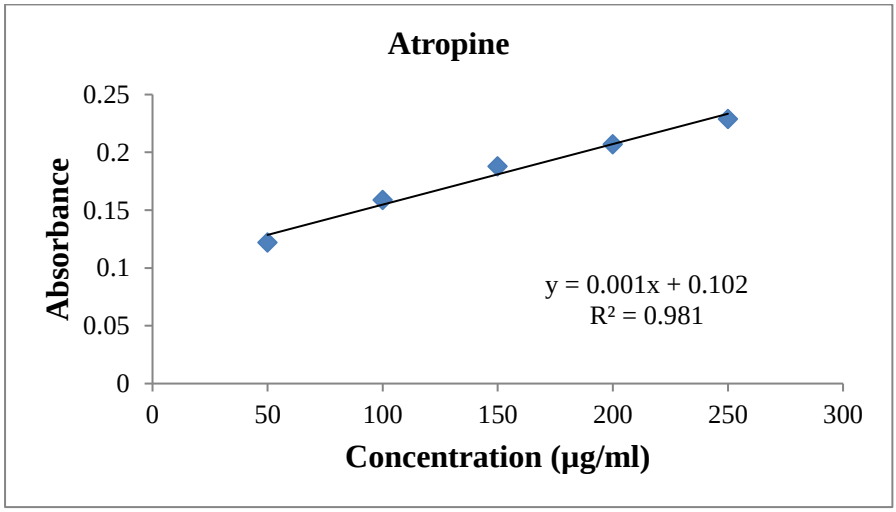


Figure 5.4: Calibration graph for atropine standard

5.6 Qualitative HPTLC study of Ursolic acid

Several reports confirm the cure of inflammation and arthritis conditions for ursolic acid [13-16]. As in the current experiment, an arthritic activity of the plant was targeted so it is becoming necessary to detect whether the plant extract is having ursolic or not. As the presence of Ursolic acid in aqueous extract may contribute a vital role in arthritic protective effect.

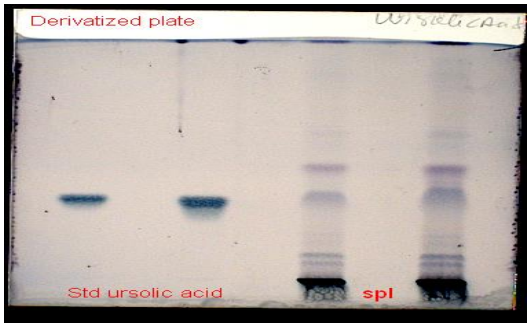


Figure 5.5: HPTLC Chromatogram of standard and sample

The presence of Ursolic acid was confirmed by performing the HPTLC of aqueous extract of *C serratum* L. along with standard Ursolic acid by using toluene: Ethyl acetate (8:2 v/v) as mobile phase and anisaldehyde sulfuric acid was used as spraying reagent. The system produces a compact spot of standard Ursolic acid with R_f value 0.38.

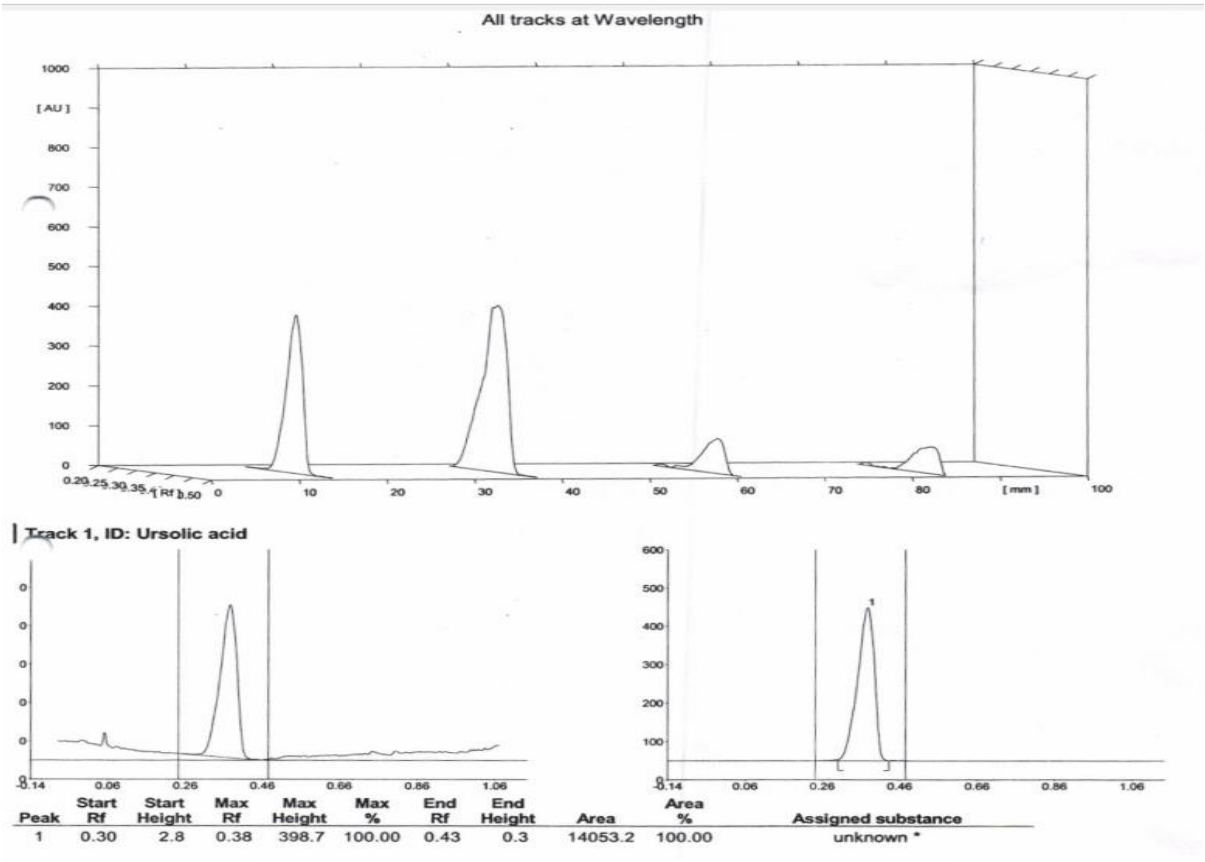


Figure 5.6: HPTLC Chromatogram of standard and sample

5.7 Phytochemical analysis of volatile principles of *C. serratum* L.

Total 15 constituents were identified & characterized from the root of *C. serratum* L (table 5.7).

Table 5.7: Identified volatile compounds from the root of *C. serratum* L.

S. No.	Peak RT (min)	Compound detected	% Peak area	Mol. Formula	Mol. Wt.	CAS No.	Activity Reported
1	4.108	3-Ethyl-3-methylheptane	9.52	$C_{10}H_{22}$	776	17302-01-01	Biomarker for urinary cell carcinoma [17]
2	5.054	Undecane	8.82	$C_{11}H24$	804	1120-21-4	Antimicrobial[18]
3	5.118	1-Heptanol, 2,4-diethyl	5.19	$C_{11}H_{24}O$	736	80192-55-8	Cosmetics[19]
4	5.354	Cyclooctane,1,4-dimethyl-, trans-	3.35	$C_{10}H_{20}$	726	13151-98-99	Vehicle hydrocarbon[20]
5	6.946	Dodecane	17.46	$C_{12}H_{26}$	832	112-40-3	Oxygen vector [21]
6	7.899	Benzene, 1,3-bis(1,1-dimethylethyl)	74.9	$C_{14}H_{22}$	901	1014-60-4	Lipid oxidation[22]
7	8.688	2-Isopropyl-5-methyl-1-heptanol	3.71	$C_{11}H_{24}O$	761	91337-07-4	Antimicrobial[23]
8	10.171	Tetradecane	26.7	$C_{14}H_{30}$	836	629-59-4	Biomedical use[24]
9	11.929	Phenol, 2,4-bis(1,1-dimethylethyl)-	35.1	$C_{14}H_{22}O$	880	96-76-4	Anti-oxidant[25]
10	12.232	1-Hexadecanesulfonyl chloride	4.97	$C16H_{33}Cl O2S$	722	38775-38-1	Antifungal and Antioxidant[26]
11	12.500	1-Decanol, 2-hexyl-	4.68	$C_{16}H_{34}O$	734	2425-77-6	Commercial[27]
12	15.614	Fumaric acid, dodecyl 2-methylallyl ester	9.19	$C_{20}H_{34}O4$	562		Not reported
13	15.791	7H-Cyclohepta[a] naphthalen-7-one, 8,9,10,11-tetrahydro-9,9-dimethyl	26.7	$C_{17}H_{18}O$	547	64184-19-6	Not reported
14	17.493	Hexadecanoic acid, methyl ester	76.5	$C_{17}H_{34}O_2$	827	112-39-0	Antibacterial & antifungal[28]
15	19.537	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	13.8	$C_{19}H_{34}O_2$	867	112-63-0	Anticancer activity [28]

Table 5.7 illustrated all the identified chemicals along with their peak percentage, retention time, molecular weight, molecular formula and CAS number. The structures of all the identified compounds are shown in figure 5.7 (a) and 5.7(b). All the compounds have been reported for the first time from the plant.

