

FORMULATION DEVELOPMENT AND EVALUATION OF POLYHERBAL ANTI- INFLAMMATORY PATCH

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MASTER OF PHARMACY

PHARMACOGNOSY



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VADODARA-390001

JULY 2021

CERTIFICATE

This is to certify that, this dissertation entitled,

FORMULATION DEVELOPMENT AND EVALUATION OF POLYHERBAL ANTI-INFLAMMATORY PATCH

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As a partial fulfilment for the award of Degree of Master of Pharmacy in Pharmacognosy, Faculty of Pharmacy, The Maharaja Sayajirao University of Baroda, Vadodara has been carried out under my supervision and guidance, the matter compiled in this thesis has not been submitted earlier for the award of any other degree or fellowship.

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DECLARATION

*I hereby declare that the topic entitled “**FORMULATION DEVELOPMENT AND EVALUATION OF POLYHERBAL ANTI-INFLAMMATORY PATCH**” which is submitted herewith to The Maharaja Sayajirao University of Baroda, Vadodara for the partial fulfilment for the award of degree of **Master of Pharmacy in Pharmacognosy** is the result of work done by me in Faculty of Pharmacy, The M. S. University of Baroda, under the Guidance of Mr. Bhavik Chauhan, Assistant Professor, Pharmacognosy, Faculty of Pharmacy, The Maharaja Sayajirao University of Baroda, Vadodara.*

I further declare that the result of this work has not previously been submitted for any degree or fellowship.

Date : 15 July, 2021

Place : Vadodara

Zuli M. Shingala

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Dedicated to my beloved
parents and my little
brother for their love,
support, endless hopes
and encouragement



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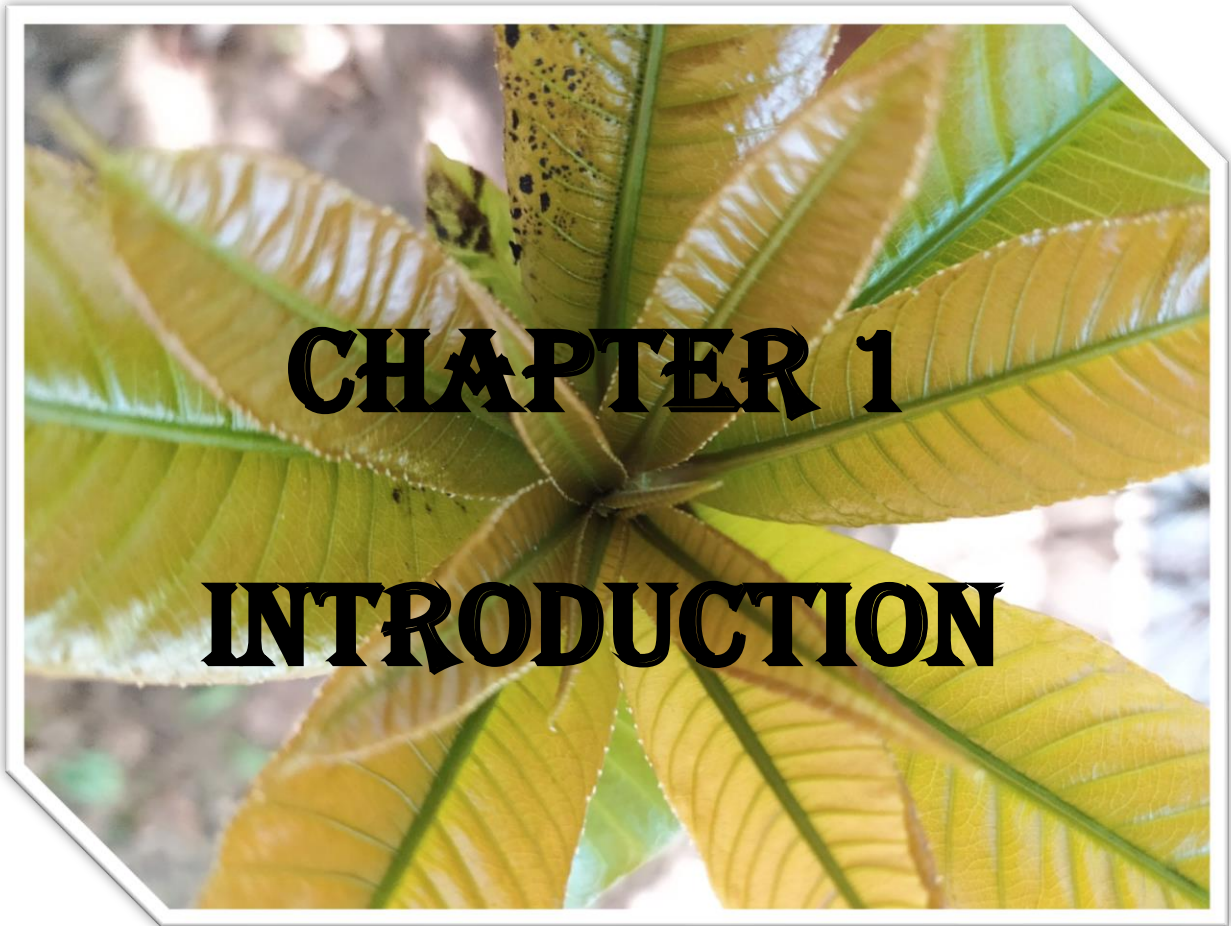
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CHAPTER 1

INTRODUCTION

1. Introduction

Diseases are born with man and drugs came into existence since a very early period to remove the pain of diseases and to cure them. Thus, the story of history of drugs is as old as mankind. Ayurveda, a traditional holistic health care system, older than any other system of medicine, has been practiced in India for more than 5000 years. In India knowledge of medicinal plants is very old. Medicinal activity of plants described in *Rigveda* and *Atharvaveda* (3500-1500 B.C.), from this two veda Ayurveda has developed [1].

Ayurveda recognized as alternative medicine now, represents the science of life and longevity originating in the vedic traditions of India, but transmitted only orally for generations. There are many traditional systems based on plants for example Indian Traditional Medicinal system (Ayurveda), Chinese Traditional Medicinal system, Siddha and Unani system of Medicine.

However, these plant based traditional medical systems generally lack adequate documentation and codification. Later on the modern science has confirmed that many of the medicinal plants traditionally used by indigenous people were indeed having the medicinal properties to cure the diseases they were used against for ages. The modern world was initially surprised over such findings. However, such a surprise seems to be misplaced in view of the intimate knowledge that the ancient man possessed about his natural environment.

These historical experiences with plants as therapeutic tools have helped to introduce single chemical entities in modern medicine.

Plants especially plants having medicinally active constituents or plants with ethnopharmacological uses, have been the primary source of medicines for early drug discovery. Current drug discovery from terrestrial plants has mainly relied on bioactivity guided isolation of phytoconstituents, for example, it led to discovery of the important phytoconstituent and potent anticancer agent Paclitaxel from *taxus brevifolia* and Camptothecin from *Camptotheca acuminata* [2].

Herbal medicine is a branch of science in which plant based formulations are used to cure the disease conditions and also as a preventive therapy. It is also known as botanical medicine or phytomedicine. “Herbal Medicines” are the medicinal products that contains an active ingredient, aerial or underground parts of the plants or other plant material or combination of thereof, whether in the crude state or as plant preparations. Plant material

includes juices, gums, fatty oils and any other materials of this nature. Herbal medicines may contain excipients in addition to active ingredient.

Herbs : is a crude plant material such as leaves, stem, roots, flowers, fruits, seeds, bark, wood, rhizomes or any plant part which may be whole part or powdered.

Herbal material : is the either whole plant or is the part of medicinal plant in the crude state, which includes herbs, fresh juice, gum, resin, oil or essential oil.

Herbal preparations : these are the fundamentals for the finished products and may include pulverized or powdered herbal materials or extracts, juice, fatty oils and processed exudates of herbal materials.

Finished herbal products : these are the final preparations made from one or more herbs, like natural active ingredients and excipients. If there is more than one herb is used to make herbal preparation, it is termed as “mixed herbal product” or “**Polyherbal formulation**”.

In the early twentieth century herbal medicine was prime healthcare system as antibiotics or analgesics were not available, but with increasing use of synthetic allopathic medicine system, herbal medicine gradually lost its popularity and usage among people. Almost a century has passed and it has witnessed limitations and side effects of synthetic allopathic system of medicine, then herbal based formulations has gained popularity. It is evident from the fact that certain herbal remedies are more effective as compare to synthetic allopathic medicines [3].

1.1 Importance of Medicinal Plants to Human Healthcare [4] [5]

Many of the modern medicines are produced indirectly from herbal medicinal plants, for example aspirin. Plants are directly used as medicines by a majority of cultures around the world, for example Chinese Traditional medicine and Indian medicine from Ayurveda. Many food crops or spices have medicinal effects, for example Garlic, Cinnamon, Turmeric. Medicinal plants are sources of phytoconstituents which can be used as new drugs. It is estimated there are more than 250,000 flowering plant species. Studying medicinal plants helps to understand plant toxicity and protect human and animals from natural poisons, disease conditions and can prevent diseases. Cultivation and preservation of medicinal plants protect biological diversity, for example metabolic engineering of plants.

Medicinal plants are important for pharmaceutical research and drug development, not only when plant or phytoconstituents are used directly as therapeutically active agents, but also as starting materials for the synthesis of drugs or as models for pharmacologically active compounds.

❖ **Advantages of herbal medicines :**

Cost effective than allopathic medicine

Less side effects

Ease of availability

Natural healing

Effective in chronic conditions

❖ **Disadvantages of herbal medicines :**

Lack of dosage indications

Poison risk and harmful effects associated with wild or toxic plants

Medication interactions

Lack of safety and regulatory profile

1.2 Quality control criteria for herbal drugs [5]

The term “Quality” is defined as the status of drug or formulation, which is determined either by identity, purity, content and other chemicals, physical or biological properties, or by the manufacturing process.

The inconsistency of the plant material is due to different growth, harvest, drying and storage conditions. Hence the cultivation procedure needs to be standardized. Natural variations and biodiversity are the problems always associated with the quality of herbal drugs.

The quality of herbal medicines and formulations has a direct impact on their safety and efficacy. Many control measures are available for herbal medicines, and the first important step is to control the quality of medicinal plants and herbal materials. However, this is a very complicated and difficult task as it involves many different areas, such as the environmental conditions and agricultural practices.

There are many contaminants and residues that may cause harm to the consumers of herbal medicines. Many are natural, such as naturally occurring radionuclides, toxic metals or bacteria. Some arise from past or present use of agents or materials that pollute the environment and subsequently medicinal plants, such as emissions from factories or the residues of certain pesticides.

Recent research has also demonstrated that herbs may absorb heavy metals during growth. This risk can be reduced by ensuring that herbal medicinal products are standardized. Herbal medicines with harmful contaminants and pesticide residues does not reach to the people, by standardizing the quality of the medicinal plants, herbal raw materials and finished herbal products before they reach to the market.

1.2.1 Why standardization required ?

In Indian system of medicine there are about 1000 single drugs and 1500 formulations. These drugs are fully documented in traditional text for their therapeutic properties. But the difficulties are arrives when identification of appropriate plant species, their geographical source, time of collection, drying and storage conditions, microbial contamination and presence of xenobiotics, amongst other factors results affects in the quality and efficacy, Because of these factors we need to develop method for standardization of raw materials as well as finished herbal products or herbal formulations. For pharmaceutical purposes, the quality of the medicinal plant materials must be as high as that of other medicinal preparations.

Most of the herbal formulations, especially the classical formulation of the traditional medicine, are polyherbal formulations. Each formulation contains 10 to 20 ingredients, a few have even 50 to 75. Many preparations are either liquids or semi solids. For such formulations it is very difficult to establish parameters for quality control, furthermore, the unique processing method followed for the manufacture of drugs turn single drug in to very complex mixture.

1.2.2 Problems associated with standardization of herbal drugs [6]

Herbal medicines have a complex chemical composition. Standardization of certain marker compound of herbal drug does not serve the purpose of standardization. Since activity of herbal drug does not depend upon one or few components.

In most of the cases, it is the result of determined activity of several active compounds as well as inert allied substances. And yet these inert components do not directly affect the activity or potency of drug, but they might affect the bioavailability and excretion of active components. Further, these inert components may also play a role in the stability of the active component and helps to reduce the rate of side effects. If there are several active components present in a herbal drug, they may have additive or potentiating effect.

1.3 WHO guidelines for Quality assessment of Herbal Drugs [7], [8]

WHO has developed a series of technical guidelines and documents relating to the safety and quality assurance of medicinal plants and herbal materials.

1. Authentication

(stage of collection, parts of plant collected, regional status, botanical identity, morphology, microscopy, taxonomical identity, etc.)

2. Foreign matter (herbs collected should be free from soil, insect parts or animal excreta, etc.)

3. Organoleptic parameters (appearance, taste, odour, texture of drug, etc.)

4. Tissues of diagnostic importance present in the drug powder.

5. Ash values and extractive values.

6. Volatile matter.

7. Moisture content determination

8. Chromatographic and spectroscopic evaluation by TLC, HPTLC, HPLC methods.

Qualitative and quantitative information about the main active constituents present in the crude drugs as chemical markers in the TLC fingerprinting evaluation of herbal drugs.

9. Determination of heavy metals – Lead, Arsenic, etc.

10. Pesticide residue – WHO and FAO (Food and Agriculture Organization) sets limits of pesticides, which are usually present in the herbs.

11. Microbial contamination – usually medicinal plants contains bacteria and molds, which are coming from atmosphere and soil. Analysis of the limits of E.coli and molds clearly indicating the harvesting and production practices. The molds known as aflatoxins will produce harmful side effects if consume along with the crude drugs.

Aflatoxins should be completely removed or should not be present.

12. Radioactive contamination – microbial growth in herbals are usually avoided by irradiation. This process may sterilize the plant material but the radioactivity of the plant samples should be checked accordingly to the guidelines of international atomic energy (IAE) in Vienna and that of WHO.

Some other tests for certain type of plant materials :

13. Volatile oil content

14. Bitter value

15. Foaming index
16. Total tannins
17. Haemolytic activity
18. Total phenolic and flavonoid content

1.4 Inflammation : [10] [11]

Inflammation is a nonspecific, defensive response of the body to tissue damage. Among the conditions that may produce inflammation are pathogens, abrasions, chemical irritations, distortion or disturbances of cells, and extreme temperatures. The four characteristic signs and symptoms of inflammation are redness, pain, heat, and swelling.

Inflammatory response has three basic stages :

1. vasodilation and increased permeability of blood vessels
2. emigration (movement) of phagocytes from the blood into interstitial fluid
3. tissue repair.

These are the substances which are contributing into the vasodilation, increased permeability and other aspects of inflammatory response- Histamine, Kinins, Prostaglandins (PGs), Leukotrienes (LT) and different components of complement system.

Two main types of inflammation are Acute inflammation, associated with increased vascular permeability, capillary infiltration and emigration of leukocytes. Chronic inflammation, associated with infiltration of mononuclear immune cells, macrophages, monocytes, neutrophils, fibroblast activation, proliferation and fibrosis.

Pain is defined as a subjective phenomenon which is associated with actual or potential damage to tissues. There are two components are found: 1. Sensory, which associated with information of noxious stimuli, and 2. emotional, which is associated with a patient reaction to painful stimuli that is often associated with psychological reactions and an individual sensitivity to pain. [12]

1.5 Topical route of administration : [13]

Topical drug administration is a localized drug delivery system anywhere in the body through ophthalmic, rectal, vaginal and skin as topical routes.

Skin is one of the most readily accessible organ on human body for topical administration of drug and is the main route of topical drug delivery system. Topical formulations such as gel, spray, patches, creams and lotions are widely accepted in both cosmetic and pharmaceuticals.

1.5.1 Advantages of topical drug delivery system : [13]

- It will avoid first pass metabolism
- Ability to deliver drug more selectively to specific site
- Improving physiological and pharmacological response
- Improve patient compliance
- Provide suitability of self-medication
- Avoidance of the GI tract incompatibility
- Can be terminate the medication easily, whenever require

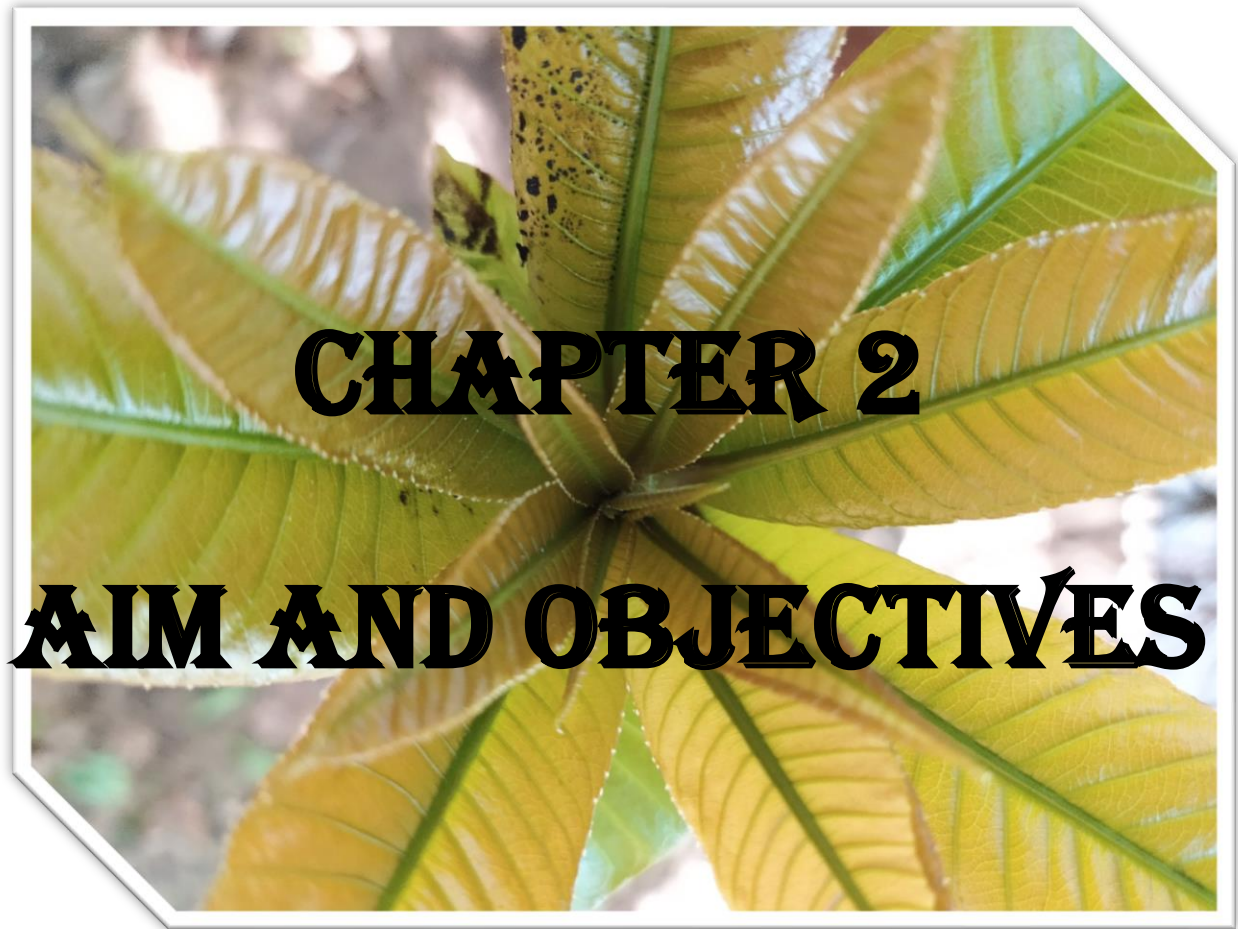
1.5.1 Disadvantages of topical drug delivery system :

- Possibility of allergic reactions
- Skin irritations may occur due to the some drug or excipients
- Drugs with poor permeability are difficult to penetrate through the skin.
- Drugs with larger particle sizes are difficult to penetrate via skin.

1.5.2 Classification of topical drug delivery system : [13]

Classification is based on type of formulation

- A. Solid formulations : Powder, Aerosol, Poultices
- B. Liquid formulations : Lotion, Solution, Emulsion, Tinctures, Liniments, etc.
- C. Semi-solid Formulations : Creams, Paste, Gels, Jelly, Suppositories, etc.
- D. Novel Drug Delivery : Hydrogel, Emulgel, Mucoadhesive formulation, Bioadhesive Formulation- patch, Liposomes, Microsponges, etc.