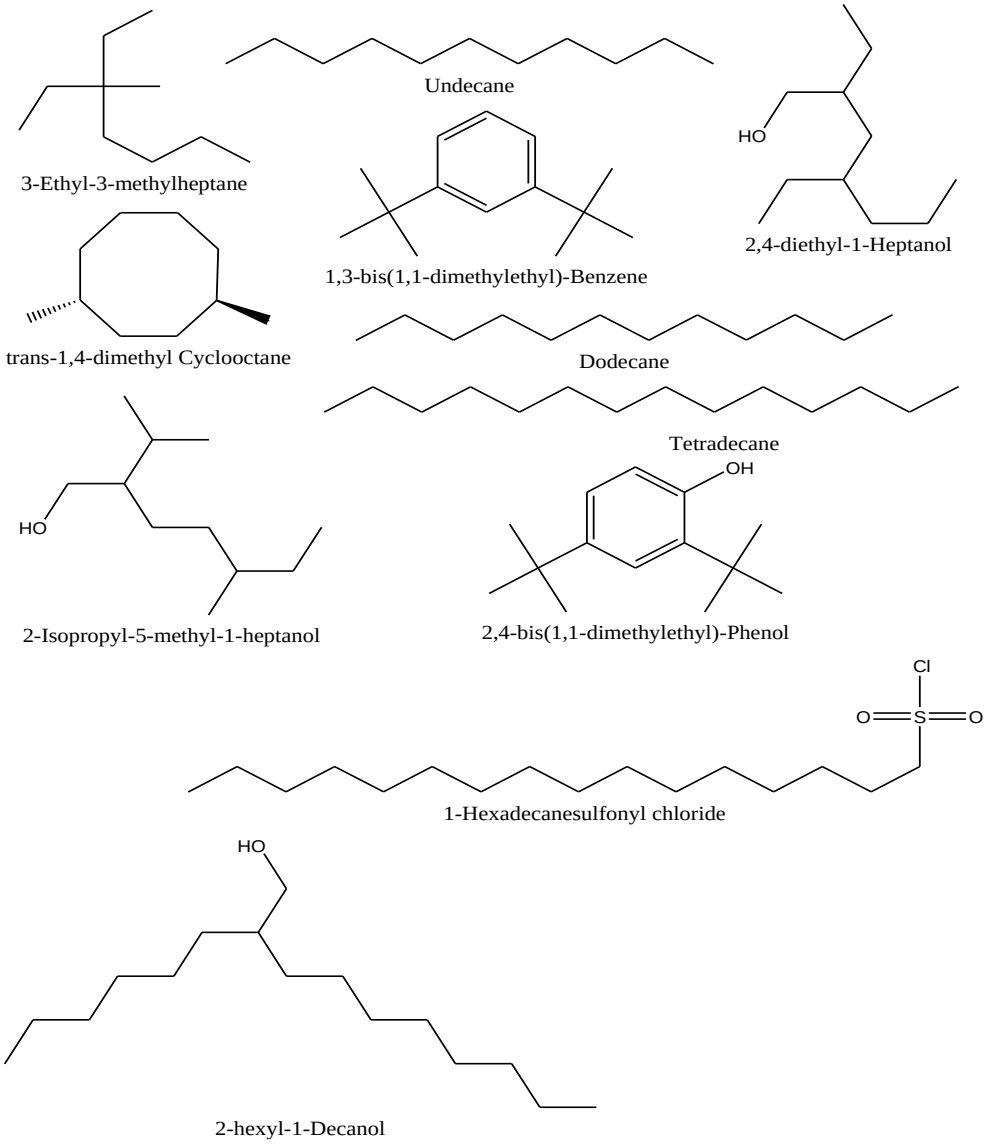
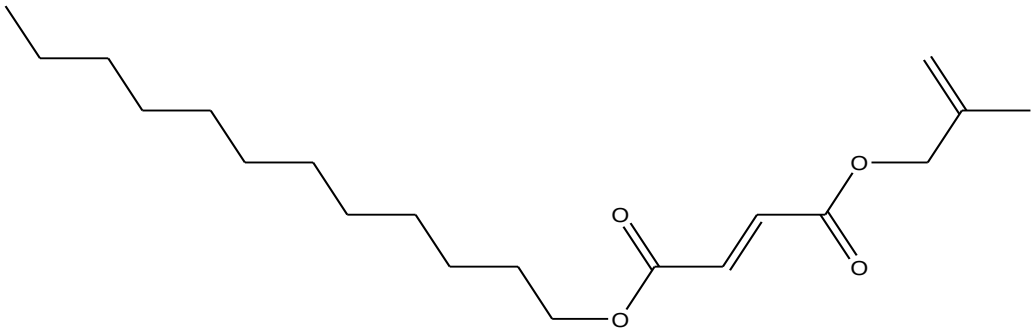
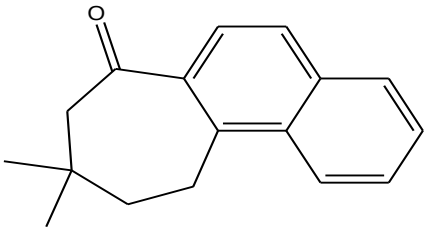


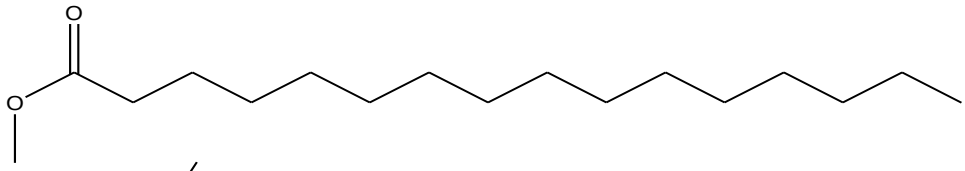
Fig. 5.7(a): Identified phytoconstituents from roots of *C. serratum* L. by GC-MS technique



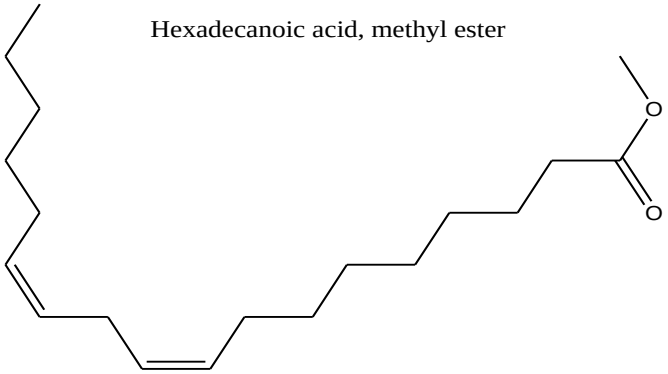
Fumaric acid, dodecyl 2-methylallyl ester



8,9,10,11-tetrahydro-9,9-dimethyl-7H-Cyclohepta[a]naphthalen-7-one



Hexadecanoic acid, methyl ester



9,12-Octadecadienoic acid (Z,Z)-methyl ester

Fig. 5.7(b): Identified phytoconstituents from roots of *C. serratum* L. by GC-MS technique

GC/MS chromatograms of n-hexane root extract of *C. serratum* L. showed several peaks indicating the presence of a wide range of volatile principles. Components were identified by using the combination of retention index value and mass spectral matching against library standards. GC-MS chromatogram for sample *C. serratum* L. is figured in 5.8, 5.9 and 5.10. In the present study, presence of bioactive entities stakes the utilization of *C. serratum* for various ailments in traditional system of medicine. The plant contains several bioactive phyto-constituents and further study is needed to discover and establish bioactivity for various compounds present in the plant.

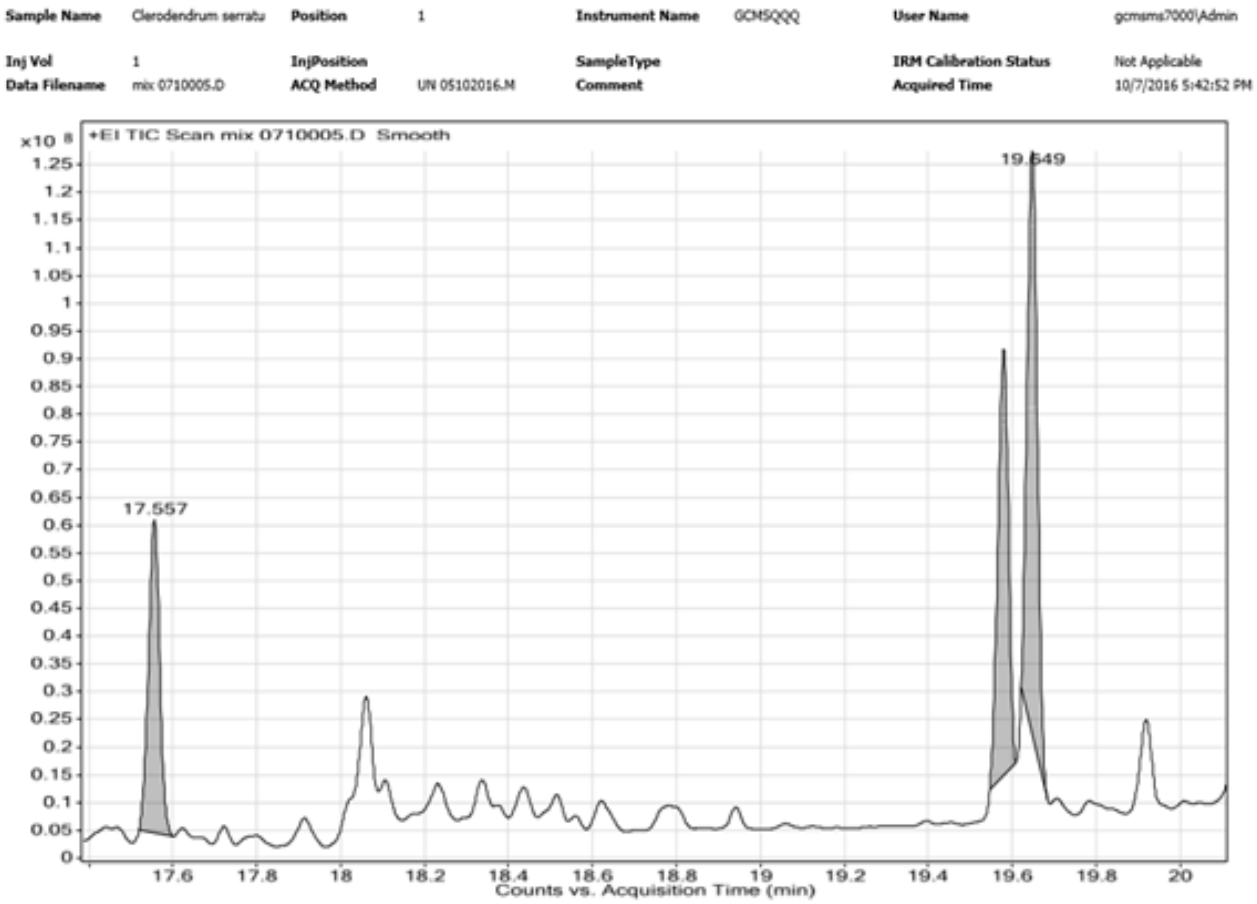


Fig 5.8: GC-MS of *Clerodendrum serratum*

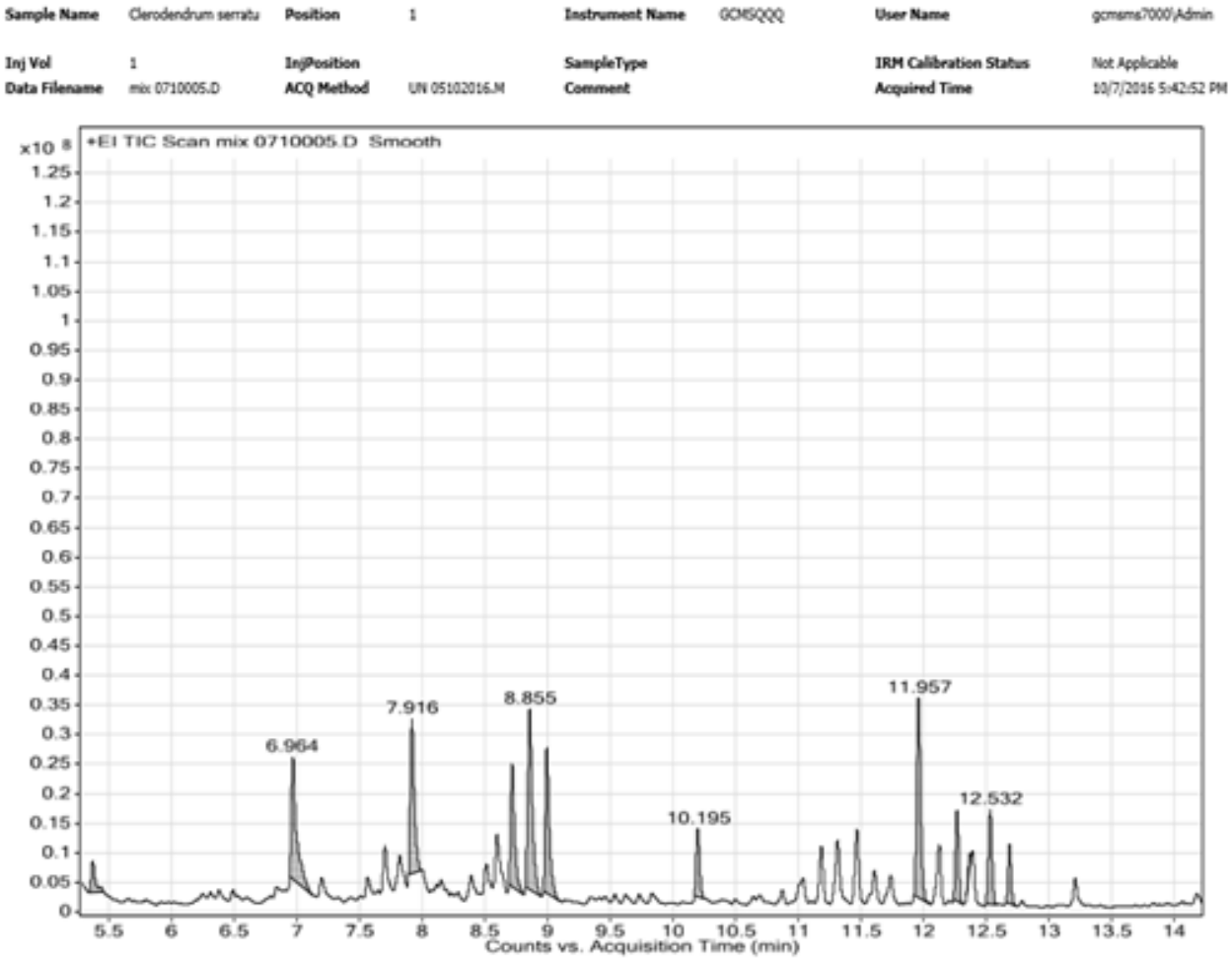


Fig 5.9 : GC-MS of *Clerodendrum serratum*

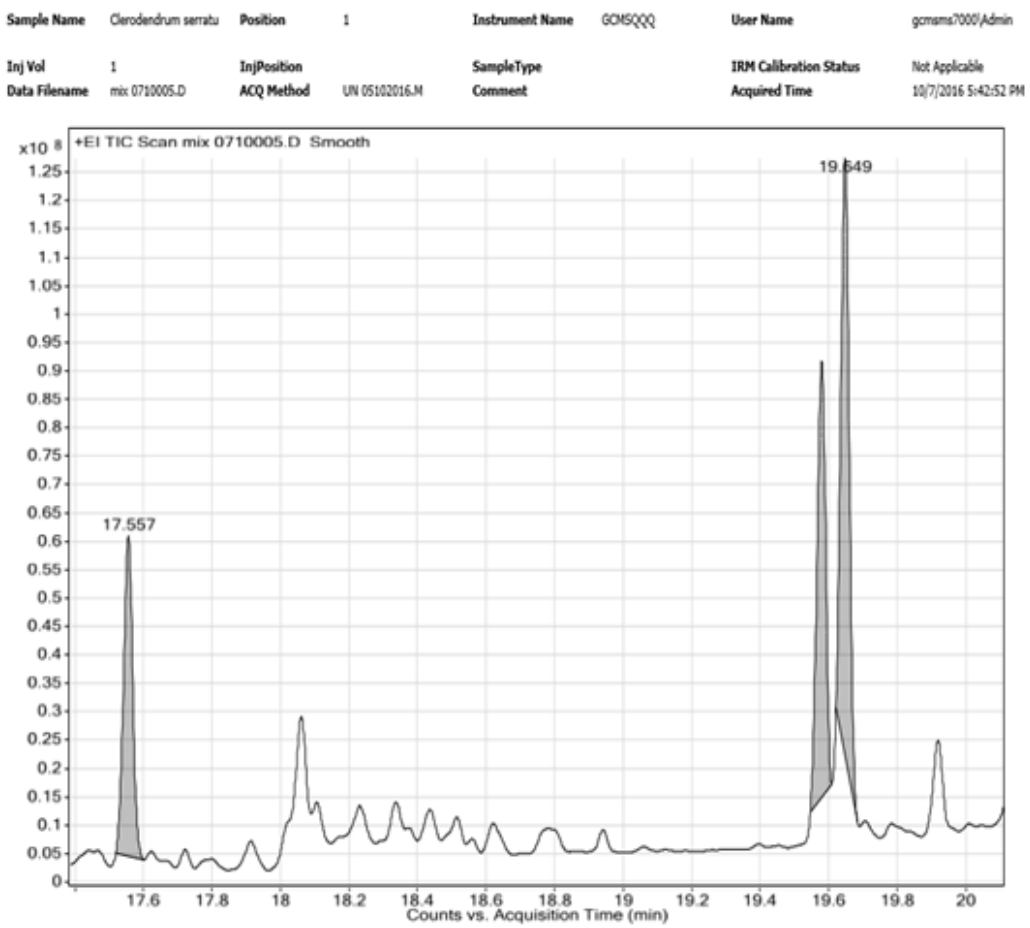


Fig 5.10 : GC-MS of *Clerodendrum serratum*

The present study provides a scientific support for the traditional use of plants as well as documents for volatile principles present in it. Volatile oil is found to have biological significance with antibacterial, antifungal, antiseptic property, etc. Bharangi has a wide range of medicinal value in regard to the constituents identified. Compound Hexadecanoic acid, methyl ester showed the highest percentage of about 76.5 possessing medicinal value of antibacterial and antifungal property, whereas Benzene, 1,3-bis (1,1-dimethylethyl) was found to be 74.9% having lipid oxidation activity. Other compounds identified were having antimicrobial, anticancer, cosmetic and commercial value. Moreover, the method can also be used as a quality control measure for bharangi plant. Activity reported for the identified compounds is mentioned in the table 5.7 for respective compounds [17-28].

5.8 HPLC method validation of plant extract, according to ICH Guidelines of extract

A plot between the concentration of a standard solution of Ursolic acid and peak response gave a straight line with a correlation coefficient 0.998 (Figure 5.14). The figure 5.14 represents the linearity curve of Ursolic acid. There was a good recovery of the substances, i.e. 95%-110% of the standards were obtained when spiked with standard at four different concentration levels. The HPLC method was found to give repeatable results at different concentrations and thereby the method was found to be reliable. Sample peak purity was checked by comparing with standard for retention time and peak area. LOD and LOQ was quantified as 33µg/ml and 0.059% respectively for the ursolic acid of test sample (extract). Validation was admitted to ICH guidelines for Linearity, Precision, Accuracy, Range, Specificity and ruggedness tabulated in table (5.21-5.23).

Acceptance criteria RSD<2.5 and RSD<5 for ruggedness.

5.8.1 Collection of reference standards

The reference standard ursolic acid was procured from with % Purity >95%

5.8.2 Collection of samples

Sample was collected as mentioned in section 5.1, Chromatogram of blank, sample and standard ursolic acid is mentioned in figure 5.11-5.13.