CHAPTER 2

AIM AND OBJECTIVES

2 AIM AND OBJECTIVE

2.1 RATIONALE

In the traditional system of Indian medicine, plant formulations and combined extract of plants are used as a drug of choice rather than the single drug. Even though the active phytochemical constituents of individual plants have been well established, they usually present in a minute amount and always, they are insufficient to achieve the desirable therapeutic effects. For this, scientific studies have revealed that these plants of varying potency when combined may theoretically produce a greater result, as compared to an individual use of plant.

In this context, the present studies have been designed to scientifically validate the traditional claims of *Boswellia serata* and *Capsicum annuum* and tried to develop new Polyherbal Anti-inflammatory topical patch formulation.

The purpose to develop a topical patch formulation is that it should give a fast and quick action/relief comparative to the other marketed formulations such as gels, creams and ointments.

The formulation consists of various components in it which when applied topically, spread uniformly and release drug into the skin.

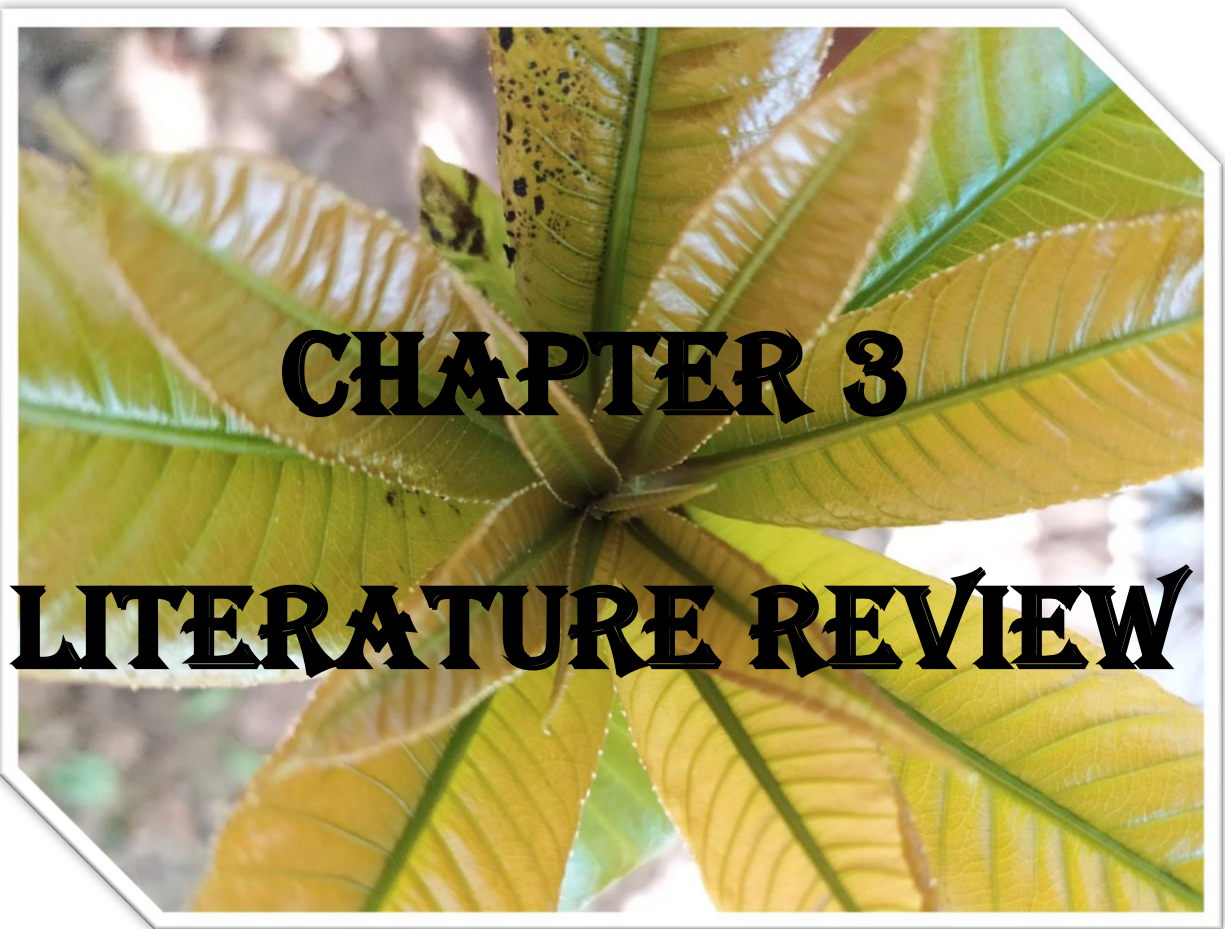
Although the physical parameters prove to be important standardization tools, the quantitative assessment of bioactive molecules has been empirically and scientifically proven to be better standardization parameter. Therefore, there is a need to develop analytical methods for quantification of the active constituents in polyherbal formulation. The present work is focused on developing new analytical technique for polyherbal patch formulation.

2.2 OBJECTIVES OF THE PRESENT STUDY

- ❖ To develop polyherbal Anti-inflammatory topical patch formulation by incorporating herbal extracts such as *Boswellia serata*, *Capsicum annuum* and Menthol as a cooling agent.
- ❖ To evaluate its Anti-inflammatory activity via *in-vivo* and *in-vitro*.
- ❖ To develop and validate HPTLC method for simultaneous estimation of Capsaicin and Boswellic acid in the developed polyherbal formulation.

2.3 PLAN OF WORK

- Collection of respective plant extracts.
- Preliminary phytochemical screening of the plant extracts.
- Qualitative and quantitative analysis of phytoconstituents.
- Development of polyherbal Patch.
- Optimization of the formulation by QbD.
- Evaluation of the developed formulation.
- Evaluation of *in-vivo* Anti-inflammatory activity (**Carrageenan induced paw edema model**) - This type of model mainly used in experiments related to skin.
- To develop and validate HPTLC method for simultaneous estimation of Capsaicin and Boswellic acid in formulation.



CHAPTER 3

LITERATURE REVIEW

3 LITERATURE REVIEW

3.1 Inflammation

Inflammation is a nonspecific, defensive response of the body to tissue damage. Among the conditions that may produce inflammation are pathogens, abrasions, chemical irritations, distortion or disturbances of cells, and extreme temperatures. The four characteristic signs and symptoms of inflammation are redness, pain, heat, and swelling [1]

Inflammation is one of the common cause in the majority of acute as well as chronic diseases and represent a main cause of morbidity in today's era of modern lifestyle. If unchecked, inflammation leads to development of rheumatoid arthritis, diabetes, cancer, Alzheimer's disease, and atherosclerosis along with pulmonary, autoimmune and cardiovascular diseases. Inflammation involves a complex network of many mediators, such as a variety of cells, and execution of multiple pathways. Current therapy for inflammatory diseases is limited to the steroidal and non-steroidal anti-inflammatory agents. The chronic use of these synthetic drugs is reported to cause severe adverse effects like gastrointestinal, cardiovascular, and renal abnormalities.

Inflammation is a complex pathophysiological process mediated by a variety of signalling molecules produced by leucocytes, macrophages and mast cells undergoing various cellular responses such as phagocytic uptake, and the production of inflammatory mediators such as nitric oxide (NO), prostaglandin E2 (PGE2) and tumour necrosis factor (TNF)- α [2], that bring about edema formation as a result of extravasation of fluid and proteins and accumulation of leucocytes at the inflammatory site. In addition, it is broadly accepted that cytokines, produced by either immune or central nervous system cells, might directly sensitize the peripheral nociceptors [3] [4].

Inflammation is an important cellular response triggered by various mechanical, chemical or immunological stress factors and it is regulated by a delicate balance between local factors that finally determine the outcome of the disease process: progression or resolution.

The inflammatory response is a complex and highly regulated sequence of events that start with an initial production of pro-inflammatory mediators that recruit professional

inflammatory cells to the site of injury to clear the offending trigger [5]. This is followed by an antiinflammatory phase, in which resident tissue cells may acquire the potential for protecting themselves from further activation and injury. More recently, inflammation was described as “the succession of changes which occurs in a living tissue when it is injured provided that the injury is not of such a degree as to at once destroy its structure and vitality” or “the reaction to injury of the living microcirculation and related tissues” [6]. Although, in ancient times inflammation was recognised as being part of the healing process, up to the end of the 19th century, inflammation was viewed as being an undesirable response that was harmful to the host. Based on visual observation, the ancients characterised inflammation by five cardinal signs, namely redness (rubor), swelling (tumour), heat (calor; only applicable to the body extremities), pain (dolor) and loss of function (functio laesa). The first four of these signs named by Celsus in ancient Rome (30-38 B.C.) and the last by Galen (A.D. 130-200) [7]. The classical description of inflammation accounts for the visual changes seen. The sensation of heat is caused by the increased movement of blood through dilated vessels into the environmentally cooled extremities. Redness is due to the additional number of erythrocytes passing through the area. Swelling (edema) is the result of increased passage of fluid from dilated and permeable blood vessels into the surrounding tissues, infiltration of cells into the damaged area, and in prolonged inflammatory responses deposition of connective tissue. Pain is due to the direct effects of mediators, either from initial damage or that resulting of sensory nerves due to oedema. Loss of function refers to either simple loss of mobility in a joint, due to the oedema and pain, or to the replacement of functional cells with scar tissue.

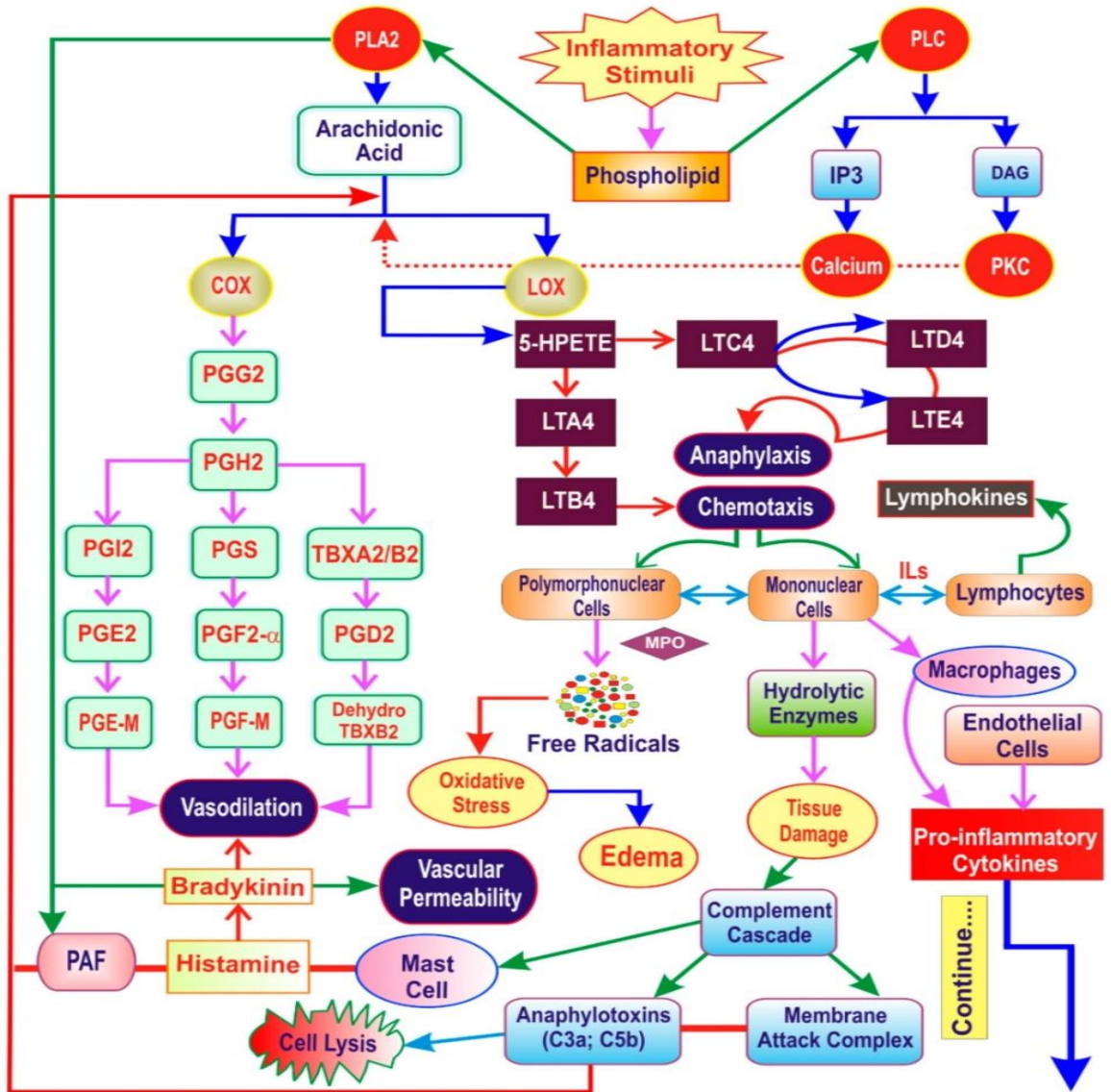
3.1.1 Types of inflammation

Inflammatory process has two phases: acute and chronic. Acute and chronic inflammations are known to be complicated processes induced by several different classes of chemical mediators, e.g. prostaglandins, leukotrienes and platelet-activating factor, etc. Anti-inflammatory agents exert their effect through a spectrum of different modes of action [8].

Acute inflammatory response is characterized by an increase in vascular permeability and cellular infiltration leading to oedema formation as a result of extravasation of fluid and proteins and accumulation of leukocytes at the inflammatory site for short time [9].

Chronic inflammation is the reaction arising when the acute response is insufficient to eliminate the pro-inflammatory agents. Chronic inflammation includes a proliferation of fibroblasts and infiltration of neutrophils with exudation of fluid. It occurs by means of development of proliferative cells which can either spread or form granuloma. Chronic inflammation may also occur due to the persistence of infection or antigen, recurring tissue injury, or a failure of endogenous anti-inflammatory mechanisms.

Chronic (or acute) inflammation is a multiple process mediated by activating inflammatory or immune cells [10], among which macrophages play a central role in managing many different immunopathological phenomena including the overproduction of proinflammatory cytokines and inflammatory mediators, generated by activated iNOS and COX-2 [11]. Under inflammatory conditions, immune cells are also stimulated by adhesion molecule activation signals in order to enhance the migration capacity to inflamed tissue and finally to form heterotypic cell clustering between the immune cells, endothelial cells and inflamed cells [12]. Macrophages in the inflammatory reaction initially requires an interaction between surface receptors such as Toll-like receptors (TLR) and stimuli [13], and subsequent up-regulation of intracellular signalling events mediated by enzymes such as phosphoinositide 3-kinases (PI3K) and mitogen activated protein kinases (MAPKs) as well as transcription factors (e.g., nuclear factor [NF]- κ B and activator protein [AP]-1) [14]. Overall, these events lead macrophages to express pro-inflammatory genes such as inducible NO synthase (iNOS) and cyclooxygenase (COX)-2. Because large amounts of macrophage-derived inflammatory mediators can cause collateral or severe damage such as septic shock, rheumatoid arthritis and arteriosclerosis, the effective blockade of these inflammatory responses is an important therapeutic target [15].



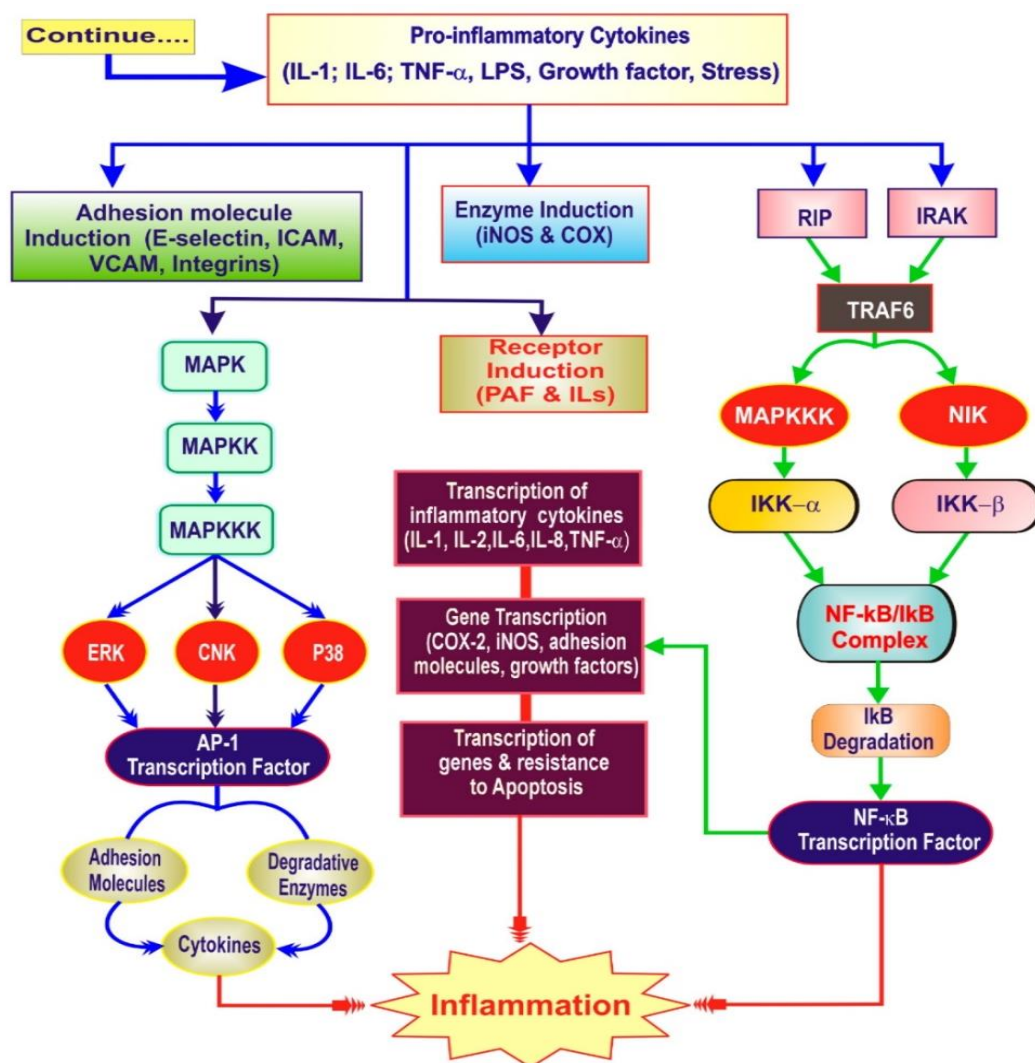


Figure 1 Inflammation Pathway

3.1.2 Inflammatory mediators :

3.1.2.1 Lipid derived mediators

Arachidonic acid (AA) is a eicosanoid precursor and a chief constituent in all the cells of body. Activation of various phospholipase enzymes, mainly phospholipase A2 (PLA2), causes release of AA from membrane phospholipid. AA is metabolized via different pathways and form multiple oxygenated products called as eicosanoids. The cyclooxygenase (COX) forms prostaglandins (PGs) and thromboxane (prostanoids) whereas the lipoxygenases (LOX) forms leukotrienes (LTs) and lipoxins (LXs). Additionally, cytochrome P450 enzymes produces epoxyeicosatrienoic acids (EETs) [16]. Eicosanoids regulate various inflammatory and homeostatic process that is linked to many diseases [17]. The proinflammatory activity of lipid-derived mediators has been well documented [18]. The PGs are connected with bronchoconstriction, mucus secretion, vasodilatation, and vascular permeability. Whereas,

leukotrienes are putative bronchoconstrictors and stimulators of vasodilatation and vascular permeability [19]. Leukotriene B₄ (LTB₄) is associated with neutrophil activation and superoxide formation. Furthermore, it increases interleukin-6 (IL-6) production and stimulates the early gene transcription of other cytokines [19] [20]. The 5-lipoxygenase (5-LOX) is vital for the leukotrienes biosynthesis and LTs are key mediators in an inflammatory and the allergic processes. Therefore, inhibition of 5-LOX is promising approach for the treatment of dermatitis and psoriasis [21]. Platelet-activating factor (PAF), generated by various inflammatory cells including macrophages, neutrophils, eosinophils, and endothelial cells is another mediator that causes bronchoconstriction, platelet activation and chemotaxis [19].