5.8.3 Collection of solvents

The details of solvents that were used throughout the experiment are given in the table no. 5.8

5.8.4 Collection of materials

Details of materials viz. glassware and membrane are given in table no. 5.9 & 5.10 respectively.

5.8.5 Collection of equipments

The details of equipments viz. analytical weighing balance, HPLC column, sonicator, water purification system, vacuum filter pump, glass vacuum mobile phase system, water bath, sample filtration and assembly were used which are given in the table 5.11-5.19 respectively.

5.8.6 Chromatographic condition

A definite chromatographic condition was maintained throughout the validation process, only column being changed in case of ruggedness.

5.8.7 Validation procedure

The estimation of Ursolic acid in *Clerodendrum Serratum* L. sextract was done by Quantification method. Their possible matrix with their details are given in the table no.5.20

Table No. 5.8: - Details on solvent used

Solvent Name	Grade	Product No	Batch No	Supplier Name
Acetonitrile	HPLC	1.44037 2.44047 3.44047	7537 6512-3 7239 6505-3 7455 6510-3	Qualigens
Methanol	HPLC	4360Q 4360Q	7701 6603-3 7790 6605-3	Qualigens
Water	Ultra pure Grade of 18 MOhm resistance	From Sartorious water purification system.		

Table No. 5.9: - Details on Glassware

Glassware	Capacity in ml	Class	Class
Volumetric flask	10ml, 100ml	A & B	B
Pipettes	1ml, 2ml, 5ml	A & B	B
Beakers	10ml, 250ml, 500ml	A & B	B
Measuring cylinder	100ml, 1000ml	A & B	B
Funnel	10 ml, 100 ml	A & B	B

Table No. 5.10: Details on Membrane Filter

Filter	Lot No	Description	Supplier Name
Nylon	05-061D000891	Ultipor® N_{66} Nylon 6,6 Membrane (0.45µm) P/N 60172	PALL. Life Sciences, Mumbai

Table No. 5.11 : Details on Analytical Weighing Balance

Balance Name	Instrument Code	Description	Supplier Name
Sartorius-BP-211D	QCD/CIF/EB/01	Weighing capacity 1mg to 210gm (max) with 0.01mg accuracy	SARTORIUS AG, GERMANY, ISO 90001
Sartorius-CP-245D	QCD/AHP/EB/01	Weighing capacity1mg to 220gm (max) with 0.1mg accuracy	

Table No. 5.12 : Details of HPLC

System	Instrumental Code	Description	Supplier Name
HPLC	QCD/CIF/HPLC/02	Shimadzu Liquid Chromatography HPLC-LC2010A	M/S.Spinco Biotech Pvt.Ltd.
HPLC	QCD/CIF/HPLC/03	Shimadzu Liquid Chromatography HPLC-LC2010A	M/s. Spinco Biotech Pvt. Ltd.

Table No. 5.13: Details Column

Column	Column code	Column No.	Description	Supplier
C_{18}	QC-C18-50	524094	HIbar®RT250-4.6 Prepacked column; Cat1.01886; Lichrospher®100; RP-18 e (5µm); Lot L54000633; NO.524094	Merck.k GaA, 64271, Germany

Table No. 14: Details Sonicator

System	Instrument Code	Description	Supplier Name
SIDILU UTRASONICS SONICATOR	QCD/AHP/SON/01	50 frequency/sec, Ultrasonic frequency 37±3,230Volt, Adjust for 1-9 min	SIDILU UTRASONICS, Bangalore
BANDELIN SONOREX	QCD/CIF/SON/01	230Volt ≈ 50/80Hz, HF-frequency 35KHz,adjust for 1-15 min	BANDELIN SONOREX, Berlin

Table No. 5.15: Details Water purification system

System	Instrument Code	Description	Supplier Name
Sartorius, arum 611 UV purifier	QCD/CIF/WP/01	Sartorius, arum 611 UV, 100Psi/6,9 bar max	Sartorius Mechatronics (I) Plt, Bangalore -58

Table No. 5.16: Details Vacuum filter pump

System	Description	Supplier Name
Aue-DLF Vacuum filter pump	Aue-LF, 1/4HP, 230Volt, 1PH, 50Hz, 1440rpm	Aue-DLF Universal limited, America

Table No. 5.17:- Details Glass vacuum mobile phase system

System	Description	Supplier name
Glass vacuum mobile phase system	Made up of borosilicate glass having filter size 47 mm diameter	Riviera Glassware Pvt.Ltd, Mumbai

Table No. 5.18: Details Water bath

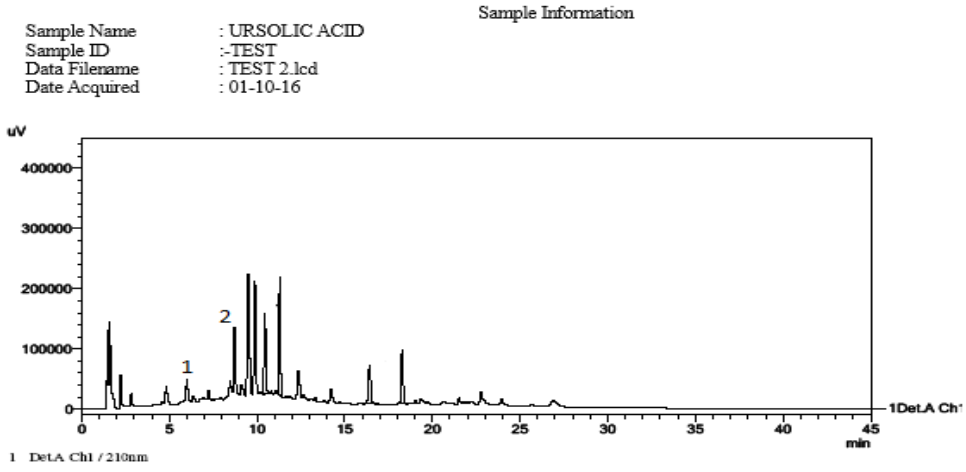
Apparatus	Description	Supplier name
Water bath	Equitron make stirred water bath, having inner chamber (LxBxDepth) 30x15x15 cm.	Serewell Instrument Pvt.Ltd, Bangalore

Table No. 5.19: - Details Sample filtration assembly (Syringe filtration assembly)

System	Description	Supplier name
Sample filtration assembl	Syringe filter filters 1-5 ml at high flow rate and higher filtration speed with a diameter 25 mm	Shandon HPLC, England

Table No. 5.20: - Details on possible matrix

Possible Matrix:	Details
Saponins,Triterpenoid acid, carbohydrates and Glycosides	Saponins, D-Mannitol,Stigmasterol,Oleanolic acid,Ferulic acid , Lupeol , Ursolic acid, 3 Serratumin A,Acteoside and Martynoside, Serratumoside-A and Myricoside



Peak#	Name	Retention time	Area	Area area %	Resolution
1	Unknown Peak	5.324	2280	0.384	0.00
2	Ursolic acid	8.058	85770	99.616	3.834

Fig 5.11 Chromatogram of HPLC(*Clereodendrum Serratum*)

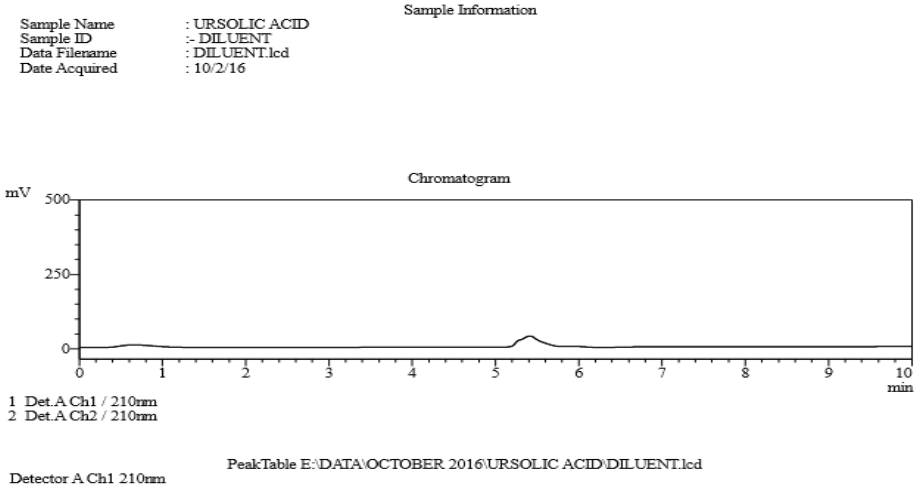
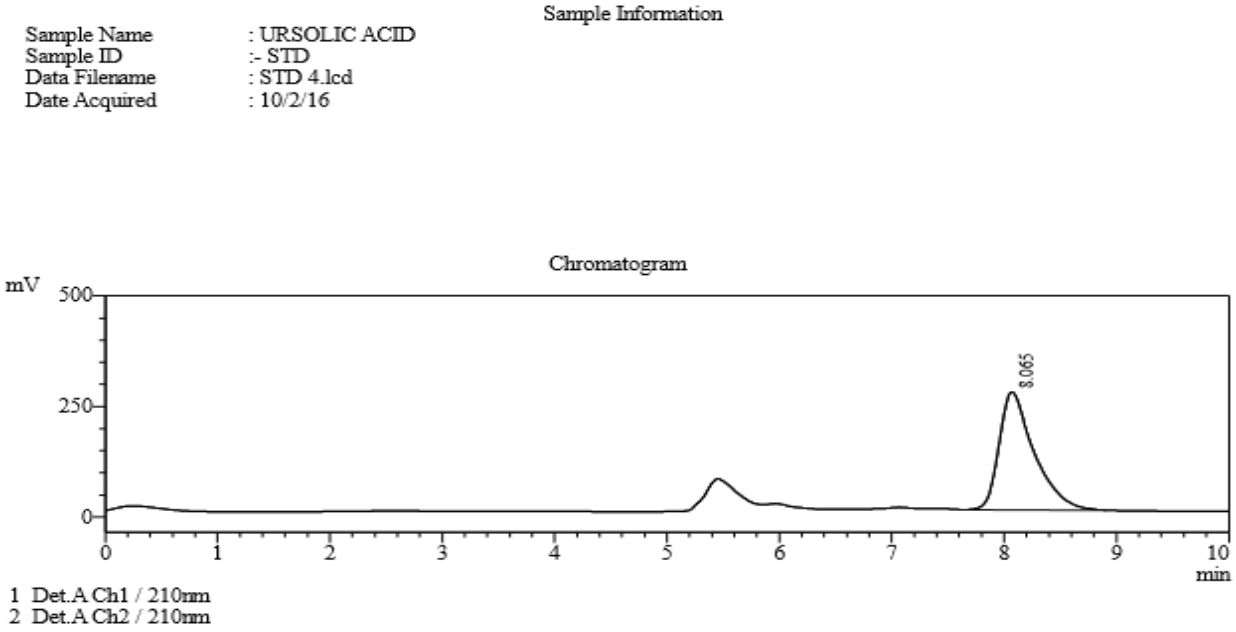


Fig 5.12 Chromatogram of HPLC(Blank)



Peak#	Name	Ret. Time	Area	Area %
1	RT8.065	8.065	5775722	100.000
Total			5775722	100.000

Fig 5.13 Chromatogram of HPLC (Ursolic acid standard)

5.8.7.1 Procedure for linearity

HPLC was run for determination of linearity by injecting the samples for 5 replicates for about 6 different concentrations within the standard working range for analytical procedure. Serial standard dilutions (I-VI) were made which are tabulated in table no. 5.21. The Chromatogram was recorded to determine the Retention time, Peak area, % peak area, assigning the name to the peak mentioned in table 5.22.