3.1.2.2 Proinflammatory Cytokines

Cytokines regulate the immune responses and inflammatory process. Tumor necrosis factors, interferons, interleukins, and colony stimulatory factors belong to the class of cytokines. Cytokines regulate adhesion molecule expression, cell growth, cell division, apoptosis, immunoglobulin production, and chemotaxis in the target cells [19]. Stimulation of monocytes and macrophages releases proinflammatory cytokines like tumor necrosis factor- α (TNF- α), IL-1 β , and IL-6. TNF- α is involved in the tumor cell metastasis and pathology of rheumatoid arthritis [31]. IL-1 β activates lymphocytes and causes bone resorption [22]. The TNF- α and IL-1 β regulate the expression of adhesion molecules and also captures the circulating leukocytes [23]. Furthermore, cytokines also initiates intracellular signaling cascades and subsequent transcription [24].

3.1.2.3 Vasoactive Mediators

Histamine, stored primarily in the mast cells and basophil leukocytes is a widely distributed and preformed proinflammatory mediator [19] [25]. Release of histamine causes a transient increase in permeability following tissue injury. Histamine causes endothelial cell contraction and allows the passage of fluid along with the proteins through the inter-endothelial junctions [25]. Along with increased vascular permeability, histamine also causes edema formation and improves the gastric acid secretion [19]. Histamine at larger concentration causes swelling of endothelial cells and leukocyte adherence. Thus, histamine is main mediator that causes early vascular changes during inflammatory response [26]. Serotonin another vasoactive amine is mainly found in the tissues of intestine, brain, and platelets which causes an increased vascular

permeability and the contraction of smooth muscles [25]. Serotonin elicits venous constriction and at higher concentration slows the capillary flow and leads to stasis [26]. Bradykinin leads to endothelial cell separation, the formation of gaps in post-capillary venules and augmented vascular permeability [25].

3.1.2.4 Hydrolytic Enzymes

During the course of inflammation, the secretion of stored proteolytic enzymes occur from stimulated proinflammatory cells. Elastin is a primary elastic component of blood vessels, lungs, and proteins including collagen, proteoglycans, and immunoglobulins. Release of human leukocyte elastase (HLE) from the stimulated polymorphonuclear leukocyte (PMNL) leads to elastin hydrolysis and endothelial migration of stimulated proinflammatory mediators [19]

3.1.2.5 Reactive Oxygen Species (ROS)

Inflammation and oxidative stress are interrelated to pathophysiological events in numerous diseases [27]. Reactive oxygen species (ROS) has the important role in cellular defense mechanisms. ROS released from inflammatory cells exaggerate the oxidative stress [27]. ROS can initiate intracellular signaling pathways and promote proinflammatory gene expression [27]. Overproduction of radicals and peroxides impair the endogenous anti-oxidative mechanisms and free radical scavengers which causes deterioration of functionally relevant structures [19].

3.1.2.6 Transcription factors

Nuclear factor-kappa beta (NF- κ B) is the chief regulator of both the immune system and the inflammatory response [28]. NF- κ B controls the transcription of genes involved in apoptosis, cell adhesion, proliferation, cellular stress response, immune response, inflammatory pathways and tissue remodeling [28]. NF- κ B regulates the transcription of inflammatory cytokines like IL-1 β , IL-2, IL-6, IL-8, and TNF- α along with the genes encoding COX-2, iNOS, cell adhesion molecules, immune-receptors and growth factor receptors. The glucocorticoids, aspirin at high doses, and sulfasalazine decrease the activation of NF- κ B. Thus, NF- κ B is an interesting therapeutic target for the pharmacotherapy of inflammatory ailments [29].

3.1.2.7 Complement System

The complement cascade activation results in the formation of anaphylatoxins C3a, C5a, and membrane attack complex. C5a is a potent chemoattractant that causes enhanced antibody production; synthesis, and release of cytokines, PGs and leukotrienes and oxidative stress. It also favors the recruitment of inflammatory cells like neutrophils, eosinophils, monocytes and T lymphocytes. Thus, complement-activated products like C5a exhibit dominant biological activities which initiate the inflammatory cascade [22].

3.2 Pain

Pain is not only an obnoxious sensation, but also a complex sensory process that essential for survival. Noxious stimuli (thermal, chemical, mechanical trauma, diseased condition, or electrical) causes the cellular damage. In response to damage, cell releases chemical mediators (histamine, bradykinin, prostaglandins, etc.), which activate nociceptive receptors via cascade of reactions and the nociceptive signal will be modulated from the periphery to the brain at all levels of the central nervous system (CNS). The International Association for the Study of Pain (IASP) defines pain as: “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage.” Though pain and coupled responses can be unpleasant and often debilitating, it implicates identification and localization of noxious stimuli, initiate withdrawal responses that limit tissue injury thereby enhance wound healing.

3.2.1 Types of pain

Pain can be classified according to several variables, including its duration (acute, chronic), its physiologic, or pathophysiologic mechanisms (nociceptive, neuropathic, physiologic), and its clinical milieu (e.g., postsurgical, malignancy related, neuropathic, degenerative, etc.).

Acute pain is limited to pain of less than 30 days duration, whereas chronic pain persists for more than 6 months.

Subacute pain comprises the interval from the end of the first month to the beginning of the seventh month of continued pain [30].

Acute pain is provoked by a specific disease or injury, serves a useful biologic purpose, is associated with skeletal muscle spasm and sympathetic nervous system activation, and is self-limited [31].

Chronic pain, in contrast, may be considered a disease state. It is pain that survives the normal time of healing, if associated with a disease or injury. Chronic pain may arise from psychological states, serves no biologic purpose, and has no recognizable end-point [32].

Nociceptive pain is experienced due to activation of nociceptors in peripheral tissues resulting from cellular damage due to traumatic or disease related injuries. Nociceptive pain has also been termed as inflammatory pain; as inflammatory mediators play role in its initiation and development [33].

Pain after surgery, pain associated with sports injury, sprains are typical examples of nociceptive pain.

Neuropathic pain results from dysfunction of the nervous tissue or nervous system lesions like in diabetic neuropathy [34]. Dysfunction of nerve tissue alters physiologic nociceptive pathways. For example, damage to inhibitory pathways or overstimulation of nociceptive pathway can change balance between nonpainful and painful sensory stimuli respectively that causes pain without activation of primary afferent nociceptors [34].

Physiologic pain is a protective mechanism of an individual to tissue injury or harmful environment, characterized by discomfort due to trauma or noxious stimuli (mechanical, thermal, or chemical) of very short duration. Physiologic pain prevents or minimizes tissue injury by initiating withdrawal reflexes and thereby alerting individual regarding potential harmful environment [33].

3.2.2 Nociception and Nociceptors

Nociception is a dynamic phenomenon in which the nociceptive signal is modulated from peripheral nervous system to brain. The nociceptive pathways can be better understood following the integration and modulation of nociceptive signal at all level of CNS [35].

Nociceptors are the relatively unspecialized, free, lightly myelinated, or unmyelinated nerve endings. Like other subcutaneous and cutaneous receptors, they detect noxious stimuli and convert the noxious stimuli into electrical signal and trigger afferent action potential into nerve endings, which in turn initiate sensation of pain.

Nociceptors, similar to other somatic sensory receptors; originate from cell bodies that are located in the dorsal root ganglia (DRG) or trigeminal ganglia [36]. The DRG or trigeminal ganglia have two axonal processes; one extends into peripheral while another extends into spinal cord or brainstem [37].

3.3 Inflammatory Diseases

Inflammation is a physiological response of a body to stimuli, including infections and tissue injury. However, excessive or persistent inflammation causes a variety of pathological conditions [38]. As the primary interface between the body and the external environment, the skin provides the first line of defense against traumatic injury and invasion by microbial pathogens. In addition to its properties as a physical barrier, the skin has many active defence mechanisms [39] and regulation of these mechanisms is crucial, as inappropriate or misdirected immune activity is implicated in the pathogenesis of a large variety of inflammatory skin disorders. While some of these conditions are easily remedied, treatments for chronic inflammatory diseases such as psoriasis and atopic dermatitis are not 100% successful. High levels of inflammatory cytokines and reactive oxygen species are proposed to contribute to the pathophysiological mechanisms associated with various inflammatory skin disorders [40]. Many degenerative diseases such as rheumatoid arthritis, shoulder tendonitis, gouty arthritis, polymyalgia rheumatica, heart disease, asthma, and inflammatory bowel disease are often associated with inflammatory processes [41]. Furthermore, oxidative and inflammatory processes are among the pathological features associated with the central nervous system in Alzheimer's disease (AD) [42]. Rheumatoid arthritis (RA) and osteoarthritis (OA) are frequent and important diseases with complex pathophysiology. There is convincing evidence that cytokines (e.g., IL-1 and TNF), prostaglandins (PG), and nitric oxide (NO) play critical roles in the development and perpetuation of inflammation and cartilage and meniscus damage in rheumatoid arthritis and osteoarthritis.

3.3.1 Conventional anti-inflammatory drugs and their side effects

Many steroids, specifically glucocorticoids and Mineralocorticoids reduce inflammation or swelling by binding to corticoid receptors. These drugs are often referred to as corticosteroids. Long-term corticosteroids use has several severe side effects eg. hyperglycemia, insulin resistance, diabetes mellitus, osteoporosis, anxiety effects etc. [43].

There are over 50 different NSAIDs available [44] and they can be divided into different groups based on their chemical structure, pharmacokinetics and selectivity towards Cox-1 or Cox-2 [45]. NSAIDs can be classified broadly into two types based on their chemical structure. Most NSAIDs are carboxylic acids; but a few, most noticeably Phenylbutazones, are Enolic acids. Carboxylic acid containing drugs include Salicylate derivatives (eg. aspirin), carbocyclic and hetrocyclic acid derivatives (eg. indomethacin), fenamic acid derivatives (eg. Ibuprofen, Ketoprofen, Fenbufen, Flurbiprofen, Suprofen and Naproxen) and phenyl acetic acid derivatives (eg. Diclofenac, Aceclofenac, etc.). Enolic acid containing drugs include oxicam derivatives (eg. Piroxicam, Tenoxicam and Meloxicam) and pyrazoles (eg. Phenylbutazone and Oxyphenbutazone). Non acidic group compounds include Nabumenton [46].

3.3.2 Adverse effects of NSAIDs

Non-steroidal anti-inflammatory drugs (NSAIDs) are one of the most widely prescribed medication in the world. Their main benefit derives from their anti-inflammatory and analgesic effect, but the use of these agents is not innocuous since they mainly increase the risk of gastrointestinal (GI) and cardiovascular complications compared with non-NSAID users. NSAIDs injures the upper and lower gut by depleting COX-1 derived prostaglandins and causing topical injury to the mucosa. The risk of upper GI complications varies, depending on the presence of one or more risk factors. Among them, the three main risk factors are prior history of peptic ulcer, the single most important risk factor, age, the most common, and concomitant aspirin use, due to their GI and cardiovascular implications. Those individuals at-risk should be considered for alternatives to NSAID therapy and modifications of risk factors. If NSAID therapy is required, patients at risk will need prevention strategies including co-therapy of NSAID with gastroprotectants (PPI or misoprostol) or the prescription of COX-2 selective inhibitors. The probable introduction of NO-NSAIDs in the market in the near future may open a new therapeutic option for patients with hypertension who need NSAIDs [47].

3.4 Inflammation as a Therapeutic Target of Phytoconstituents

Inflammation itself is a source of discomfort and major cause of the pathophysiological processes involved in the initiation and progression of the many diseases [48]. Systematic investigations of phytoconstituents for its anti-inflammatory activity can provide the safer and efficacious remedies to treat the inflammatory diseases [49]. Over the centuries, the treatment of inflammatory disorders are achieved through the use of medicinal plants. The phytoconstituents present in these medicinal plants are recognized to be responsible for their anti-inflammatory activities. The capability of phytoconstituents to act on several steps of pathophysiological processes is responsible for their anti-inflammatory activity. The phytoconstituents exhibit potential anti-inflammatory activities by interaction with important cellular targets including the inflammatory pathways or specifically with certain components of the pathways like the proinflammatory mediator production, complement cascade activation, and leukocyte migration [48]. Different biomolecules like matrix-degrading enzymes, proinflammatory cytokines, and the components of signaling pathways are the promising therapeutic targets in chronic inflammatory diseases [22]. Regulation of pro-inflammatory substance gene expression is the key target of phytoconstituents during an inflammatory process [49]. Safayhi et al. [28] proposed that anti-inflammatory compounds might act by one or more of the several mechanisms. Blockade of proinflammatory mediator biosynthesis, reduced expression of key enzymes, inhibition of mediator release, blockade of the interaction between mediator and its receptors are few to summarize.

3.4.1 Anti-Inflammatory Drug Discovery from Phytoconstituents:

Current Status and Systemic Approach

Majority of drugs existing in the market constitute the natural products and even the semi-synthetic or synthetic drugs have originated from the natural sources. The therapeutic potential of medicinal plants used in several traditional systems has been established through scientific studies from across the globe. The interest of the scientific community in correlating the phytoconstituents of a plant and their botanical properties with its pharmacological activity has increased [50]. The plant extracts and isolated phytoconstituents have expressively contributed to the new drug discoveries. Finding viable, robust, and druggable lead candidates is a challenging task in drug discovery and development of pharmaceuticals for use in humans. As

it involves the transformation of screening hits to the drug candidate, it demands for both experience and expertise [51]. The new drug development is a very expensive, time-consuming, and complex task. Usually, it takes about 12 years from the discovery of new lead to its appearance in the clinic as a therapeutic agent. The diminution in the new drug approvals and escalating development cost are major challenges in the new drug discovery. Although the arrival of combinatorial chemistry has rationalized the drug discovery process, it does not increase the success rate. Primarily, drug discovery focus on the identification of new chemical entities possessing potential characteristics of druggability [52]. Natural products have formed the basis of useful therapeutic agents for centuries. Plants have continued to serve mankind with discoveries of new remedies [53]. Several phytoconstituents like flavonoids, triterpenoids, alkaloids, steroids, and phenols have been documented to possess interesting anti-inflammatory properties. Many phytoconstituents exhibited potent activities at micromolar concentrations against well-established biomarkers of inflammation. The active components obtained from natural products used as traditional medicines appear to be the main sources of drug discovery in modern medicines. Despite the advances in the allopathy field, plants are still continue to be the source of potential therapeutic agents in the modern and traditional system of medicine. Therefore, the isolation of pure compounds from the natural sources, and characterization of pharmacologically active compounds have been continued [53]. The chemical diversity and advancements in new technologies have transformed the drug discovery from natural sources. Traditional limitations of the natural products have been overcome by the novel technologies. It has created an avenue to establish the value of natural products as a drug discovery leads [51]. Structural interpretation of phytoconstituents has enabled the medicinal chemist to synthesize the compounds by total synthesis instead of their isolation from the plants. This has been resulted in the decreased production cost and improved potency of natural leads [54].

3.5 Anti-inflammatory Herbal Drugs

Herbal medicine is one of the important aspects of complementary medicines. Herbal drugs have been used for prolong period of time for prevention as well as treatment of diseases including inflammation. Many people are now using herbal remedies for their day to day life as phytonutrients of nutraceuticals, because of that herbal drugs and phytonutrients or nutraceuticals continues to expand rapidly across the world. According to World Health Organisation (WHO) three quarters of people rely on traditional and plant based medicine for their day to day healthcare. Herbal drugs now in more demand, as they have lesser side effects

than the synthetic one. There are many medicinal plants available, which possesses anti-inflammatory properties, some of them are used since ancient's time and also some of them mentioned in Ayurveda and Traditional Chinese medicines. Some medicinal plants with anti-inflammatory activity listed below in Table no.1 with their biological name, common name, plant part used as anti-inflammatory and chemical constituent from particular plant part responsible for anti-inflammatory activity.

Table 1 Anti-inflammatory Medicinal Plants

Sr. No.	Plant name (Biological source)	Common name	Plant part used	Type of extract	Marker compound	References
1.	Aegle marmelos	Bael	Roots, fruits	Aqueous, Ethyl acetate	Marmelosin	[55] [56]
2.	Albizia lebeck	Shirish	Leaves, bark	Ethanol chloroform, ether	Catechin	[57] [58] [59]
3.	Allium cepa	Onion	Leaves and bulb	Methanolic	Quercetin	[60]
4.	Allium sativum	Garlic	Leaves and cloves	Garlic clove powder	Allin, Allicin	[61] [62]
5.	Aralia cachemirica	Kashmir spikenard	Whole plant	Hydroalcoholic extract (70 % alcohol)	Octadec-6-enoic acid	[63]
6.	Azadirachta indica	Neem	Leaves	Methanolic extract	Azadirachtin, Nimbin	[64]
7.	Borago officinalis	Borage	Seed oil	Seed oil	Gamma-linoleic acid	[65]
8.	Boswellia serrata	Salai guggul	Gum resin	Hydroalcoholic	α -boswellic acid β -boswellic acid	[66]
9.	Bryophyllum pinnatum	Goethe plant	Leaves	Ethanol extract	Rutin, Luteolin	[67]
10.	Butea monosperma	Flame of the forest tree, Palash	Flowers	Methanol extract	Butrin, Butein	[68] [69]
11.	Camellia sinesis	Green tea	Leaves	Ehtanol extract	Catechin, Epigallocatechin	[70]
12.	Capsicum annum	Chilli	Fruits	Ethyl acetate extract	Capsaicin	[71]
13.	Cassia fistula	Golden shower tree	Flowers	Isolated rhein	Rhein	[72]
14.	Cinnamomum camphora	Camphor tree	Leaves	Methanolic extract	Camphor, Linalool, Cineole	[73]

15.	<i>Commiphora mukul</i>	Guggul	Gum resin	Hydroalcoholic	Guggulsterone	[74]
16.	<i>Curcuma longa</i>	Turmeric	Rhizomes	Dichloromethane	Curcumin	[75] [76]
17.	<i>Elaeagnus angustifolia</i>	Russian olive, Silverberry	Fruits	Methanol extract	Catechin, Epicatechin	[77] [78]
18.	<i>Eucalyptus globulus</i>	Nilgiri	Oil from leaves	Oil	1,8-cineole	[79]
19.	<i>Garcinia cambogia</i>	Malabar tamarind	Fruits	Ethanol extract	Hydrocitric acid (HCA)	[80]
20.	<i>Gaultheria procumbens</i>	American Wintergreen	Leaves and oil	Hydroalcoholic extract	Quercetin, Catechin	[81]
21.	<i>Zingiber officinalis</i>	Ginger, Adrakh	Rhizomes, Oil	Essential oil	Gingerol	[82]
22.	<i>Glycyrrhiza glabra</i>	Licorice	Roots	Ethanol	Glycyrrhizin	[83]
23.	<i>Harpagophytum procumbens</i>	Devil's claw	Roots	Water	Harpagoside	[84]
24.	<i>Hibiscus tiliaceus</i>	Bhola	Leaves and Bark	Aqueous Methanol (90%)	Tiliaceic acid A α -Glucosidase	[85] [86]
25.	<i>Linum usitatissimum</i>	Flaxseed, Linseed	Seeds	Oil from seeds	α -Linolenic acid	[87]
26.	<i>Madhuca longifolia</i>	Mahudo	Seeds Leaves	Oil from seeds Aqueous extract of leaves	Oleic acid	[88] [89]
27.	<i>Mentha piperita</i>	Pudina, Mint leaves	Leaves	Ethanol extract	Menthol	[90]
28.	<i>Moringa oleifera</i>	Drumstick plant	Leaves, Seeds, Roots	Ethanol, Hydroalcoholic extract	β -carotene,	[91] [92]
29.	<i>Ocimum sanctum</i>	Tulsi	Leaves	Essential oil from leaves	Eugenol	[93]
30.	<i>Oenothera biennis</i>	Evening primrose	Aerial parts	Oil, Methanolic extract	Linoleic acid	[94] [95]
31.	<i>Olea europea</i>	Olive	Fruits	Methanolic extract	Oleuropein	[96]
32.	<i>Panax ginseng</i>	Chinese ginseng	Roots, Calyx	Ethanol, Water-Methanol	Protopanaxadiol protopanaxatriol	[97] [98]
33.	<i>Persea americana</i>	Avocado	Fruit, Seed	Lipid extracted from fruits and seeds	Palmitic acid Oleic acid Linoleic acid	[99]
34.	<i>Pinus roxburghii</i>	Chir pine	Bark	Alcoholic extract	α -pinene β -pinene	[100]
35.	<i>Pluchea indica</i>	Camphorweed	Leaves Roots	Ethanol Chloform	Quercetin	[101] [102]