Acceptance criteria: Coefficient of correlation < 0.99 r^2 mentioned in figure 5.14. Sketching the graph concentration on X-axis and mean peak area on the Y-axis Co-rrelation coefficient was calculated.

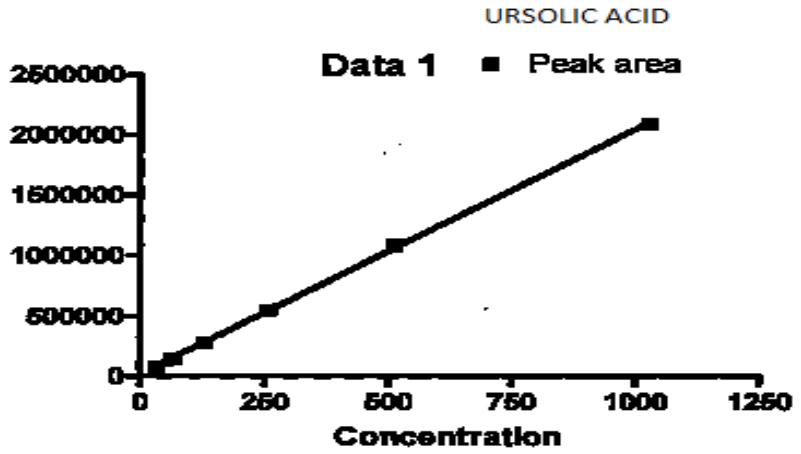


Fig 5.14 Calibration curve (Ursolic acid)

Table 5.21: Dilution for standard

	Analyte	Concentration (µg/ml) Dilutions					
		I	II	III	IV	V	VI
1	Ursolic acid	1070	535	267.5	133.75	66.87	33.43

Table 5.22: Peak area for standard

Replicates	I	II	III	IV	V	VI
1	5881787	2940893	1470446	745222	367612	183806
2	5788907	2880893	1420444	742228	369620	182810
3	5822254	2820893	1450452	740230	365812	185809
4	5775722	2850893	1440454	739223	363615	180811
5	5783395	2800893	1450462	735254	367624	184807
Average	5810413	2858893	1446452	740431	366856	183608

5.8.7.2 Procedure for specificity

The Retention time, resolution and relative retention time (RRT) was obtained from chromatographic spectra and the RRT was calculated tabulated in table 5.23 to check the peak purity for confirmation of ursolic acid in a test sample compared to standard for specificity of the analyte.

Acceptance criteria: - Resolution > 2%.

Table 5.23: Retention time and resolution of Ursolic acid

Parameters	Analyte
	Ursolic acid
Retention time	10.7686
RRT	1
Resolution	2.5

5.8.7.3 Procedure for precision

Precision is a measurement to get accurate results complies for repeatability and reproducibility.

Acceptance criteria: RSD<2.5% for peak area and retention time.

5.8.7.3.1 Repeatability (for the system) and reproducibility:

Precision is determined by injecting the test sample (5 times) within the working range concentration**.** The chromatogram was recorded to determine the peak area, retention time and assay content. The mean, standard deviation and relative standard deviation for precision is tabulated in table 5.24-5.26 and reproducibility in table5.27 & 5.28.

Table no. 5.24: Peak area of Ursolic acid

Replicate	Ursolic acid
1	1470446
2	1420444
3	1450452
4	1440454
5	1450462
Average	1446452
SD	16249.21
RSD	1.1233

Table no. 5.25: Retention time of Ursolic acid

Replicate	**Ursolic acid**
1	8.065
2	8.002
3	8.049
4	8.065
5	8.078
Average	8.0518
SD	0.026544
RSD	0.3296

Table no. 5.26: Assay content of Ursolic acid

Replicates	**Ursolic acid**
1	0.059%w/w
2	0.060%w/w
Mean	0.059
SD	0.00
RSD	0.83

Table no. 5.27: Reproducibility for method (By same analyst)

Test sample	Analyte	Retention time	Retention time	RSD
500mg	Ursolic acid	8.031	8.045	0.087086
100mg	Ursolic acid	8.058	8.054	0.111815

Table no. 5.28: Reproducibility for method (By Different analyst)

Test sample	Theoretical value	Practical value	RSD
400mg	8.065	8.058	0.043416
200mg	8.065	8.031	0.211233

5.8.7.4 Validation of accuracy

Accuracy was done by spiking the test sample with standard to check the recovery of ursolic acid in the extract in different concentration from (50% to 200%) of the analytical standard range. The sample was analyzed according to the method and the assay value, % recoveries were reported tabulated in table 5.29 & 5.30.

Assessment: Accuracy is determined by the spike recovery method.

Acceptance Criteria: Assay>10 %< 95% between 95-110%

Assay >0.5 %< 7.5% between 85-120%

Table 5.29 : Assay content

Batch No	Observed value
(1502.3mg) Ursolic acid	0.059%

Table 5.30: Spiking and recovery

Substance	Amount (%) of standard spiked in the sample	Theoretical content	Amount of substance recovered	% of substance recovered
Ursolic acid	0	0.059 %	0.057%	96.61
	50	0.063%	0.065%	103.17
	100	0.068%	0.071%	104.41
	150	0.073%	0.076%	104.41

5.8.7.5 Validation of range

Assessment: Linearity studies were used to determine the exact range within which the method is validated for analytical method**.** Response factor was concluded for all the validation parameters.

Acceptance criteria: RSD < 2.5

The response factor of ursolic acid is tabulated in the table no.5.31. The RSD for response factor is tabulated in the table no.5.32. The range of the ursolic acid was tabulated in the table no.5.33.

Table 5.31 : Response factor for Ursolic acid

Replicate	I	II	III	IV	V	VI
1	5881787	2940893	1470446	745222	367612	183806
2	5788907	2880893	1420444	742228	369620	182810
3	5822254	2820893	1450452	740230	365812	185809
4	5775722	2850893	1440454	739223	363615	180811
5	5783395	2800893	1450462	735254	367624	184807
Average	5810413	2858893	1446452	740431.4	366856.6	183608.6
Response factor	543029	534373	541742	556715	555844	556391

Table 5.32: RSD for response factor for Ursolic acid

Sr.no	Ursolic acid
1	543029
2	534373
3	541742
4	556715
5	555844
6	556391
Avg	548016
SD	8731.744
RSD	1.593338

Table 5.33: Range for Ursolic acid

Reference standard	Concentration range
Ursolic acid	1070 to 33 mcg/ml

5.8.6 Validation of ruggedness

Assessment: The objective of ruggedness in regard to validation is to provide confirmation of analytical procedure by varying different conditions like chromatographic parameters of the HPLC run (Column, pH, mobile phase) etc.

The degree of obtained results is compared to the precision value obtained without variable conditions tabulated in table 5.34.

Acceptance criteria: RSD < 5

Table 5.34 : Ruggedness for Ursolic acid

Sample	%Purity(Ruggedness)	%Purity (Precision)	RSD
Test sample	0.048%	0.059%	0.031

5.9 Evaluation of *in-vitro* antioxidant activity

In vitro anti-oxidant activity of the aqueous extract of root of *Clerodendrum Serratum* L. was determined by evaluating the DPPH*, Superoxide scavenging assay and FRAP assay. The experiment was done in triplicate for both the sample The IC_{50} values are 85.43 µg/ml and 107.59 µg/ml for DPPH radical scavenging and Superoxide scavenging assay, respectively, whereas FRAP showed significantly reduced activity with increased concentration of the sample

mentioned in figure 5.35-5.36 and figure 5.15. Rutin was used as reference standard. Calculation of percentage of inhibition was done by using following formula:

% inhibition= [(A_0-A_1/A_0)] X 100

Where, A_0 is the control absorbance (blank, without extract) and A_1 the extract or standard absorbance. The mean percentage inhibition by each concentration was plotted against the log concentration. The 50% inhibitory dose (IC_{50} value) was found by interpolation by using graph pad (prism 6 software) and compared with standard [27].

Table 5.35: DPPH radical scavenging assay

SL No	Concentration (µg/ml)	Inhibition (%)		IC50 (µg/ml)	
		Rutin	Sample	Rutin	Sample
1	20	52.86±0.089	31.42±0.399	10.10	85.43
2	40	55.50±0.358	36.42±0.224		
3	60	58.30±0.016	40.97±0.145		
4	80	61.68±0.092	45.81±0.083		
5	100	68.57±0.342	56.53±0.347		

Table 5.36: Superoxide scavenging assay

SL No	Concentration (µg/ml)	Inhibition (%)		IC50 (µg/ml)	
		Rutin	Sample	Rutin	Sample
1	20	51.95±0.026	25.61±0.377	11.18	107.59
2	40	57.30±0.226	34.05±0.038		
3	60	66.77±0.234	38.06±0.029		
4	80	72.42±0.261	42.28±0.290		
5	100	74.07±0.062	47.73±0.186		

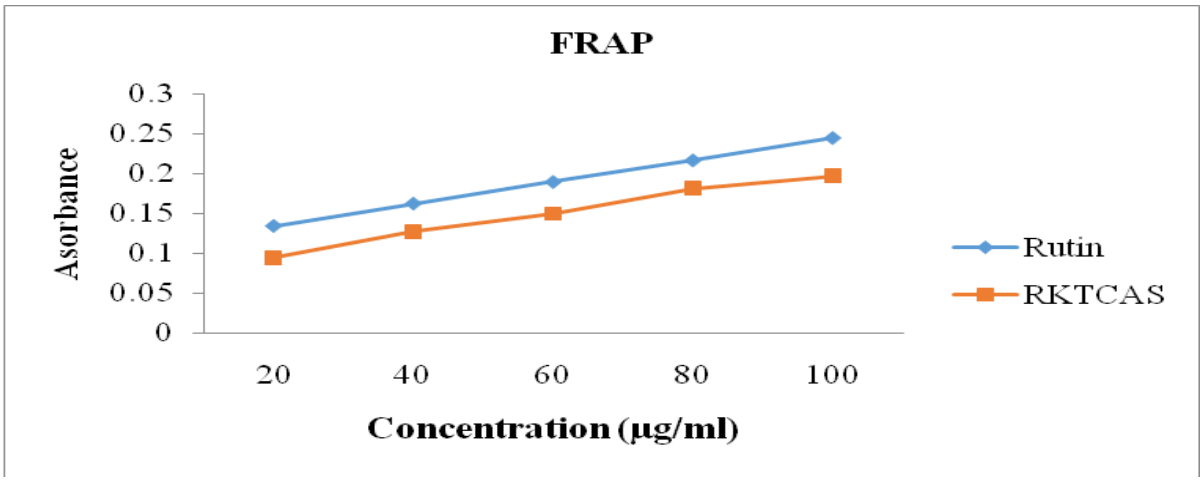


Fig. 5.15: FRAP assay showing the antioxidant capacity to reduce ferric ions of sample with increase in absorbance of sample and standard.

The *Clerodendrum serratum* aqueous extract has significant antioxidant activity against DPPH scavenging assay, Superoxide scavenging assay and Ferric reducing antioxidant power (FRAP). Plant exhibits the antioxidant activity through the scavenging of free radicals and FRAP showed significant reducing power activity with increased concentration of the sample.

5.10 *In-vitro* Anti inflammatory Activity

5.10.1 Hypotonic solution –Induced haemolysis or membrane stabilizing activity

Plant derived drug have been demonstrated to contain secondary metabolites which enhances the stability of biological membranes when stressed under lysis. The *Clerodendrum serratum* aqueous extract was found to possess membrane stabilizing property which is one of the preliminary steps involved in the screening of anti-inflammatory property. Membrane of RBC structurally resembles the lysosomal membrane, the effect of any substance on stabilization of RBC membrane may be extrapolated to the stabilization of lysosomal membrane tabulated in table 5.37.

Table 5.37 Hypotonic solution –Induced haemolysis or membrane stabilizing activity

Sr. no.	Conc.(µg/ml)	% Inhibition	
		Standard ASA	**Sample RKTCAS**
1	100	37.93±0.247	8.67±0.276
2	200	42.97±0.310	12.40±0.310
3	300	47.14±0.117	16.95±0.276
4	400	51.48±0.117	20.76±0.193
5	500	57.23±0.089	22.90±0.234

4.10.2 Effect of Protein Denaturation

Anti-arthritic effect of *Clerodendrum serratum* was studied significantly by using *in-vitro* inhibition of protein denaturation and it can be concluded that *Clerodendrum serratum* extract can serve as an anti-arthritic agent as tabulated in table 5.38.

Table 5.38: Protein Denaturation

Sr. no.	Conc.(µg/ml)	% Inhibition	
		Standard ASA	**Sample RKTCAS**
1	100	67.73±0.448	39.62±0.448
2	200	73.49±0.356	44.25±0.625
3	300	79.89±0.178	47.98±0.448
4	400	82.80±0.205	51.66±0.719
5	500	85.23±0.178	55.93±0.544

5.11 Evaluation of *In- vivo* anti arthritic study

Complete Freund's adjuvant (CFA) induced arthritis protocol was adopted. Vehicle/drug treatment was continued for the duration of 20 more days. Arthritic parameters like arthritic score, Joint diameter and Paw diameter were recorded, tabulated in table 5.39, 5.40 & 5.41,5.42 & 5.43. Blood was collected from the terminal part of animals. Level of TNF-α, COX-2 was estimated by ELISA assay. The ankle joints part was further processed for histological studies mentioned in figure 5.16 showed a significant result at the dose of 200 mg/kg body weight.

Table 5.39 : Paw Diameter(mm)

Groups	0 day	3day	7day	14 day	21 day
Group 1(Vehicle)	3.44±0.109	3.56±0.113	3.50±0.104	3.52±0.131	3.52±0.101
Group 2 (positive control)	3.58±0.164	5.01±0.164	5.23±0134	5.89±0.150	6.060.123
Group 3 (Standard)	3.50±0.118	4.08±0.089	4.64±0.105	4.89±0.120	4.17±0.188
Group 4 (RKT-100 mg/kg)	3.51±0.064	4.74±0.125	5.06±0.086	5.46±0.063	4.89±0.155
Group 5 (RKT-200 mg/kg)	3.54±0.097	4.42±0.108	4.91±0.093	5.17±0.136	4.43±0.162

RKT = Aqueous extract of Clerodendrum serratum; n=6; Data = Mean±SEM; **P<.01(G2 Vs G4); αP<0.001 (G2 Vs G3, G4 & G5)

Table 5.40 : Joint Diameter(mm)

Groups	0 day	3day	7day	14 day	21 day
Group - 1 (Vehicle)	6.10±0.605	6.140.593	6.10±0.571	6.12±0.557	6.12±0.576
Group - 2 (positive control)	6.17±0.528	7.43±0.522	7.86±0.521	9.51±0.523	9.67±0.515
Group - 3 (Standard)	6.17±0563	6.71±0.572	7.18±0.542	7.31±0.504	6.65±0.490
Group - 4 (RKT-100 mg/kg)	6.24±0.464	7.55±0.534	7.81±0.500	8.21±0.522	7.62±0.461
Group - 5 (RKT-200 mg/kg)	6.24±0.445	7.18±0.487	7.57±0.491	7.77±0.484	7.10±0.495

RKT = Aqueous extract of Clerodendrum serratum; n=6; Data = Mean±SEM; **P<.01(G2 Vs G4); αP<0.001 (G2 Vs G3, G4 & G5)

Table 5.41 : Arthritic Index

Groups	Arthritic score
Group - 1 (Vehicle)	0.00±0.00
Group - 2 (positive control)	3.50±0.548
Group - 3 (Standard)	1.33±0.516
Group - 4 (RKT-100 mg/kg)	2.33±0.516
Group - 5 (RKT-200 mg/kg)	1.67±0.516

RKT = Aqueous extract of Clerodendrum serratum; n=6; Data = Mean±SEM; *P<.05(G2 Vs G4); αP<0.001 (G2 Vs G3 & G5)

Table 5.42 : Body Weight (gm)

Group	INITIAL	FINAL
Group - 1 (Vehicle)	192.83±6.555	217.17±6.047
Group - 2 (positive control)	198.67±6.218	210.83±6.555
Group - 3 (Standard)	203.17±10.998	223.00±7.772
Group - 4 (RKT-100 mg/kg)	196.17±7.935	213.67±8.756
Group - 5 (RKT-200 mg/kg)	194.00±6.870	213.50±10.232