					Chlorogenic acid	
36.	<i>Pluchea lanceolata</i>	Rasna	Aerial parts	Ethanol extract	Quercetin Quercitrin	[103] [104]
37.	<i>Podophyllum emodi</i>	Mayapple	Roots and rhizomes	Isolated podophyllotoxin derivatives	Podophyllotoxin	[105]
38.	<i>Ribes nigrum</i>	Blackcurrant	Berries Buds Leaves	Acetone/water/ acetic acid(70:28:2) Ethanol extract	Cyanidin-3-O-glucoside Delphinidin-3-O-glucoside	[106] [107] [108]
39.	<i>Ricinus coumaris</i>	Castor bean Castor oil plant	Roots Leaves Seed oil	Methanol, Acetone, Hexane	Ricicolic acid Linoleic acid Kaempferol-3-O-beta-D-rutinoside	[109] [110] [111]
40.	<i>Rosa canina</i>	Dog rose	Rose hip	Hydroalcoholic extract	Linoleic acid Alpha linoleic acid	[112] [113]
41.	<i>Rosmarinus officinalis</i>	Rosemary	Aerial parts Essential oil	Ethanol extract	Caffeic acid Rosmarinic acid Carnosol	[114] [115] [116]
42.	<i>Salix alba</i>	Willow	Bark	Ethanol extract	Salicin	[117]
43.	<i>Salvia officinalis</i>	Sage	Oil from aerial parts	Chloform extract	Borneol Camphor Caryophyllene Cineole	[118]
44.	<i>Sesamum indicum</i>	Sesame	Oil from seeds	Oil	Sesamol Ferulic acid	[119] [120]
45.	<i>Solanum xanthocarpum</i>	Kantakari	Fruits	Aqueous extract	Campesterol Chlorogenic acid	[121] [122]
46.	<i>Symphytum officinale</i>	Comfrey	Leaves Roots	Ethanol extract	Allantoin Rosmarinic acid	[123]
47.	<i>Tinospora cordifolia</i>	Guduchi Galo	Whole plant	Ethanol, Aqueous extract	β -sitosterol sigmasterol	[124] [125] [126]
48.	<i>Thymus vulgaris</i>	Thyme	Essential oil	Essential oil	Thymol, Carvacrol	[127] [128] [129]
49.	<i>Trigonella foenum-graecum</i>	Fenugreek	Seeds	Petroleum Ether Extract	Linolenic acid Galactomannan	[130] [131]
50.	<i>Vitex negundo</i>	Nirgundi	Leaves	Methanol, Petroleum Ether extract	Caryophyllene epoxide	[132] [133] [134]
51.	<i>Withania somnifera</i>	Ashwagandha	Roots	Aqueous extract	Withanolides	[135] [136] [137]

3.6 Selected Medicinal Plants :

1. *Boswellia serrata*
2. *Capsicum annum*
3. Menthol

3.6.1 *Boswellia serrata* (Monograph) [138]

It consists of exudate of *Boswellia serrata* Roxb., belonging to Family Burseraceae, a moderate sized, deciduous tree, upto 18 m in height and upto 2.4 m in girth, commonly found in the dry forests from Punjab to West Bengal and in peninsular India.

SYNONYMS

Sanskrit : Sallaki

Assamese : Sallaki

Bengali : Luban, Salai, Salgai

English : Indian frankincense

Gujrati : Shaledum, Saleda, Saladi, Gugal, Saledhi

Hindi : Salai, Labana

Kannada : Madimar, Chilakdupa, Tallaki, Maddi

Kashmiri : Kunturukkam, Samprani

Marathi : Salai cha dink

Punjabi : Salai Gonda

Tamil : Parangi Sambrani

Telugu : Parangi sambrani, Anduga, Kondagugi tamu

Urdu : Kundur

DESCRIPTION

A) Macroscopic

Drug occurs in stalactitic, transparent, tears forming agglomerates of various shapes and sizes, brownish-yellow, upto 5 cm long, 2 cm thick, fragrant, fracture brittle; fractured surface waxy

and translucent; burns readily and emanates an agreeable characteristic, balsamic resinous odour; taste, aromatic and agreeable.

B) Microscopic

Debris of fibres, rectangular cork cells, very few yellowish oil globules and numerous, small or large, oval to round or rhomboidal crystalline fragments present.

Identification

Trituration with water forms an emulsion; when immersed in alcohol (90%) a tear of Kunduru is not altered much in form but becomes almost opaque and white; when a drop of con. H₂S₀₄ is added on a freshly fractured surface, it becomes cherry red which, when washed with water changes to a white emulsion, then turn to a buff colour.

Fluorescence Test

Brownish-yellow colour in day light; aqueous extract under U.V. light (366 nm) light green and in (254 nm) shows dark blue colour; alcoholic extract under U.V. light (366 nm) is colourless and in (254 nm) shows light green colour.

IDENTITY, PURITY AND STRENGTH

Foreign matter Not more than 5 %

Total Ash Not more than 10 %

Acid-insoluble ash Not more than 8 %

Alcohol-soluble extractive Not less than 45 %

Water-soluble extractive Not less than 28 %

T.L.C. Profile

T.L.C. of alcoholic extract on Silica Gel 'G' using Toluene : Ethylacetate (9: 1) shows under U.V. (366nm) four fluorescent zones at Rf. 0.23 (light blue), 0.79 (light blue), 0.91 (blue) and 0.96 (blue).

On exposure to Iodine vapour nine spots appear at Rf. 0.08, 0.23, 0.29, 0.41, 0.47, 0.55, 0.82, 0.91 and 0.96 (all yellow).

On spraying with Vanillin Sulphuric acid reagent and heating the plate for ten minutes at 110°C tailing with four conspicuous spots appear at Rf. 0.23, 0.55, 0.79 and 0.91 (all violet).

CONSTITUENTS - Oleo-gum-resins.

PROPERTIES AND ACTION

Rasa : Madhura, Katu, Tikta

Guna : Guru, Snigdha, Tiksna

Virya : Usna

Vipaka : Madhura

Karma : Balya, Kaphahara, Vj̄tahara, Kaphapittahara, Rakta Stambhahar, Svedahara

IMPORTANT FORMULATIONS - Karpuradyarka, Jirakadi Modaka, Bala Tila, Bala Guducyadi Taila

THERAPEUTIC USES - Jvara, Pradara, Svasa, Pittabhisyandu, Sarkarameha, Vrsana Sula, Mukharoga, Uka

DOSE - 1-3 gm.

CHEMICAL CONSTITUENTS [139], [140]

The gum resin typically contains 30% boswellic acids, while ethanol extracts contain 43% boswellic acids. Some commercial sources contain up to 65% boswellic acids. Oral doses of 200-400 mg are often standardized to contain 37.5% boswellic acids per dose. The standardized boswellia products Sallaki (India) and H15 (Switzerland) contain 11-keto- β -boswellic acid (1.8%), acetyl-11-keto- β -boswellic acid (1.4%), and acetyl- β -boswellic acid/ β -boswellic acid (2%).¹² The standardized boswellia product S-compound® contains 11-keto- β -boswellic acid (0.63%), acetyl-11-keto- β -boswellic acid (0.7%), acetyl- β -boswellic acid/ β -boswellic acid (1.5%).

PHARMACOLOGICAL ACTIVITY

In vitro and rat studies have reported that acetyl-11-keto- β -boswellic acid from boswellia inhibits the enzyme 5-lipoxygenase, which produces 5-hydroxyeicosatetraenoic (5-HETE) and leukotriene B4 (LTB4) [141], [142]. These products are involved with the induction of bronchoconstriction, chemotaxis, and vascular permeability [142], [140].

Additional studies have found that boswellia inhibits human leukocyte elastase (HLE), which is involved in the pathogenesis of emphysema, cystic fibrosis, chronic bronchitis, and acute

respiratory distress syndrome. Multiple pentacyclic triterpenic acids have been isolated from boswellia [143] [144].

Anti-inflammatory effects of boswellic acids have been reported in animal studies [145]. Doses of 50-200 mg/kg given orally to mice, after intra-pleural injection of carrageenan, inhibited polymorphonuclear leukocyte (PMN) infiltration into the pleural cavity. This response was similar to indomethacin (1.25 to 5 mg/kg). Alcoholic extracts of boswellia in doses of 50-200 mg/kg orally inhibited carrageenan-induced paw edema in rats similar to phenylbutazone (50-100 mg/kg), and improved blood supply to joint tissues [146].

Mixed acetylboswellic acids extracted from the gum resin of *Boswellia serrata* significantly inhibited ionophore-stimulated release of leukotrienes B₄ and C₄ from intact human PMNs [147]. Boswellic acids have demonstrated anti-inflammatory and anti-arthritic activity in chronic models of adjuvant-induced polyarthritis and formaldehyde arthritis in rats, [146] and in BSA-induced arthritis in rabbits [148].

Boswellic acids produced a protective effect in sodium urate gouty arthritis in dogs, reduced exudate volume and inhibited leukocyte migration in carrageenan-induced pleurisy in rats, and was antipyretic in rats and rabbits [146].

In animals, ingestion of defatted alcoholic extracts of boswellia decreases PMN infiltration and migration, decreases primary antibody synthesis, and inhibits the classical complement pathway [149], [148], Humoral responses are also inhibited by oral boswellia extract 25-200 mg/kg in mice (similar to the effect of azathioprine 100 mg/kg orally) [146]. Prolonged administration of boswellic acids (25-100 mg/kg for 21 days) increases body weight and total leukocyte counts in rats.^{30,31} The non-phenolic fraction of *Boswellia serrata* gum resin (20-300 mg/kg) exhibits an analgesic effect in rats similar to morphine (4.5 mg/kg), and a sedative effect (55-300 mg/kg) comparable to chlorpromazine (7.5 mg/kg) [150]. In biochemical studies, boswellic acids have acted to reduce arthritis-associated elevated enzymes such as glutamic pyruvic transaminase, glycohydrolase, and β -glucuronidase [151]. Inhibition of glycosaminoglycan (GAG) synthesis and urinary excretion of connective tissue metabolites by boswellic acids have been proposed as support for the purported beneficial effects of boswellia in preventing the degradation of connective tissue in inflammatory arthritic conditions. In anti-hyperlipidemic studies performed in rats, boswellic acids have been found to reduce serum cholesterol and triglycerides. Boswellic acids have not been found to act as antioxidants [140].

3.6.2 CAPSICUM [152], [153], [154]

Capsicum consists of the dried, ripe fruits of *Capsicum minimum* and *Capsicum annuum* Linn., belonging to family Solanaceae.

SYNONYMS

Chillies; cayenne pepper; red peppers, Spanish pepper, mirch (Hindi), capsicum fruits, Fructus Capsici.

Geographical Source

Capsicum is native of America and cultivated in tropical regions of India, Japan, southern Europe, Mexico, Africa (Kenya, Tanzania, and Sierra Leone), and Sri Lanka.

Cultivation and Collection

Capsicum is cultivated mostly as a rainfed crop. In the Gangetic area, it is a cold weather crop. The crop is raised on a variety of soils, for example, ordinary red loams, black soils and clayey loams. Good drainage is essential and water-logging is detrimental. Seedlings are first raised in a nursery. Seeds obtained from selected pods and mixed with ashes are sown by broadcasting. Germination occurs in about a week. The field is ploughed and manured with compost. The field is irrigated once a day until the plants are established. Flowering starts when the plants are 2.5–3.5 months old. Dew and heavy rain at flowering time are injurious. Ripe and nearly ripe fruits are picked at intervals of 5, 10, and 20 days. The fruits are picked as they become fully ripe. The quality of the drug is in part determined by its colour. The unripe fruits fade to pale buff upon drying. The fruits are dried in sun, graded by colour; occasionally oil is rubbed on the fruits to give glossiness to the pericarps. Most of the calices and pedicels are removed.

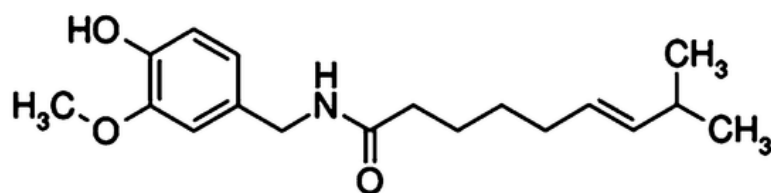
Characteristics

Capsicum is 5–12 cm long, 2–4 cm wide, globular, ovoid, or oblong in shape, pericarp is shriveled, orange or red in colour, pedicel is prominent and bent. The calyx is toothed. The amount of calices and pedicels should not exceed beyond 3%. Internally the fruits are divided into two half parts by a membranous dissepiment to which the seeds are attached. The seeds are reniform, flattened, 3–4 mm long, with a coiled embryo and oily endosperm. Capsicum has characteristic odour and an intense pungent taste.

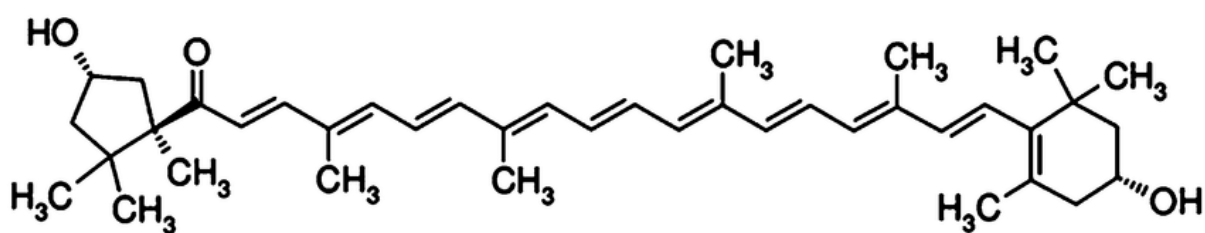
Chemical Constituents

Capsicum contains fixed oils (4–16%), oleoresin, carotenoids, capsacutin, capsico (a volatile alkaloid), thiamine, volatile oil (1.5%), and ascorbic acid (0.2%). The resin contains an extremely pungent principle, capsaicin, (decylenic vanillyl amide) (about 0.5%).

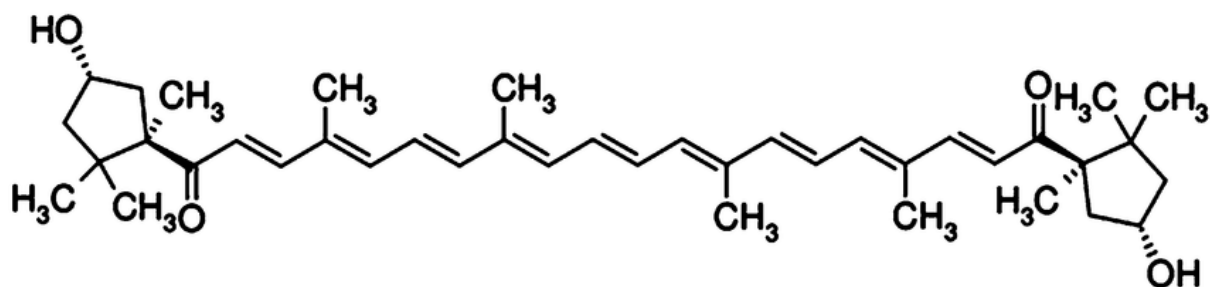
Capsaicin retains its characteristic pungency in a dilution of 1 part in 10 million parts with water. Capsanthin is the main carotenoid of red fruits. It also occurs as monoester and diester along with cryptocapsin. Other carotenoids include zeaxanthin, capsorubrin, rubixanthin, phylofluene, capsanthin-5,6-epoxide, capsanthin-3,6-epoxide, lutein, cryptoxanthin, α - and β -carotenes, capsorubin, and few xanthophylls. The carbohydrates reported in chilies are fructose, galactose, sucrose, etc. Tocopherol (vitamin E) is present in trace amounts (~2.4 mg/100 g).



Capsaicin



Capsanthin



Capsorubin

Uses

Capsicum has been used externally as stimulant, counter irritant, rubefacient, in sore throat, scarlatina, hoarseness, and yellow fever; internally it is used as carminative, stomachic, dyspepsia, and flatulence. In the form of ointment, plaster and medicated wool it is used for the relief of rheumatism and lumbago. Capsaicin is used for the treatment of migraine and cluster headache, and for some patients with neurogenic ladder dysfunction.

ISOLATION OF CAPSAICIN [152]

Capsaicin is the pungent principle known as capsicum oleoresin obtained from the dried, ripe fruits of *Capsicum annum* var. *minimum* and small-fruited varieties of *C. frutescens*; family Solanaceae. Capsaicin is mostly present in the dissepiment of fruit.

Chemically it is 8-methyl-N-vanillyl non-6-enamide. It is present to the extent of 0.02–0.14%.

Dried, ripe fruits of capsicum are coarsely powdered for the extraction of oleo-resin. It is extracted with hot acetone or alcohol (90%). The extract obtained is concentrated and dried. The dried residue is further extracted with cold alcohol (90%) and the alcohol is removed by evaporation. Capsicum oleoresin thus obtained contains not less than 8% of capsaicin

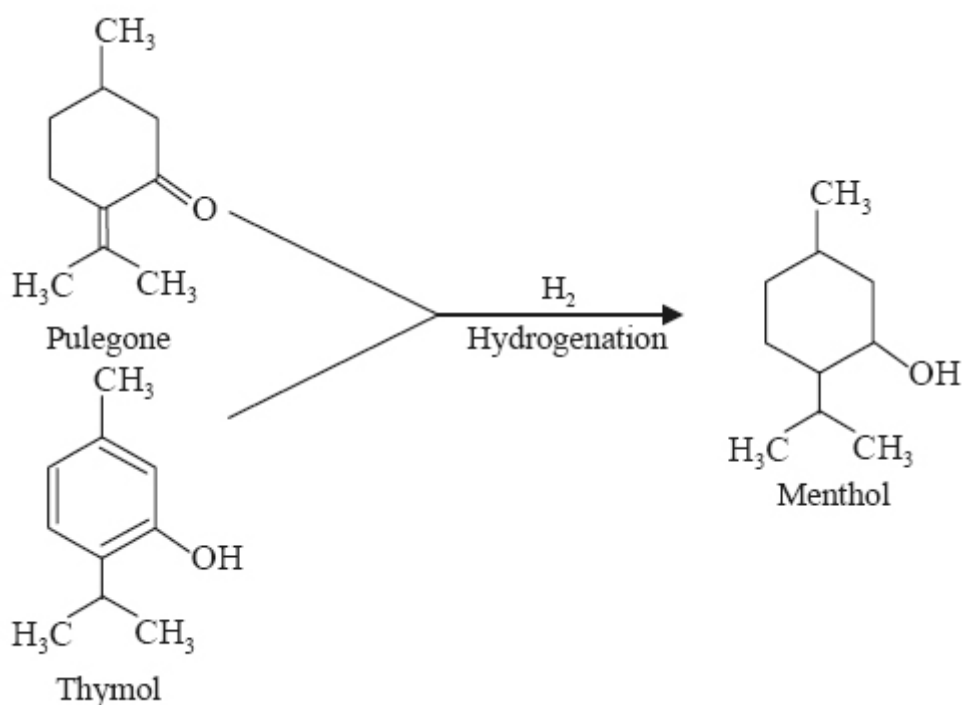
Melting point: 57–66°C

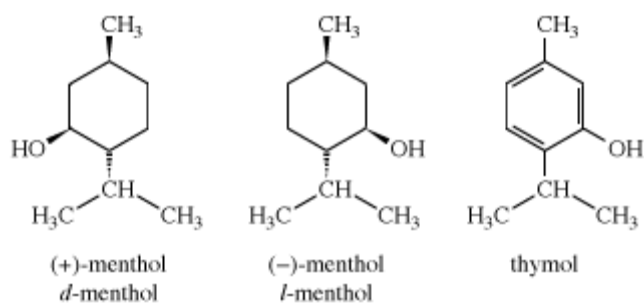
Thin Layer Chromatography of Capsaicin

The oleo-resin 1 mg/ml is dissolved in alcohol and spotted on silica gel-G plate. The plate is eluted in the solvent system containing a mixture of benzene-methanol (9:1). Spray the dried plate with a 0.5% solution of 2,6-dibromoquinone-chlorimide in methanol and allow to stand in a chamber containing ammonia fumes. Blue colour and the R_f value 0.31 of the principal spot corresponds to the spots of the standard solution.

3.6.3 MENTHOL [152]

Menthol is a monoterpene alcohol obtained from diverse types of mint oils or peppermint. The sources of mint oil include black peppermint. *Mentha piperita* Var. *vulgaris*; white peppermint, *M. piperita* Var. *officinalis*; *M. arvensis*; *M. canadensis* Var. *piperascens* etc. Peppermint contains about 1–3% of volatile oil. First two species contains not less than 45% of menthol while the later species contains menthol up to about 70–90%. Along with menthol the oil contains (+) neomenthol, (+) isomenthol, menthone, menthofuran, menthyl acetate and cineol. The menthol obtained from the natural sources is. Levorotatory (l-menthol) or racemic (dl-menthol). Menthol can be synthetically prepared by hydrogenation of thymol.





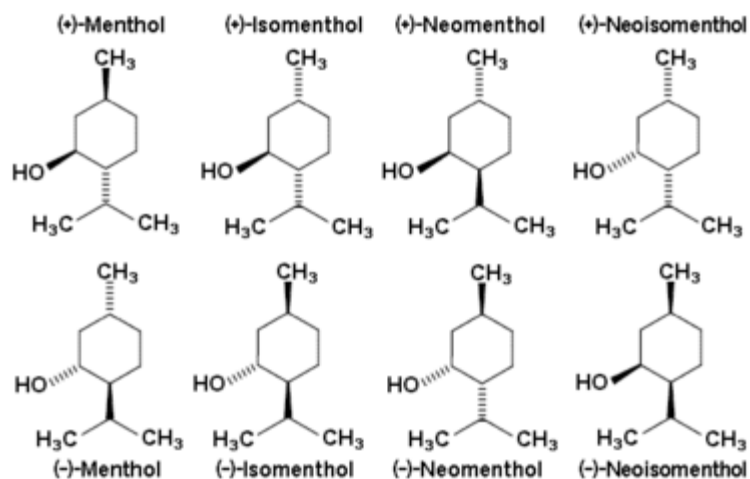
Isolation

Mentha oil is obtained from the hydrodistillation or steam distillation of fresh above-ground parts just before flowering. For (-) menthol isolation from peppermint oil the oil is subjected to cooling. The crystals of menthol crystallize out from the oil which is separated by centrifugation. Cornmint oil obtained from the steam distillation of the flowering herb *Mentha arvensis* contains about 70–80% of free (-) menthol. Cornmint oil is cooled and the crystals of menthol produced are separated by centrifugation. Since the crystalline product contains traces of cornmint oil, this menthol has a slightly herbaceous minty note. Pure (-) menthol is obtained by re-crystallization from solvents with low boiling points. Dementholized corn mint oil from which (-) menthol is removed by crystallization and which still contains 40–50% free menthol can also be reused for producing (-) menthol.

Melting point: 41–44°C

Thin Layer Chromatography of Menthol

Dissolve about 1 mg of menthol in about 1 ml of methanol. Apply the spot on silica gel-G plate and elute it in pure chloroform. Spray the dried plates with 1% vanillinsulphuric acid reagent and heat the plate at 110°C for 10 min. Menthol gives R_f value 0.48–0.62 in case of normal chamber saturation at 24°C.



TOPICAL MENTHOL'S MECHANISMS OF ACTION

Menthol, along with other substances such as capsaicin and camphor, are counter-irritants. They first activate nociceptors on the skin (which is why they are sometimes used to act as surrogates for pain in clinical trials) and then desensitize them [155]. Menthol is also a vasodilator; however, the exact mechanism by which this is mediated remains unclear. It has been suggested (but not universally endorsed) that menthol acts as a vasorelaxant [156]. Differences in findings may be due to species differences or in the vascular beds of test subjects [156]. In a study of ten healthy adult volunteers, menthol produced significant vasodilation vs baseline at all concentrations of 100 mM menthol or greater, and cutaneous vascular circulation was attenuated at all application sites compared to controls [156]. This vasodilation increased in dose-dependent fashion. Inhibitors (lidocaine, among others) could effectively block menthol-related vasodilation. The sensations associated with topical menthol were significant at five minutes to 60 minutes after application compared to baseline. Cutaneous vasodilation with menthol could be measured at 15 minutes after menthol application and remained elevated up to 45 minutes after application, but returned to baseline at 60 minutes.

Transient receptor potential melastatin-8 (TRPM8) has been nicknamed the menthol receptor. TRPM8 is activated by temperatures below 26°C as well as by a variety of chemical agents, including menthol, cubebol (a sesquiterpene alcohol) and icilin (a synthetic TRPM8 agonist) [157]. TRPM8 is part of a larger family of TRPs. The transient receptor potential vanilloid type I (TRPV1) was first elucidated in the 1990s, TRPV1 is the receptor activated by capsaicin, the active ingredient of hot pepper. At least six subfamilies of transient receptor potentials have now been identified: vanilloid (TRPV), canonical (TRPC), melastatin (TRPM),

ankyrin (TRPA), polycystin (TRPP) and mucolipin (TRPML), along with 28 associated gene products [158]. All of these are non-selective cation channels at the nerve endings or along the axons of neurons involved in pain sensation or perception. Their role is to transduce a range of stimuli, including (but not limited to) temperature [158]. The channels promote the propagation of action potentials which travel via the spinal cord to brain pain processing centres [159].

Clinical profile: the role of topical menthol in the treatment of specific painful conditions

Topical products containing menthol are used in patients with a wide range of conditions, including the management of pain associated with muscle strain, back pain, joint pain, arthritis-related painful conditions and so on. These products offer both a cooling sensation, which can offer comfort, and pain relief [160].

3.7 TRANSDERMAL DRUG DELIVERY SYSTEM (TDDS)

During the past few years, interest in the development of novel drug delivery systems for existing drug molecules has been renewed. The development of a novel delivery system for existing drug molecules not only improves the drug's performance in terms of efficacy and safety but also improves patient compliance and overall therapeutic benefit to a significant extent [161]. Transdermal Drug Delivery System (TDDS) are defined as self contained, discrete dosage forms which are also known as "patches" [162], [163]. When patches are applied to the intact skin, deliver the drug through the skin at a controlled rate to the systemic circulation [164]. TDDS are dosage forms designed to deliver a therapeutically effective amount of drug across a patient's skin.

The main objective of transdermal drug delivery system is to deliver drugs into systemic circulation into the skin through skin at predetermined rate with minimal inter and intra patient variation [163]. Currently transdermal delivery is one of the most promising methods for drug application [165]. It reduces the load that the oral route commonly places on the digestive tract and liver. It enhances patient compliances and minimizes harmful side effects of a drug caused from temporary over dose and is convenience in transdermal delivered drugs that require only once weakly application [166].

3.7.1 ADVANTAGES OF TRANSDERMAL DRUG DELIVERY SYSTEM [161], [167]

- Improves bioavailability,
- More uniform plasma levels,
- Longer duration of action resulting in a reduction in dosing frequency,
- Reduced side effects and improved therapy due to maintenance of plasma levels up to the end of the dosing interval compared to a decline in plasma levels with conventional oral dosage forms .
- Provides controlled, constant administration of drugs,
- Allows continuous input of drugs with short biological half lives and eliminates pulsed entry into systemic circulation, which often causes undesirable side effects.
- Several important advantages of transdermal drug delivery are limitations of hepatic first pass metabolism,
- Enhancement of therapeutic efficacy and maintenance of steady plasma level of drug.

The developments of TDDS is a multidisciplinary activity that encompasses fundamental feasibility studies starting from the selection of drug molecule to the demonstration of sufficient drug flux in an ex vivo and in vivo model followed by fabrication of a drug delivery system that meets all the stringent needs that are specific to the drug molecule (physicochemical, stability factors), the patient (comfort and cosmetic appeal), the manufacturer (scale up and manufacturability) and most important economy [166].

The first transdermal system, Transderm SCOP was approved by FDA in 1979 for the prevention of nausea and vomiting associated with travel. Most transdermal patches are designed to release the active ingredient at a zero order rate for a period of several hours to days following application to the skin. This is especially advantageous for prophylactic therapy in chronic conditions [168]. The evidence of percutaneous drug absorption may be found through measurable blood levels of the drug, detectable excretion of the drug and its metabolites in the urine and through the clinical response of the patient to the administered drug therapy [169].

3.7.2 Anatomy and Physiology of Human Skin [1], [170], [171]

The skin is largest organ in the body and has surface area about 1.5 to 2 sq. meter in adult and is includes glands, hair and nails. There are two main layers the epidermis and the dermis.

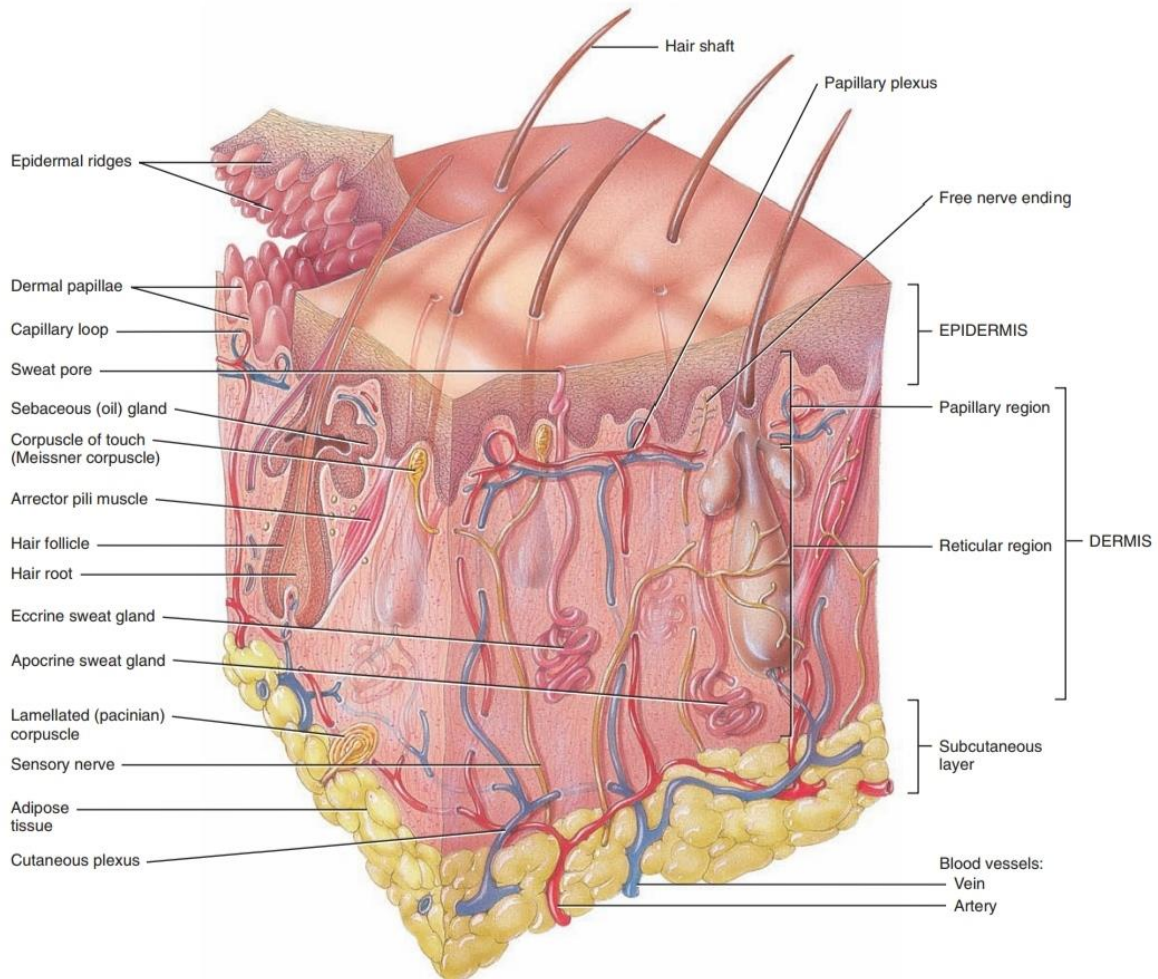


Figure 2 Structure and layers of skin

EPIDERMIS

The epidermis the most superficial layer of the skin and is composed of stratified keratinisquamous epithelium which varied in thickness in different part of the body. It is thickest on the palms of the hands and soles of the feet. There are blood vessel or nerve ending in the epidermis, but its differ layers are bathed in interstitial fluid from the dermis which, provides oxygen and nutrient, and drains away as lymph.

The maintenance of healthy epidermis depends upon three processes:

- Desquamation of the keratinized cell from the surface
- Effective keratinisation of the cell approaching surface
- Continual cell division in the deeper layers with newly formed cells being pushed to the surface.

DERMIS

The dermis is tough and elastic. It is formed from connective tissue and the matrix contains collagen fibres interlaced with elastic fibres. Rupture of elastic fibres occurs when the skin is overstretched, resulting in permanent striae, or stretch marks, that may be found in pregnancy and obesity. Collagen binds water and gives the skin its tensile strength but as this ability declines with age, wrinkles develop. Fibroblasts, macrophages and mast cells are found in the dermis. Underlying its deepest layer is areolar tissue and varying amounts of adipose tissue. The structures in the dermis are blood vessels, lymph vessels, sensory nerve endings, sweat glands and their ducts, hairs, arrector pili muscles and sebaceous glands.

HYPODERMIS

The hypodermis or subcutaneous fat tissue supports the dermis and epidermis. It serves as a fat storage area. This layer helps to regulate temperature, provides nutritional support and mechanical protection. It carries principal blood vessels and nerves to the skin and may contain sensory pressure organs.

For transdermal drug delivery, a drug has to penetrate through all these three layers and reach into systemic circulation while in the case of topical drug delivery, only penetration through the stratum corneum is essential and then retention of drug in skin layers is desired.

Functions of the Skin [2]

1. Protection
2. Regulation of the body temperature
3. Heat production
4. Heat loss
5. Synthesis of Vitamin D

6. Controlled body temperature

7. Absorption

8. Excretion

3.7.3 Types of TDDS [172]

Matrix system

Drug in adhesive system In this type, the drug reservoir is formed by dispersing the drug in an adhesive polymer and then spreading the medicated adhesive polymer by solvent casting or melting on an impervious backing layer. On top of the reservoir un-medicated adhesive polymer layer are applied for protection purpose.

Matrix dispersion system

In this type, the drug is dispersed homogeneously in hydrophilic or lipophilic polymer matrix. This drug containing polymer disc is fixed on to an occlusion base plate in a compartment fabricated from a drug impermeable backing layer. Instead of applying the adhesive on the face of drug reservoir. It is spread along with the circumference to form a strip of adhesive rim.

Micro-reservoir system

In this type the drug delivery system is combination of reservoir and matrix dispersion system. The drug reservoir is formed by first suspending drug in an aqueous solution of water soluble polymer and when dispersed in the solution homogeneously in lipophilic polymer to form thousands of unreachably small, microscopic spheres of drug reservoir. This thermodynamically unstable dispersion is stabilized quickly by immediately cross linking the polymer in situ by using cross linking agent.

3.7.3.1 Physicochemical and biological properties of drug for TDDS [173], [174]

The drug should have a molecular weight less than 1000 Daltons Drug should be very potent, i.e. it should be effective in few mg/day The drug should have affinity for both lipophilic and Hydrophilic

The drug should have short biological half-life. Extreme partitioning characteristics are not conducive to successful drug delivery via the skin.

Tolerance to the drug must not develop under near zero order release profile of transdermal delivery.

Along with these properties the drug should be potent, having short half-life and be non-irritating.

The drug should not be irritant and non-allergic to human skin.

The drug should have low melting point.

The drug should be stable when contact with the skin Dose is less than 50 mg per day, and ideally less than 10 mg per day.

They should not stimulate an immune reaction to the skin.

3.7.3.2 Advantages of transdermal drug delivery system

Avoid GIT absorption.

Avoid FP hepatic metabolism of drugs.

More improved and convenient patient compliance Drug with long half-life cannot be formulated in TDDS. Self-medication is possible.

Reduces frequency of doses.

Possible for sustained or controlled release drugs.. Minimizing undesirable side effects.

Provide utilization of drug with short biological half-lives, narrow therapeutic window It cannot achieve high drug levels in blood.

Inter and intra patient variation.

Termination of therapy is easy at any point of time.

Provide suitability for self-administration skin.

They are noninvasive,avoiding the inconvenience of parental therapy.

3.7.3.3 Disadvantages

Large daily dose is not possible.

Local irritation is major problem.

Barrier of the physiological differ in the functions.

It cannot achieve high drug levels in blood.

Transdermal drug delivery system cannot deliver ionic drugs.

It cannot deliver drugs in a pulsatile fashion.

It cannot develop if drug or formulation causes irritation to skin.

Possibility of local irritation at site of application.

3.7.4 BASIC COMPONENTS OF TDDS [172]

1. Drug

The drug is in direct contact with release liner.

Example: Nicotine, Methotrexate and Oestrogen.

Some of the desirable properties of a drug for transdermal delivery are as follows:

- The drug molecule should possess an adequate solubility in oil and water.
- The drug should have molecular weight less than approximately 1000 Daltons.
- The drug should have low melting point.
- The drug molecule would require a balanced partition coefficient to penetrate the stratum corneum.

2. Polymer matrix

- These polymers control the release of the drug from the drug reservoir Natural polymer: Shellac, gelatin, waxes, gums, starch etc.
- Synthetic polymer: Polyvinyl alcohol, polyamide, polyethylene, polypropylene, polyurea, polymethylmethacrylate etc.

3. Permeation enhancer

Substances exist which temporarily diminish impermeability of the skin are known as accelerants or sorption promoters or penetration enhancers. This include water, pyrrolidones, fatty acids and alcohol, ozone and its derivatives, alcohol and glycols, essential oils, terpenes and derivatives, sulfoxides like dimethylsulfoximide and their derivatives urea and surfactants.

4. Adhesive

Serves to add to the skin for systemic delivery of drug Examples: silicones, polysobutylene

5. Backing layer

Backing layer protect patch from outer environment. Example: cellulose derivatives, polypropylene silicon rubber.

3.7.5 FACTORS AFFECTING TDDS [175]

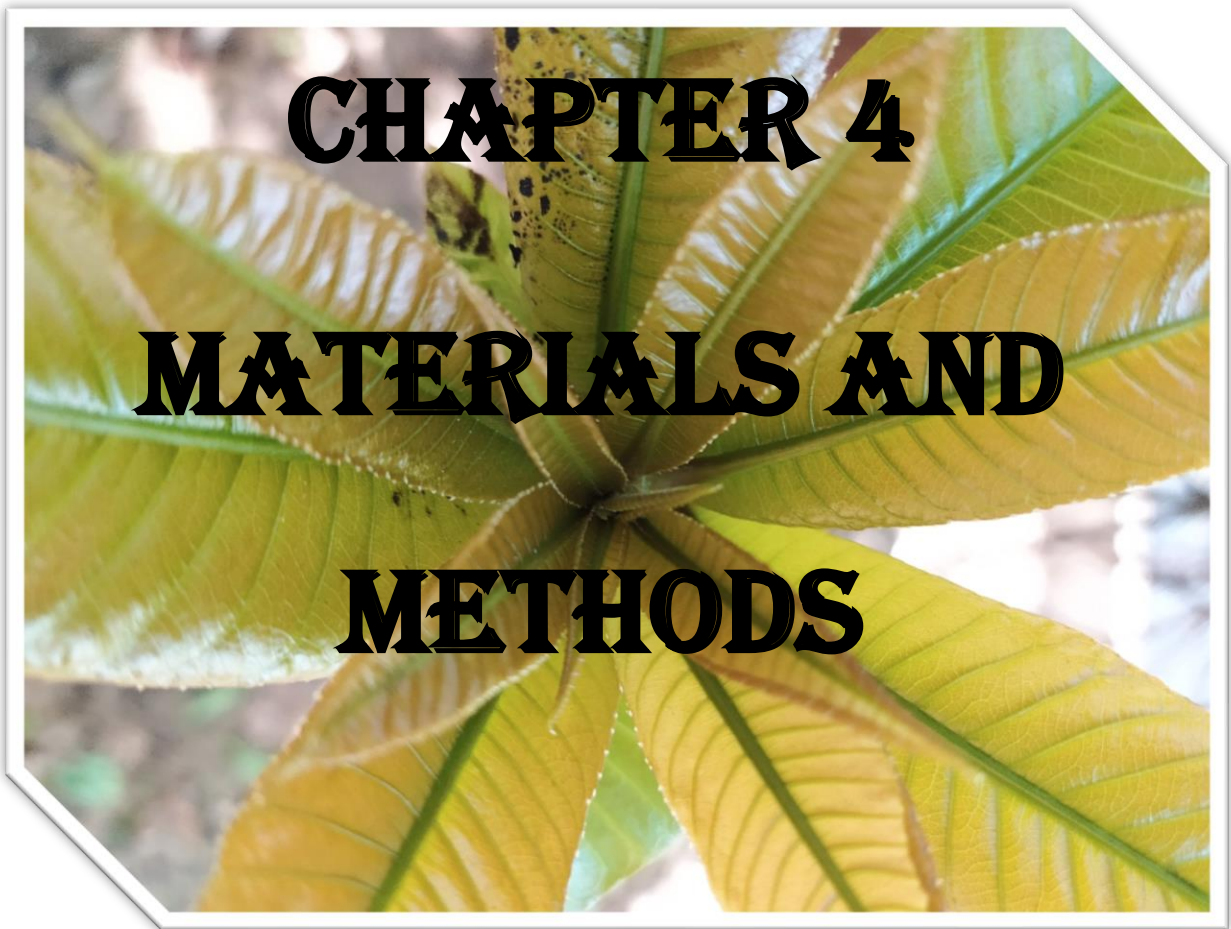
- Not all drug substance are suitable for transdermal delivery. Among the factors playing a part in percutaneous absorption are the physical and chemical properties of the drug, including its molecular weight solubility partitioning coefficient and dissociation constant, (pka), the nature of the carrier vehicle, condition of skin

Drug concentration is an important factor. Generally, the amount of drug percutaneously absorbed per unit of surface area per time interval increases with increase in the concentration of drug in the TDDS.