Table 5.43 : TNF-α and COX-2 Estimation

Groups	TNF-α (ng/ml)	COX-2 (ng/ml)
Group - 1 (Vehicle)	3.13±0.659	5.73±0.093
Group - 2 (positive control)	13.46±0.848	9.86±0.101
Group - 3 (Standard)	5.03±0.612	6.40±0.108
Group - 4 (RKT-100 mg/kg)	10.63±0.707	8.08±0.129
Group - 5 (RKT-200 mg/kg)	8.40±0.188	7.14±0.0.86

RKT = Aqueous extract of Clerodendrum serratum; n=6; Data = Mean±SEM; αP<0.001 (G2 Vs G3, G4 & G5)
For COX 2: **P<.01(G2 Vs G4); αP<0.001 (G2 Vs G3 & G5)

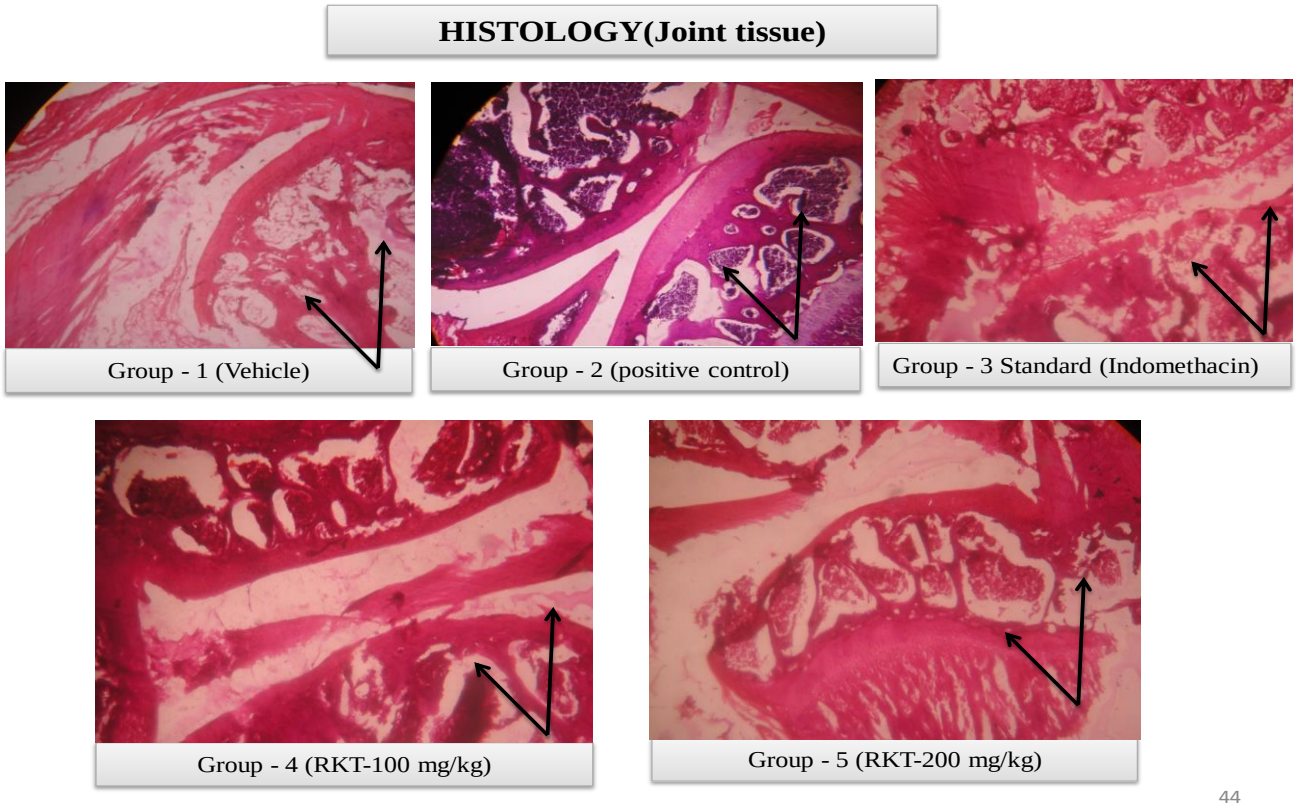


Figure 5.16 : Histology of ankle joint

Arthritic parameters along with histopathological study indicate that *Clerodendrum serratum* possesses significant arthritis activity. The aqueous extract of *Clerodendrum serratum* possess anti– inflammatory and anti-arthritic activity observed in parameters like Paw edema, Arthritic index and Joint diameter. The anti arthritic activity of *Clerodendrum serratum* was observed by inhibition of COX-2 and TNF-α. The potential activity of various ingredients in *Clerodendrum serratum* acting synergistically and working in concert for overall anti-arthritic activity.

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CHAPTER 6

CONCLUSIONS

The thrust in the area of development of new benefits and alternative treatment for arthritis is day by day increasing due to the several existing unavoidable side effects and limitations of currently available drugs.

India being one of the 12 mega bio-diversity, culturally firm and have strong knowledge about the use of plants and people live in their own style in different communities. On the basis of observation for the traditional claim, current investigation was done to check out, the richness of ethno-medicinal plant species with a proper scientific approach, used by the different communities for arthritis disorders.

Roots of *Clerodendrum serratum* L. (Verbenaceae) was purchased from the local market of Faizabad Uttar Pradesh, India. The taxonomical recognition was done from Department of Agronomy, Aacharya Narendra Dev Agriculture Technical University, Dist. Faizabad, Uttar Pradesh.

The collected brown colored roots (100gm) were extracted (decoction) with water (500ml) at a temperature not exceeding 110^0C for 2hrs. The extract was allowed to cool, filtered & lyophilized. The practical yield of the aqueous extract of the plant was 10%w/w.

Phytochemical analysis of aqueous root extract indicated the presence of triterpenoids, volatile oil, sterols, alkaloid and glycoside. HPTLC analysis reveals the presence of Ursolic acid. Further GC-MS analysis of hexane root extract indicated the presence of 15 compounds which were reported in *Clerodendrum serratum* L. The root extract was standardized by HPLC and the analytical method was further validated in accordance with ICH guidelines. The validated HPLC method is established for analyzing ursolic acid in *Clerodendrum Serratum.* Quantitative estimation revealed 0.059% Ursolic acid present in the extract determined from the regression equation. A simple RP-HPLC is validated for the quantitative estimation of Ursolic acid in *Clerodendrum serratum* extracts.

The established HPLC method provides a quantitative estimation of ursolic acid in extracts of *Clerodendrum serratum* is standardized and validated within the range. Accordingly the proposed HPLC method can be utilized for for the quality control and assay of the markers in *Clerodendrum serratum* for ursolic acid.

The aqueous extract of the root was subjected to *in-vitro* and *in-vivo* anti arthritic activity. IC_{50} values for was around 85.43 µg/ml and 107.59 µg/ml for DPPH radical scavenging and Superoxide scavenging assay for extract, respectively,whereas FRAP showed significant reducing power activity with increasing concentration of the sample.

The extract showed around 8% and 40% inhibition for membrane stabilizing and protein denaturation with 100 µg/ml concentrations. The results of the present experiments indicate that *Clerodendrum serratum* possesses anti-inflammatory and anti-arthritic activity revealed in arthritic parameters like paw edema, arthritic index and joint diameter. The anti-arthritic activity of *Clerodendrum serratum* was observed by inhibition of COX-2 and TNF-α.

Anti-arthritic activity of standardized aqueous extract and validated method of *Clerodendrum Serratum* was established by Freund's Complete in rats. FCA is established experimental model worldwide for the study of arthritis. This anti-arthritic effect is due to inhibition of COX-2 inhibition and TNF-α level as compared to identical effect of Indomethacin. Moreover, the results obtained in the study show favorable effects of plants from the restoration of arthritis by including body weight, arthritic score along with clinical observations like histopathological examination.

The current study established scientific basis of ethnomedicinal use of *Clerodendrum serratum* for arthritis. The study also reveals the medicinal importance of the plant as of anti-inflammatory and free radical scavenger. The plant has potential to be commercialized in the future. The study also documents the use and significance of *Clerodendrum serratum* as ethnomedicinal plant and also suggest a possible alternate solution for arthritis to overcome limitation and drawbacks of current medicines.

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