- The larger the area of application (the larger the TDDS), the more drug is absorbed
- The drug should have greater physicochemical attraction to the skin than to the vehicle so that the drug will leave the vehicle in favor of skin.
- Drug with molecular weight of 100 to 800 and adequate lipid and aqueous solubility can permeate lipid and aqueous solubility can permit the skin. The ideal molecular weight of a drug for transdermal drug delivery is believed to be 400 or less.
- Hydration of skin generally favors percutaneous absorption. The TDDS acts as an occlusive moisture barrier through which sweat cannot pass, increasing skin hydration.
- Percutaneous absorption appears to be greater when the TDDS is applied to a site with a thin horny layer than with a thick one.
- Generally, the longer the medicated application is permitted to remain in contact with the skin, the greater is the total drug absorption.

3.8 TRANSDERMAL PATCH [175]

The system for passive transdermal delivery, two areas of formulation research is focused on adhesives and excipients. Adhesive research focuses on customizing the adhesive to improve skin adhesion over the wear period, improve drug stability and solubility, reduce lag time, and increase the rate of delivery. Because a one-size-fits-all adhesive does not exist that can accommodate all drug and formulation chemistries, customizing the adhesive chemistry allows the transdermal formulator to optimize the performance of the transdermal patch.



CHAPTER 4

MATERIALS AND

METHODS

4 Materials and method

4.1 Materials

4.1.2 Herbal drugs

Boswellia serrata
gum resin extract

- Received as Gift Sample from Sunpure Extracts pvt. Ltd. Kerala, India

Capsicum
annuum fruit
extract

- Procured from Vital Herbs, Delhi, India

Menthol

- Procured from Loba Chemie, India

Marker : Capsaicin standard compound purchased from TCI Chemicals India.

4.1.3 Chemicals and Reagents:

Chloroform AR grade, Potassium bismuth iodide, Sodium Picrate, Mayer's reagent, iodine, Potassium Iodide, Ferric chloride, Sodium Nitroprusside, Conc. H₂SO₄, Conc.HCl, Pyridine, Potassium permanganate, Lead acetate, Methanol, Toluene, Ethyl acetate, Formic acid, Ninhydrine, Aluminium coated TLC plate 254 F (10x10 cm), saturation chamber etc.

The plant extracts were subjected to various qualitative tests to determine presence of various phytochemical classes.

4.2 METHOD

4.2.1 Organoleptic evaluation

The colour, odour, taste, surface etc of the plant extract were studied.

4.2.2 Proximate Analysis of Each Plant Extracts:

4.2.2.1 Alkaloids:

a. Dragendroff's test for alkaloids

Dissolve extract in chloroform. Chloroform was evaporated and the residue was acidified by adding few drops of Dragendroff's reagent (Potassium bismuth iodide). Appearance of orange red precipitate indicated presence of alkaloids.

b. Hager's test for alkaloids

Dissolve extract in chloroform. Filter it and concentrate to 2-3 mL filtrate. Add Hager's reagent (picric acid solution) to filtrate. Appearance of yellow precipitate indicated presence of alkaloids.

c. Mayer's test for alkaloids

Dissolve extract in chloroform. Filter it and concentrate to 2-3 mL filtrate. Add Mayer's reagent (mercuric chloride) to filtrate. Appearance of cream coloured precipitate indicated presence of alkaloids.

d. Wagner's test for alkaloids

Dissolve extract in chloroform. Filter it and concentrate to 2-3 mL filtrate. Add Wagner's reagent (iodine and potassium iodide) to filtrate. Appearance of buff red precipitate indicated presence of alkaloids.

4.2.2.2 Glycosides:**a. Keller-Killiani's test for steroidal glycosides**

A total of 1 mL of glacial acetic acid, few drops of ferric chloride solution and conc. H₂SO₄ (Slowly through the sides of the test tube) were added to the extract. Appearance of reddish brown ring at the junction of the liquids indicated the presence of de-oxysugars.

b. Legal's test for lactones or cardiac glycosides

To the extract mixtures add sodium nitroprusside and pyridine. Then the mixture was treated with NaOH. Appearance of deep red colour indicated the presence of lactones.

c. Foam test for Saponins Glycosides

Small amount of extract was taken in a test tube with little quantity of water and shake vigorously. Appearance of foam persisting for 10 min indicated presence of saponins.

d. Sodium picrate test for Cyanogenetic Glycosides:

Soak filter paper strip first in 10% picric acid, then in 10% sodium carbonate, dry. In a conical flask place moistened powdered drug. Cork it and place the above filter paper strip in the slit in cork. The paper turns brick red or maroon indicating presence of Cyanogenetic glycosides.

e. Alkaline ammonia or Fluorescence test for Coumarin Glycosides

Alcoholic extract when made alkaline, shows blue or green fluorescence.

4.2.2.3 Carbohydrates:

a. Molisch's test for carbohydrates

To 2-3 ml of aqueous extract add few drops of alpha naphthol in alcohol and add concentrated sulfuric acid from the side of the test tube. Violet ring is formed at the junction of two liquids.

4.2.2.4 Steroids:

a. Salkowski reaction test for phytosterols

To 0.5 mL chloroform extract in a test tube add 1 mL of concentrated (conc.) H_2SO_4 from the sides of the test tube. Appearance of reddish brown colour in chloroform layer indicates presence of phytosterols.

b. Liebermann-Burchard's test for triterpenoids

Extract was treated with few drops of acetic anhydride, boil and cool. Conc. sulphuric acid was added from the sides of the test tube which showed a brown ring at the junction of two layers, and formation of deep red colour indicated the presence of triterpenoids.

4.2.2.5 Fats and oils:

a. Filter paper test

Powdered material is pressed firmly in double layered filter paper; permanent stain is obtained indicating presence of essential fixed oil.

4.2.2.6 Flavonoids:

a. Lead acetate test

To the alcoholic solution of the extract add few drops of 10% lead acetate solution. Appearance of yellow precipitate indicated presence of flavonoids.

b. Shinoda test

To dry powder or extract, add 5 mL 95% ethanol, few drops of conc. HCl and 0.5 g magnesium turnings. Pink colour is observed.

4.1.3.7 Proteins:

a. Biuret test

To aqueous extract add 4% sodium hydroxide solution and two drops of one percent copper sulphate solution. The appearance of violet colour indicates that the presence of protein.

b. Millon's test

5 ml of Millon's reagent was added to the aqueous extract gives white precipitates which on heating convert in to brick red colour .

c. Precipitation test

The aqueous extract gives white colloidal precipitates with following reagents (a) absolute alcohol (b) 5% mercuric chloride solution (c) 5% lead acetate (d) 5% ammonium sulphate.

4.2.2.8 Amino acids:**Ninhydrin test**

Heat aqueous extract and add 3 drops of 5% Ninhydrin solution in boiling water for 10 min. The appearance of pink or purple colour indicates that the presence of proteins, peptides or amino acids

4.2.2.9 Tannins and Phenolic compounds**Ferric chloride test**

To aqueous extract add 5% ferric chloride solution gives deep blue-black colour.

Lead acetate test

To aqueous extract add lead acetate solution, gives white precipitates.

KMnO₄ test

Aqueous extract when treated with dilute potassium permanganate solution, it decolourised the solution.

4.2.3 Thin Layer Chromatography of each Plant Extracts [1], [2], [3]

Thin Layer chromatography is effective standardization technique through which the presence of marker compound is determined in relevant plant extracts.

Executed steps for TLC were given below

- Activation of TLC plate by heated in oven at 121⁰ C for 15-20 minutes.
- After drying sample was applied on lower surface of it.
- Then TLC plate was run under saturated mobile phase up to 70-80% of height.
- Then further allowed to air dry and derivatised with Anisaldehyde Sulphuric acid reagent, followed by heating the plate at 110 °C for 10-15 min.

- Obtained spot/band was calculated and reported as Rf value.
- Rf value = Distance travelled by solute/distance travelled by solvent

Table 2 TLC profile of Herbal extracts

Extract	Mobile Phase	Detection wavelength	Rf value of standard	Rf value of taken extract
<i>Boswellia serrata</i> extract	Toluene : Ethyl acetate : Methanol (8:1.5:0.5)	254 nm	Boswellic acid	
		α -boswellic acid	0.71	0.73
		β -boswellic acid	0.50	0.54
<i>Capsicum annum</i> extract	Toluene : Ethyl acetate : Methanol (8:1.5:0.5)	254 nm	Capsaicin	
			0.67	0.69

4.2.4 Microbial Analysis :

The plant extracts were procured from different industries and Certificate Of Analysis of each extracts were provided so from that all data were gathered and compared with the standard limit. Standard value for total microbial count is 10^5 CFU/gm as per Ayurvedic standard.

4.2.5 Determination of Moisture content:

The procedure used to determines the amount of water and volatile matter present in plant extract. It is only applicable when drug/extract which contains any volatile matter of water in it.

4.2.6 Heavy metal Analysis:

Heavy metals such as arsenic, lead, cadmium, mercury etc. were analysed by the Atomic Absorption Spectrophotometer (AAS) in plant extracts; which was done by company from where we collected the extracts and certificate of analysis also provided by them. (COA attached in Appendix)

4.2.7 Determination of pH of aqueous solution (2% w/v)

2 g powdered drug material (extract) was suspended in 100 mL distilled water and pH was measured in triplicates by using calibrated and validated pH meter.

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4.2.8 Estimation of Secondary metabolites [4]

4.2.8.1 Determination of Total Phenolic content:

The concentration of phenolics in plant extracts was determined using spectrophotometric method. Folin-Ciocalteu assay method was used for the determination of the total phenol content. The reaction mixture consists of 1 ml of extract and 9 ml of distilled water was taken in a volumetric flask (25 ml). One millilitre of Folin-Ciocalteu phenol reagent was treated to the mixture and shaken well. After 5 minutes, 10 ml of 7 % Sodium carbonate (Na_2CO_3) solution was treated to the mixture. The volume was made up to 25 ml. A set of standard solutions of gallic acid (4,6,8,10 and 12 $\mu\text{g}/\text{ml}$) were prepared in the same manner as described earlier. Incubated for 90 min at room temperature and the absorbance for test and standard solutions were determined against the reagent blank at 550 nm with an Ultraviolet (UV) /Visible spectrophotometer. Total phenol content was expressed as mg of GAE/gm of extract.

4.2.8.2 Determination of Total tannin content:

The tannins were determined by Folin - Ciocalteu method. About 0.1 ml of the sample extract was added to a volumetric flask (10 ml) containing 7.5 ml of distilled water and 0.5 ml of Folin-Ciocalteu phenol reagent, 1 ml of 35 % Na_2CO_3 solution and dilute to 10 ml with distilled water. The mixture was shaken well and kept at room temperature for 30 min. A set of reference standard solutions of gallic acid (1,2,3,4,5 and 6 $\mu\text{g}/\text{ml}$) were prepared in the same manner as described earlier. Absorbance for test and standard solutions were measured against the blank at 725 nm with an UV/Visible spectrophotometer. The tannin content was expressed in terms of mg of GAE /g of extract.

4.2.8.3 Determination of Total flavanoid content:

Total flavonoid content was measured by the aluminium chloride colorimetric assay. The reaction mixture consists of 1 ml of extract and 4 ml of distilled water was taken in a 10 ml volumetric flask. To the flask, 0.30 ml of 5 % sodium nitrite was treated and after 5 minutes, 0.3 ml of 10 % aluminium chloride was mixed. After 5 minutes, 2 ml of 1M Sodium hydroxide was treated and diluted to 10 ml with distilled water. A set of reference standard solutions of

quercetin (10, 20, 30, 40 and 50 $\mu\text{g/ml}$) were prepared in the same manner as described earlier. The absorbance for test and standard solutions were determined against the reagent blank at 510 nm with an UV/Visible spectrophotometer. The total flavonoid content was expressed as mg of QE/g of extract.

4.2.8.4 Determination of Total alkaloid content [5]

The percentage of total alkaloids in sample was determined by titrimetric method. It was dissolved in 2.0 ml acetone and 10.0 ml ether. The solution was quantitatively transferred to a 250 ml conical flask and 5 ml bromocresol green indicator was added. This solution was carefully titrated with N/20 H_2SO_4 until the appearance of light green colour (till end point). The volume of acid used was noted.

Each ml of acid used is equivalent to 0.00725 gm of total alkaloid.

Subsequently the percentage of alkaloids in the powdered sample was calculated with reference to the original weight of the sample used.

4.2.9 ESTIMATION OF CAPSAICIN IN CAPSICUM EXTRACT BY UV SPECTROSCOPY

Preparation of Capsaicin stock solution

25 mg of Capsaicin dissolved in 25 ml Methanol to make 1000 $\mu\text{g/ml}$ stock solution.

Dilute 1 ml in 10 ml Methanol to obtain 100 $\mu\text{g/ml}$ solution. Accordingly make solution range between 10, 20, 40, 80, 160 $\mu\text{g/ml}$.

Take absorption at 280 nm.

Preparation of sample solution

1 gm of *Capsicum annum* extract dissolved in 50 ml methanol.

Stirr for 15 min on magnetic stirrer at moderate speed, then filter the undissolved particles.

Take the filtrate and make up the volume upto 100 ml with Methanol.

Take absorbance of above prepared solutin at 280 nm.

DESIGN AND DEVELOPMENT OF POLYHERBAL PATCH FORMULATION

4.3 LIST OF MATERIALS USED

Table 3 List of Materials

	Material	Name	Supplier of material
1.	Herbal Drugs	Boswellia serrata extract	Gift sample from Sun pure pvt. Ltd.
		Capsicum annum extract	Procured from Vital herbs ltd. Delhi.
		Menthol	Procured from Loba Chemie Pvt ltd.
2.	Polymer	HPMC K100m PVP	Gift sample from Ipca Lab pvt. Ltd. Gift sample from Ipca Lab pvt. Ltd.
3.	Penetration Enhancer	Propylene glycol	Procured from Loba Chemie pvt. Ltd.
4.	Plasticizer	PEG-400	Procured from Loba Chemie pvt. Ltd.
5.	Solvents	Methanol	Rankem Chemical
		Distilled water	Prepared in lab.

4.4 LIST OF EQUIPMENTS USED

Table 4 List of Equipments

Sr. No.	Name	Company's name
1.	Weighing balance	Aczet pvt Ltd.
2.	Magnetic stirrer	Remi equipments pvt. Ltd.

4.5 PATCH FORMULATION

The system for passive transdermal delivery, two areas of formulation research is focused on adhesives and excipients. Adhesive research focuses on customizing the adhesive to improve skin adhesion over the wear period, improve drug stability and solubility, reduce lag time, and increase the rate of delivery. Because a one-size-fits-all adhesive does not exist that can accommodate all drug and formulation chemistries, customizing the adhesive chemistry allows the transdermal formulator to optimize the performance of the transdermal patch.

4.5.1 Selected herbal drug and their dose

Table 5 Selected Herbal drugs

Sr. No.	Extract's name	Dose	Reference
1.	<i>Boswellia serrata</i>	0.25 – 0.75 %	[1]
2.	<i>Capsicum annuum</i>	0.025 – 1 %	[4]
3.	Menthol	1 – 10 %	[5]

4.5.2 Polymer

Basic criteria

- should adhere onto the skin surface.
- should be non-irritant on skin.
- Synthetic polymers majorly used for topical patch formations are the wide range of HPMC(hydroxy propyl methyl cellulose) series from HPMC to HPMC Iv grade, PVP, PVA, Ehtyl Cellulose.
- Natural polymers includes sodium alginate, eudragit, chitosan, cellulose & Pectin.
- HPMC K100m and Polyvinyl Pyrrolidone (PVP) grade polymer was ultimately selected based on the solubility in the hydroalcoholic solvent.

4.5.3 Selection of excipients

Table 6 Selection of Excipients

Sr. No.	Excipients	Purpose
1.	HPMC K100m	<ul style="list-style-type: none"> • Thickening properties • Film forming agent
2.	PVP	<ul style="list-style-type: none"> • Film forming agent
3.	Water	<ul style="list-style-type: none"> • To increase the solubility of extracts and excipients
4.	PEG 400	<ul style="list-style-type: none"> • Acts as plasticizer.
5.	Methanol	<ul style="list-style-type: none"> • To increase the solubility of extracts and excipients
6.	Propylene glycol	<ul style="list-style-type: none"> • Penetration Enhancer

Polymers	<ul style="list-style-type: none">• Hydroxypropyl methylcellulose(HPMC),• Polyvinyl pyrrolidone(PVP)
Plasticizer	<ul style="list-style-type: none">• Polyethylene glycol 400
Penetration enhancer	<ul style="list-style-type: none">• Propylene glycol
Solvent	<ul style="list-style-type: none">• Methanol, Water

4.6 Preparation of patch formulation by solvent casting method

- Prepare homogenous solution of polymer in Methanol : Water
- Add plasticizer and Penetration enhancer and stir well.
- Put a mixture on magnetic stirrer (moderate speed) to make uniform mixture.
- Dissolve the respective herbal drugs in desired quantity and add to above mixture.
- Stir on magnetic stirrer for 5 minutes.
- Put aside on stand for another 5 minutes to remove air bubble.
- Pour this prepared mixture in clean and dried petri plate.
- Let it dry for 24 hours at room temperature, then remove it from petri dish and store in cool and dry place.

4.7 OPTIMIZATION OF FORMULATION PARAMETERS

Almost all the reported methods of preparation of transdermal patch have been the same but some formulation conditions are varied. Hence it is necessary to optimize the certain parameters like plasticizer concentration, drying method and polymer ratio.

The influence of these factors on patch properties and drug release was investigated initially. Film forming polymer was used alone to prepare a patch, but patch shows best results in combination with a another polymer. Various batches of formulations were prepared by varying one parameter and keeping the others constant. As the concentration of polymer increases the patch thickness and weight increases, therefore for uniform thickness of the patch and weight, the total polymer or solid content weight was kept 700 mg.

4.7.1 Optimization of Polymer ratio (Batch 1)

Table 7 Optimization of Polymer ratio

Batch number	Polymer	Quantity (mg)	Taken	Solvent (ml)
A1	HPMC : PVP	500 : 200		Methanol:Water (15:5)
A2	HPMC : PVP	350 : 350		Methanol: Water
A3	HPMC : PVP	400 : 300		Methanol: Water
A4	HPMC : PVA	500 : 200		Methanol: Water
A5	HPMC : PVA	350 : 350		Methanol: Water
A6	HPMC : PVA	400 : 300		Methanol: Water
A7	HPMC : Ethyl Cellulose	500 : 200		Methanol: Water
A8	HPMC : Ethyl Cellulose	350 : 350		Methanol: Water
A9	HPMC : Ethyl Cellulose	400 : 300		Methanol: Water
A10	HPMC	500		Methanol:Water (15:5)
A11	HPMC	400		Methanol:Water (15:5)
A12	Pectin : Sodium Alginate	350 : 350		Water (15)

A13	Pectin : Sodium Alginate	500 : 200	Water (15 ml)
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4.7.2 Optimization of Plasticizer concentration (Batch 2)

Table 8 Optimization of Plasticizer concentration

Batch Number	Constant Parameter	Quantity taken	Plasticizer	Quantity taken (ml)
B1	HPMC : PVP	350 : 350	PEG 400	0
B2	HPMC : PVP	350 : 350	PEG 400	2
B3	HPMC : PVP	350 : 350	PEG 400	3
B4	HPMC : PVP	350 : 350	PEG 400	5

4.7.3 Optimization of Solvent ratio (Batch 3)

Table 9 Optimization of solvent ratio

F No.	HPMC (mg)	PVP (mg)	Methanol (ml)	Water (ml)	PEG 400 (ml)	Propylene Glycol(ml)
1.	400	400	13	7	7	0.5
2.	350	350	15	5	5	1.25
3.	350	350	15	5	5	1.25
4.	400	400	17	3	7	2
5.	300	300	13	3	3	0.5
6.	350	350	15	5	5	1.25
7.	300	400	13	3	3	2
8.	400	300	13	7	3	0.5
9.	300	300	13	7	7	2
10.	400	300	17	7	3	2

11.	300	300	17	7	7	0.5
12.	400	300	17	3	3	0.5
13.	400	300	13	3	7	2
14.	300	400	17	7	3	2
15.	300	400	17	5	7	0.5

Design Summary

Factors: 6 Replicates: 1
 Base runs: 15 Total runs: 15
 Base blocks: 1 Total blocks: 1

4.7.4 OPTIMIZED FORMULA OF THE FORMULATION

Table 10 Optimized formula

Sr no.	Placebo	Quantity taken
1.	Boswellia serrata	-
2.	Capsicum annum	-
3.	Menthol	-
4.	HPMC	350 mg
5.	PVP	350 mg
6.	Methanol	13 ml
7.	Water	7 ml
8.	PEG 400	3 ml
9.	Propylene Glycol	0.5 ml

4.7.5 FORMULA OF DRUG LOADED PATCH FORMULATION

Table 11 Formula of drug loaded patch formulation

Sr. no	Ingredients	Optimized Quantity taken			
		F1	F2	F3	F4
1.	Boswellia serrata	250 mg	500 mg	750 mg	1 gm
2.	Capsicum annum	25 mg	50 mg	75 mg	100 mg
3.	Menthol	10 mg	50 mg	70 mg	100 mg
4.	HPMC	350 mg			
5.	PVP	350 mg			
6.	Methanol	13 ml			
7.	Water	7 ml			
8.	PEG 400	3 ml			
9.	Propylene glycol	0.5 ml			

4.8 Evaluation of patch formulation

4.8.1 Folding endurance

A strip of specific area (2 cm*2 cm) was cut evenly and repeatedly folded at the same place till it broke. The number of times the film was folded at the same place without breaking gave the value of the folding endurance.

4.8.2 Thickness

Patch thickness was measured using micrometer screw gauge at three different places, and the mean value was calculated.

4.8.3 Tensile strength

The tensile strength of the patch was evaluated by using the texture analyzer. It consists of two load cell grips. The lower one was fixed and upper one was movable. Film strips with

dimensions of 2*2 cm were fixed between these cell grips, and force was gradually applied till the film broke. The tensile strength was taken directly from the dial reading in kg.

4.8.4 Percentage Moisture content

The prepared films were weighed individually and kept in a desiccator containing fused calcium chloride at room temperature for 24 h. After 24 h, the films were reweighed and determined the percentage moisture content from the below mentioned formula:

$$\% \text{ moisture content} = [\text{Initial weight} - \text{Final weight}] / \text{Final weight} \times 100.$$

4.8.5 Percentage Moisture uptake

The weighed films were kept in a desiccators at room temperature for 24 h containing saturated solution of potassium chloride in order to maintain 84% RH. After 24 h, the films were reweighed and determine the percentage moisture uptake from the below mentioned formula:

$$\% \text{ Moisture uptake} = [\text{Final weight} - \text{Initial weight}] / \text{Initial weight} \times 100.$$

4.8.6 In vitro drug release study

In Vitro drug release studies were performed by using a Franz diffusion cell with a receptor compartment capacity of 60 mL. The cellulose acetate membrane was used for the determination of drug from the prepared transdermal matrix-type patches. The cellulose acetate membrane having a pore size 0.45 μ was mounted between the donor and receptor compartment of the diffusion cell. The prepared patch was placed on the cellulose acetate membrane and covered with aluminum foil. The receptor compartment of the diffusion cell was filled with phosphate buffer pH 7.4. The whole assembly was fixed on a hot plate magnetic stirrer, and the solution in the receptor compartment was constantly and continuously stirred using magnetic beads, and the temperature was maintained at 32 ± 0.5 °C, because the normal skin temperature of human is 32 °C. The samples were withdrawn at different time intervals and analyzed for drug content spectrophotometrically. The receptor phase was replenished with an equal volume of phosphate buffer at each sample withdrawal.

4.8.7 Drug content uniformity

A specified area of patch (2 cm*2 cm) was dissolved in 100 mL methanol and shaken continuously for 24 h. Then the whole solution was ultrasonicated for 15 min. After filtration, the drug was estimated spectrophotometrically at wavelength of 280 nm and determined the drug content.

4.9 HPTLC METHOD DEVELOPMENT AND VALIDATION

4.9.1 DEVELOPMENT OF HPTLC METHOD

HPTLC INSTRUMENTATION

Table 12 HPTLC Instrumentation

Sample Applicator	Automated TLC sample applicator Linomat V with Nitrogen as inert gas
Syringe	Camag 100 µl HPTLC syringe
Development Chamber	Camag twin through chamber (10 x10 cm)
Scanner	Camag TLC Scanner
Software	Wincats software

4.9.1.1 Preparation of sample solution

Sample 1 : Boswellia serrata extract

0.25 gm of B. serrata gum resin extract was dissolved in 25 ml of Methanol. 1 ml diluted to 10 ml Methanol.

Sample 2 : Capsicum annum extract

0.25 gm of Capsicum annum fruit extract was dissolved in Methanol.

Sample 3 : Test solution of prepared formulation

4.9.1.2 Preparation of Capsaicin stock solution

10 mg of Capsaicin dissolved in 10 ml of Methanol.

Optimized Mobile phase : **Toluene : Ethyl Acetate : Methanol**

(8 : 1.5 : 0.5)

Rf Value = Distance travelled by solute/Distance travelled by solvent

4.9.2 Validation of HPTLC method:

The method was validated according to ICH guidelines for Linearity, Precision, Accuracy, Limit of Detection and Limit of Quantification.

Linearity:

Linearity of the method was performed by analyzing both the markers in combination as following concentration range three times.

Precision

Precision of an analytical method is usually expressed as the standard deviation. The repeatability studies were conducted by estimating the response of 2 markers in six times.

Reproducibility of methods was checked by performing intra-day precision (three times a day) and inter-day precision (repeated triplicates for three consecutive days). Results are expressed in terms of standard deviation and % Relative standard Deviation (RSD).

Intraday precision was determined by estimation of mixture of standard markers solution in lower, middle and higher concentration in triplicates on the same day.

Interday precision was determined by estimation of mixture of standard markers solution in lower, middle and higher concentration on three different days.

Robustness

Robustness of the method was investigated under a variety of conditions including changes of composition of mobile phase, saturation time and different detection wavelength. This deliberate change in the method has no effect on the peak shape and good resolution. Therefore the method was found to be robust.

Limit of Detection (LOD):

The LOD can be defined as the lowest amount of analyte that can be detected but not quantified.

LOD can be calculated as per following equation:

$$\text{LOD} = 3.3 \sigma/S$$

Where σ is standard deviation of regression line and S is slope of calibration curve

Limit of Quantification:

Quantification limit of an individual analytical procedure is the lowest amount of analyte that can be quantitatively determined with suitable precision and accuracy.

$$\text{LOQ} = 10 \sigma/S$$

Where σ is standard deviation of regression line and S is slope of calibration curve

Accuracy

The accuracy was determined by calculating % recoveries of 2 markers (Spiking method). It was carried out by adding known amounts of each analyte corresponding to three concentration levels (80, 100, and 120%) of the labeled claim to the excipients. At each level, two determinations were performed, and the accuracy results were expressed as percent analyte recovered by the proposed method.

Application of HPTLC method on developed formulation:

Applicability of proposed method for both the laboratory based formulations was quantified for the marker components – Catechin and Curcumin. The content of both markers were determined by applying the prepared laboratory sample as per proposed chromatographic condition. The concentrations of markers were determined by following equation.

$$\% \text{ Assay} = \frac{\text{Area of sample} \times \text{Std wt taken} \times \text{Sample dilution}}{\text{Area of std} \times \text{Std dilution} \times \text{Sample wt taken}} \times 100$$

4.10 In-vivo evaluation of Anti-inflammatory activity by Carrageenan induced paw edema model

PURPOSE AND RATIONALE

Among the many methods used for screening of antiinflammatory drugs, one of the most commonly employed techniques is based upon the ability of such agents to inhibit the edema produced in the hind paw of the rat after injection of a phlogistic agent. Many phlogistic agents (irritants) have been used, such as brewer's yeast, formaldehyde, dextran, egg albumin, kaolin, Aerosil, sulfated polysaccharides like carrageenan or naphthoylheparamine. The effect can be measured in several ways. The hind limb can be dissected at the talocrural joint and weighed. Usually, the volume of the injected paw is measured before and after application of the irritant and the paw volume of the treated animals is compared to the controls. Many methods have been described how to measure the paw volume by simple and less accurate and by more sophisticated electronically devised methods. The value of the assessment is less dependent on the apparatus but much more on the irritant being chosen. Some irritants induce only a short lasting inflammation whereas other irritants cause the paw edema to continue over more than 24 h.

PROCEDURE

Wistar rats will be divided into 5 groups, with 6 rats in each. The first group of rats had no inflammation and received no treatment. The second group was inflamed by carrageenan injection and did not undergo any treatment. The inflammation of the third group, use as reference which will be treated with diclofenac patch and that of the fourth group was treated by a polyherbal anti-inflammatory patch and fifth group will be treated with herbal marketed formulation, respectively, 1 hour after the carrageenan injection. The dose of polyherbal formulation will proportional in the size of the edema and covered the whole swelling. In all treated groups, the size of the edema will be measured before and after the inflammatory injection using a digital caliper.

Edema is expressed as the relative increase in paw volume induced by the inflammation injection (i.e., the edema was proportional to the volume difference between 0 hours and the other times, 1 hour, 2, 3, 4, and 5 hours, after carrageenan injection).

EXPERIMENTAL DESIGN TO EVALUATE IN-VIVO ANTI-INFLAMMATORY ACTIVITY

Table 13 Experimental design for in-vivo anti-inflammatory activity

Groups		Administered samples	No. of animals
1.	Normal control	Normal control	6
2.	Model control	1 % freshly prepared carrageenan in distilled water into the subplantar region of the right hind paws	6
3.	Standard control	Diclofenac patch	6
4.	Test Treatment	Polyherbal patch formulation	6
5.	Herbal marketed formulation	Viopatch herbal pain relief patch	6

Total number of Animal = 30

INFLAMMATION INDUCING AGENT AND DOSE

Table 14 Inflammatory inducing agent and dose

Substances	1 % carrageenan solution in distilled water
Site/ Route of administration	subplantar region of the right hind paws
Volume	0.1 ml carrageenan injection

Percentile edema inhibition is calculated according to the following formula:

$$\text{Percentile inhibition} = [1 - (VT/V0)] \times 100.$$

Where, *VT* represents the edema volume in the drug treated group.

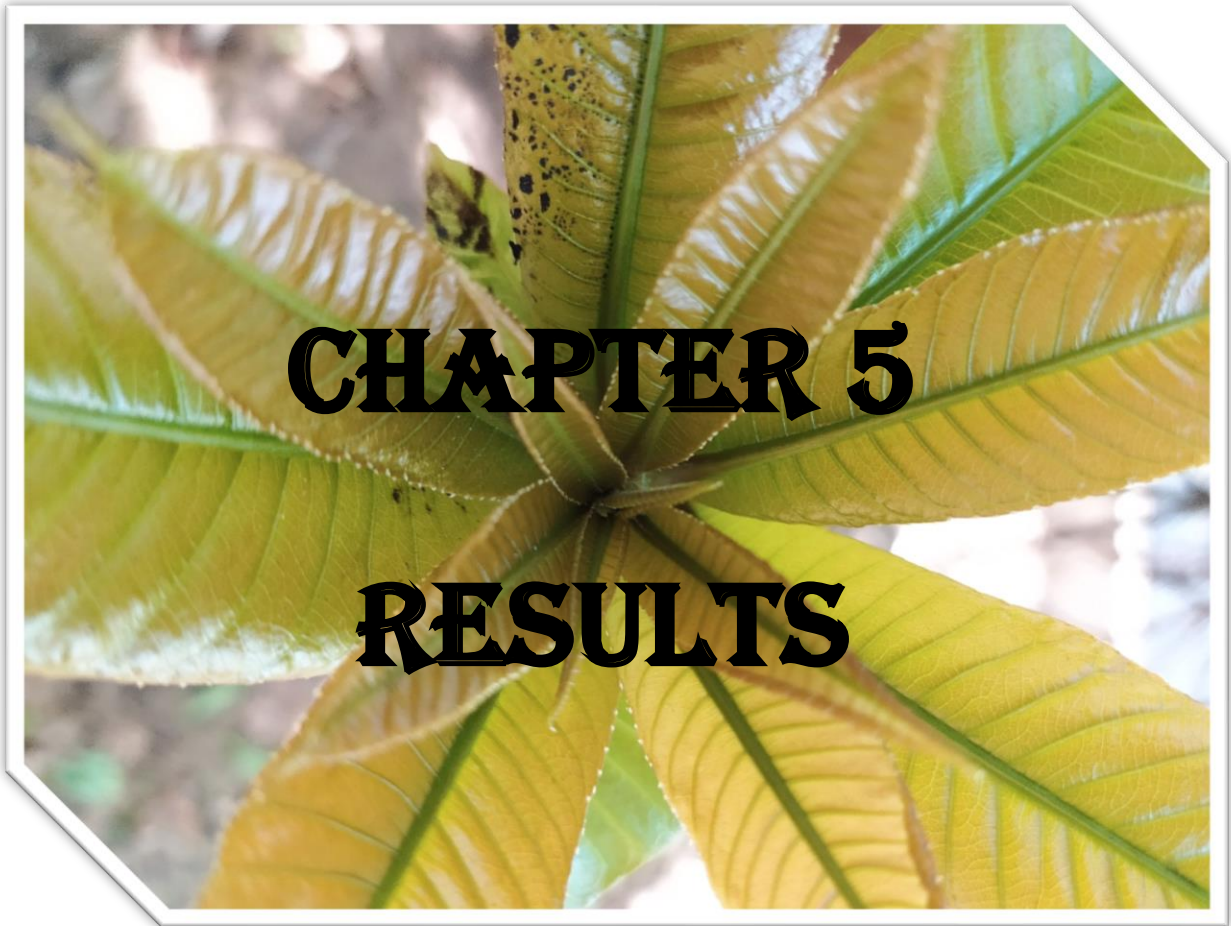
V0 represents the edema volume in the Carr group.

The degree of inflammation induced will be evaluated according to the following formula:

$$\text{Percentile inflammation} = (PT - P0)/P0 \times 100.$$

Where, *PT* represents the volume of the right hind paw after Carrageenan treatment.

P0 represents the volume of the right hind paw before Carrageenan treatment.



CHAPTER 5

RESULTS

5 Results

5.1 Standardization of plant extract as per WHO guidelines

5.1.1 ORGANOLEPTIC EVALUATION

Table 15 Organoleptic evaluation

	<i>Boswellia serrata</i> extract	<i>Capsicum annum</i> extract	Method
Colour	Off white to pale yellow	Green to brown	Visual
Odour	Odourless	Pungent	Organoleptic
Appearance	Powder	Fine Powder	Organoleptic

APPEARANCE

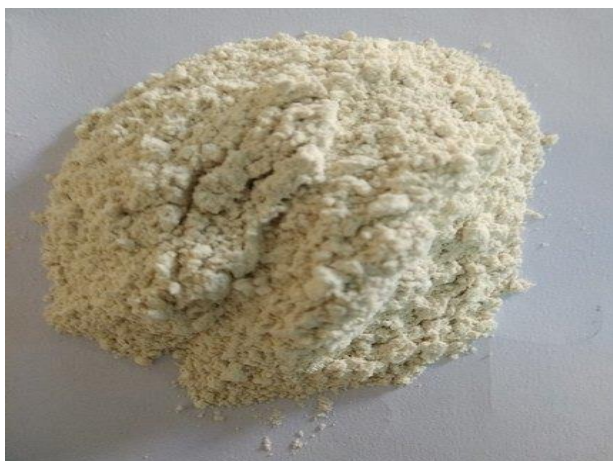


Figure 3 *Boswellia serrata* extract



Figure 4 *Capsicum annum* extract



Figure 5 Menthol crystals

5.1.2 Analytical assay

Table 16 Analytical assay

Description	<i>Boswellia serrata</i> extract	<i>Capsicum annum</i> extract
Herb : Extract ratio	6 : 1	10 : 1
Solubility	Methanol, Chloroform	Water, Methanol
Moisture	1 %	2.89 %
Extraction solvent	Ethyl acetate	Methanol
Particle size range	100 % through 30#	Pass 80 #
Tapped density	0.54 gm/ml	-
Bulk density	0.30 gm/ml	49.2 gm / 100 ml
Pesticide residue	Complies with EP	Complies with EP

5.1.3 Microbial analysis

Table 17 Microbial analysis

Description	<i>Boswellia</i> extract extract	<i>Capsicum annum</i> extract
Total plate count(CFU/gm)	10 Cfugm	NMT 5000 Cfug
(Limit)	NMT 10000 Cfugm	
Yeast & Mould Count(CFU/gm)	10 Cfugm	Less than 100Cfugm
(Limit)	NMT 100 Cfugm	
E.coli	Absent	Absent
Salmonella	Absent	Absent

5.1.4 Heavy metal analysis

Table 18 Heavy metal analysis

Heavy Metals	Limit(ppm)	<i>Boswellia serrata</i> extract	<i>Capsicum annum</i> extract
Arsenic(ppm)	NMT 1	Complies	Complies
Mercury (ppm)	NMT 0.1	Complies	Complies
Lead(ppm)	NMT 3	Complies	Complies
Cadmium(ppm)	NMT 1	Complies	Complies

5.1.5 pH

Table 19 pH

	<i>Boswellia serrata</i> extract	<i>Capsicum annum</i> extract
pH	7.6	6.3

5.1.6 Moisture content

Table 20 Moisture content

	<i>Boswellia serrata</i> extract	<i>Capsicum annum</i> extract
%LOD	1.00 %	2.89 %

5.2 Preliminary phytochemical screening

Table 21 Preliminary phytochemical screening

Sr. no.	Phytochemical test	Boswellia extract	Capsicum extract	Observation
1.	For carbohydrates 1. Molisch's test	+	+	Violet ring observed
	2. Fehling's test	--	--	No red precipitate
	3. Barfoed's test	--	--	Red precipitate not observed
2.	For proteins 1. Biuret test	--	--	No pink colour observed
	2. millon's test	+	+	White precipitate observed
	3. Xanthoproteic tests	--	--	Yellow precipitate not observed
3.	Amino acids Ninhydrin test	--	--	Purple colour not observed
4.	For phytosterols 1. Salkowski reaction	+	+	Golden yellow colour at the bottom
	2. Libermann-Burchard reaction	+	+	Formation of green blue colour
5.	For glycoside 1. Borntrager's test	--	--	No pink colour observed
	2. Modified Borntrager's test	--	--	No red/pink colour observed
	3. Keller-killiani test for cardiac glycoside	--	--	No blue colour observed
6.	For flavonoid 1. NaOH test	+	+	Emulsion formed

	2. FeCl ₃ test	+	+	Dark green/bluish green colour observed
	3. Conc. Sulphuric acid test	+	--	Orange colour observed
	4. Ammonia test	--	+	Yellow/green colour observed
7.	For alkaloids 1. Dragendroff's test	+	+	Reddish brown colour observed
	2. Mayer's test	--	+	Creamy white precipitate
	3. Wagner's test	+	+	Reddish brown precipitate
	4. Hager's test	+	+	Creamish white precipitate
8.	For tannins 1. 5% Ferric Chloride test	+	+	Bluish black colour observed
	2. Braymer's test	+	+	Blue to green colour observed
9.	For gum and mucilage 1. Alcohol tests	+	--	Powder gets cloudy in alcohol
10.	For resin 1. Acetic anhydride test	+	--	Yellow/orange colour observed
	2. Turbidity test	+	--	Turbidity formed
11.	Test for carotenoids	--	+	Blue/green colour, changes to red

Table 5.7 Preliminary Phytochemical screening

5.3 Thin Layer Chromatography of Plant extract

Table 22 Thin Layer Chromatography of plant extracts

Extract	Mobile Phase	Detection wavelength	Rf value of standard	Rf value of taken extract
<i>Boswellia serrata</i> extract	Toluene : Ethyl acetate : Methanol (8:1.5:0.5)	254 nm	Boswellic acid	
		Derivatization with Anisaldehyde Sulphuric Acid reagent	0.71	0.73
			0.50	0.54
<i>Capsicum annum</i> extract	Toluene : Ethyl acetate : Methanol (8:1.5:0.5)	254nm Derivatization with Anisaldehyde Sulphuric Acid reagent	Capsaicin	
			0.67	0.69

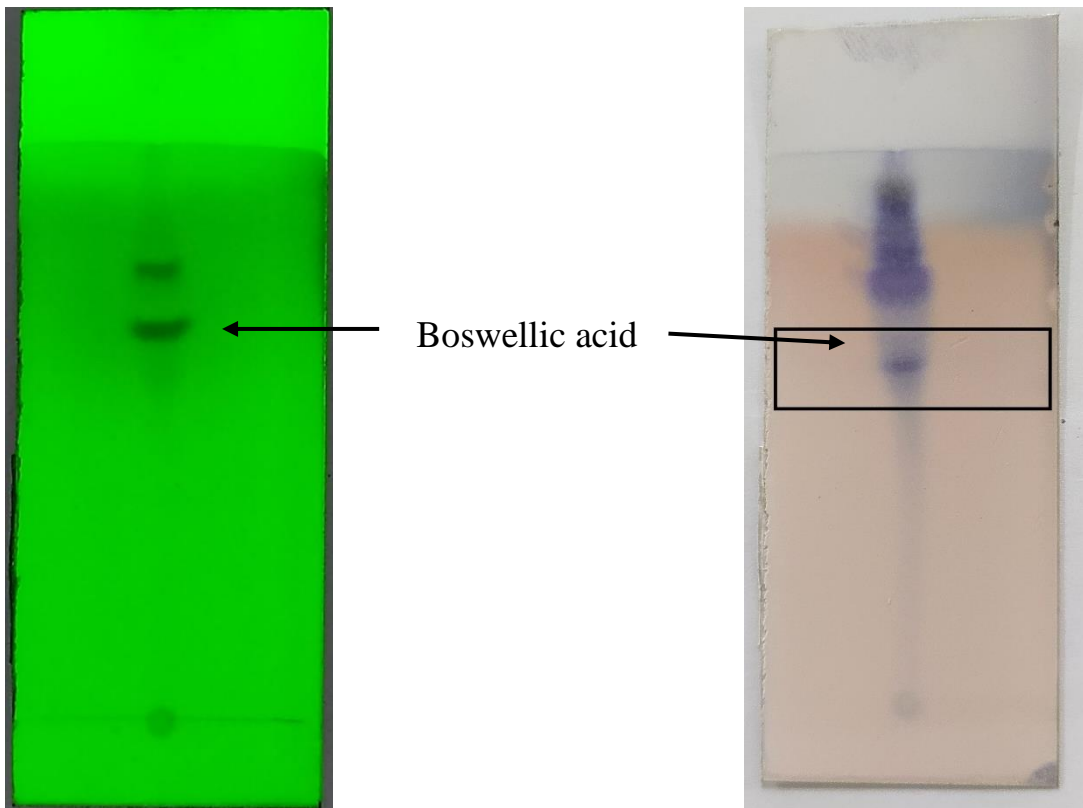


Figure 6 *Boswellia serrata* extract TLC

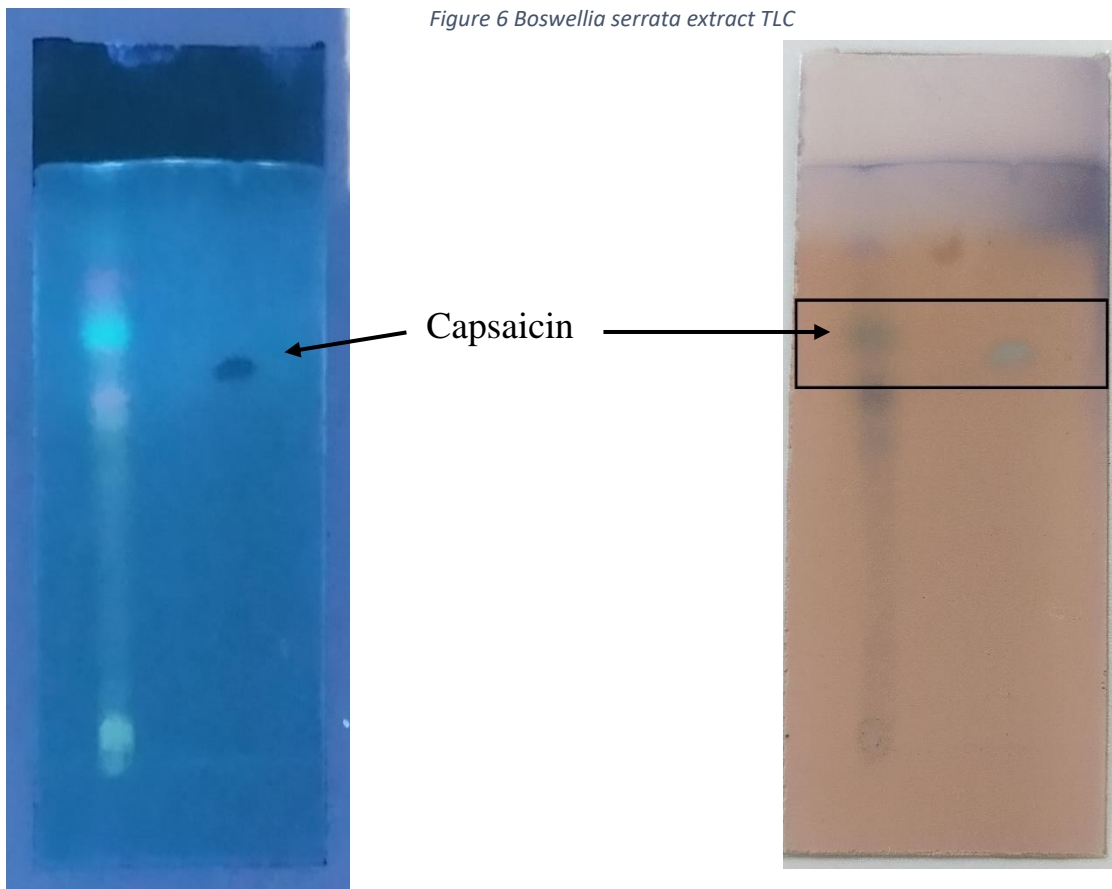


Figure 7 *Capsaicin annum* extract TLC

5.4 Estimation of secondary metabolites

5.4.1 Total Phenolic content

Phenolic compounds such as Gallic acid, which reacts with folin ciocalteu reagent to form blue coloured complex and determined by measured the absorbance of this blue coloured complex at 550 nm. The total phenolic content was expressed in terms of % of Gallic acid.

Table 23 Total Phenolic content

Concentration ($\mu\text{g/ml}$)	Absorbance
2	0.21808
4	0.42122
6	0.631
8	0.78861
10	1

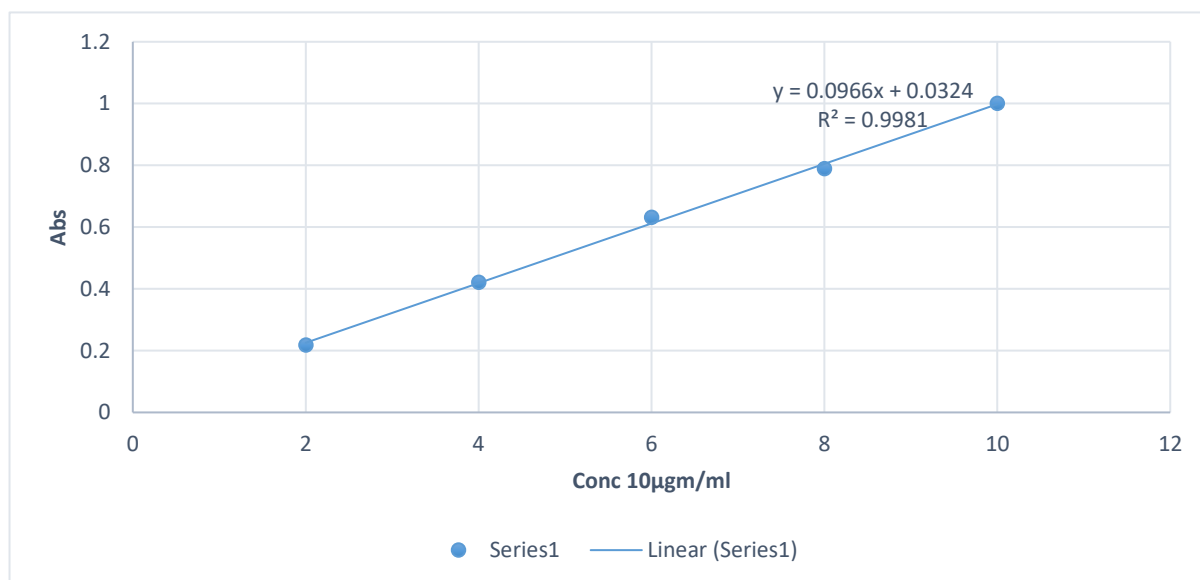


Figure 8 Calibration curve of Gallic acid

The total phenolic content in two extract *Boswellia serrata* and *Capsicum annum* were found as below

Plant extract	Total phenolic content (%)
<i>Boswellia serrata</i>	3.1 %
<i>Capsicum annum</i>	7.45 %

5.4.2 Total flavanoid content

Flavonoid compound such as quercetin react with Aluminium chloride to formed yellowish orange coloured complex and determined the absorbance of yellow coloured complex at 510 nm. Total flavonoid content was expressed in terms of % Quercetin.

Table 24 Total flavanoid content

Concentration (µg/ml)	Absorbance
1	0.02699
2	0.30122
3	0.55931
4	0.8703
5	1.02745

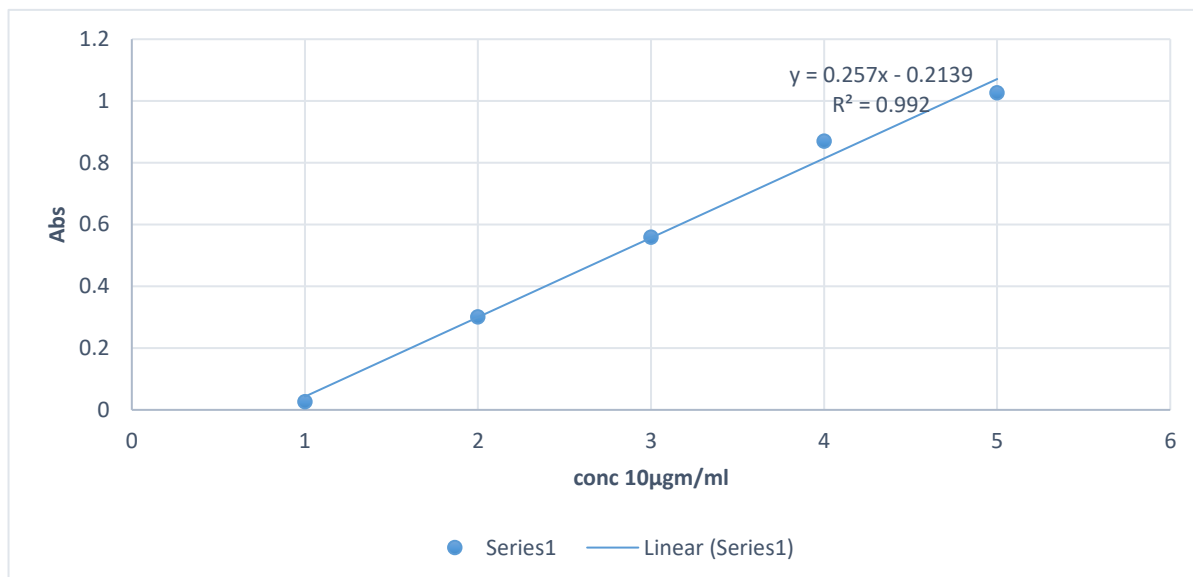


Figure 9 Calibration curve of Quercetin

The total flavanoid content in two extract *Boswellia serrata* and *Capsicum annum* were found as below

Plant extract	Total flavanoid content (%)
<i>Boswellia serrata</i>	0.18 %
<i>Capsicum annum</i>	2.76 %

5.4.3 total alkaloid content

Total alkaloid content was determined by titrimetric method. Alkaloid compound such as Atropine reacts with bromocresol green indicator and produce green coloured produced with 0.05 N H₂SO₄.

The total alkaloid content in two extract *Boswellia serrata* and *Capsicum annum* were found as below

Plant extract	Total alkaloid content (%)
<i>Boswellia serrata</i>	0.23 %
<i>Capsicum annum</i>	3.05 %

5.4.4 Total tannin content

Total tannin content was also determined by folin coicalteu method, where tannin was also form coloured complex with folin coicalteu reagent and was determined by measured absorption uof green colour complex at 725 nm. Total tannin content was expressed in terms of % Gallic acid.

Table 25 Total Tannin content

Concentration ($\mu\text{g/ml}$)	Absorbance
1	0.196
2	0.262
3	0.314
4	0.373
5	0.425
6	0.489

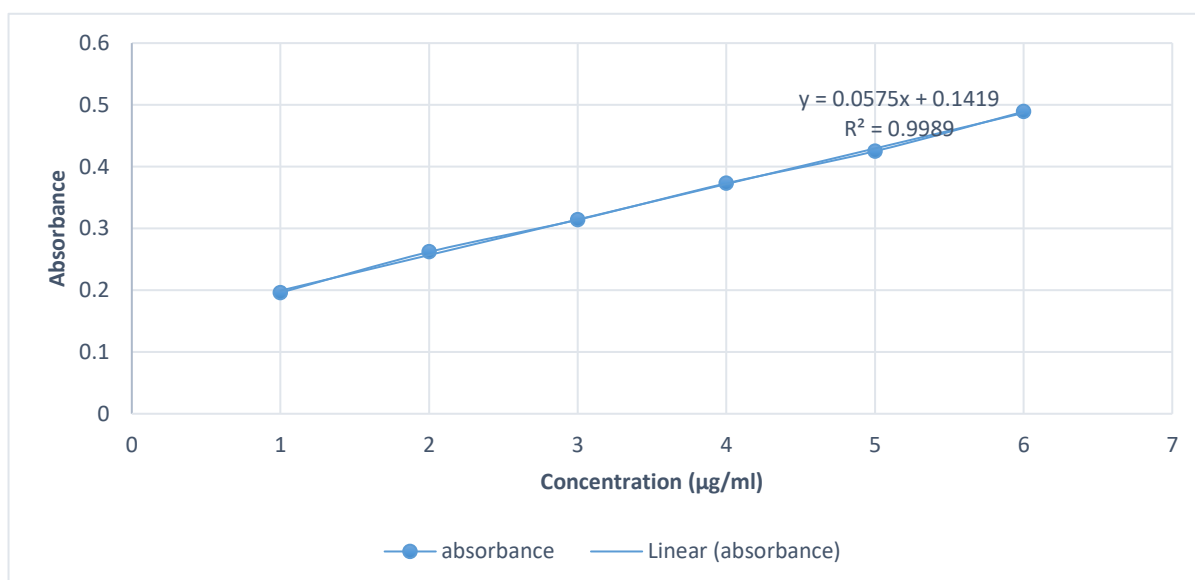


Figure 10 Calibration curve of Gallic acid for total Tannin content

The total tannin content in two extract *Boswellia serrata* and *Capsicum annum* were found as below

Plant extract	Total tannin content (%)
<i>Boswellia serrata</i>	0.106 %
<i>Capsicum annum</i>	1.04 %

5.5 Estimation of capsaicin in capsicum extract by uv spectroscopy

Table 26 Estimation of Capsaicin in *Capsicum annum* extract

Concentration ($\mu\text{g/ml}$)	Absorbance
1	0.101
2	0.19
4	0.393
8	0.725
16	1.31

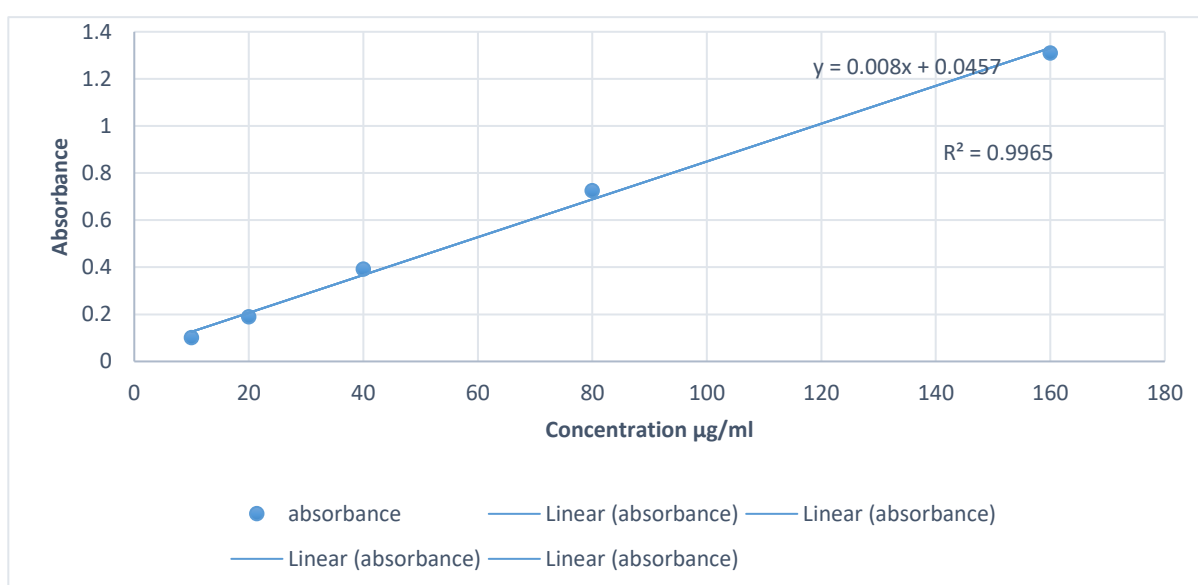


Figure 11 Calibration curve of Capsaicin

Concentration of Capsaicin in *Capsicum annum* extract was found to be 103.28 $\mu\text{g/ml}$.

5.6 Evaluation of Patch formulation

5.6.1 Optimization of Batch 1 for selection of polymer

Table 27 Results of Optimization of Batch for selection of Polymer

Formulation number	Polymer	Folding endurance
A1	HPMC : PVP	>80
A2	HPMC : PVP	
A3	HPMC : PVP	
A4	HPMC : PVA	<55
A5	HPMC : PVA	
A6	HPMC : PVA	
A7	HPMC : Ethyl Cellulose	Result was not obtained
A8	HPMC : Ethyl Cellulose	
A9	HPMC : Ethyl Cellulose	
A10	HPMC	<20
A11	HPMC	
A12	Pectin : Sodium Alginate	Result was not obtained
A13	Pectin : Sodium Alginate	

5.6.2 Optimization of plasticizer concentration

Plasticizers are the agents which affect the plasticity of the patch formulation. Plasticizers increase the flexibility of the polymers. They also affect the patch properties like folding capacity, penetration properties and drying time. A higher concentration of plasticizer will form a sticky, semisolid patch which is unacceptable and also affect the hydration of patch.

Plasticizer PEG 400 was used in different quantities to optimize the final concentration.

Table 28 Results of optimization of Plasticizer concentration

Formulation number	Quantity taken	Results
B1	0	Patch was very dry like plastic sheet

B2	2	Folding endurance 72
B3	3	Folding endurance 87
B4	5	Patch was not obtained, after 34 h, it was gel like formulation

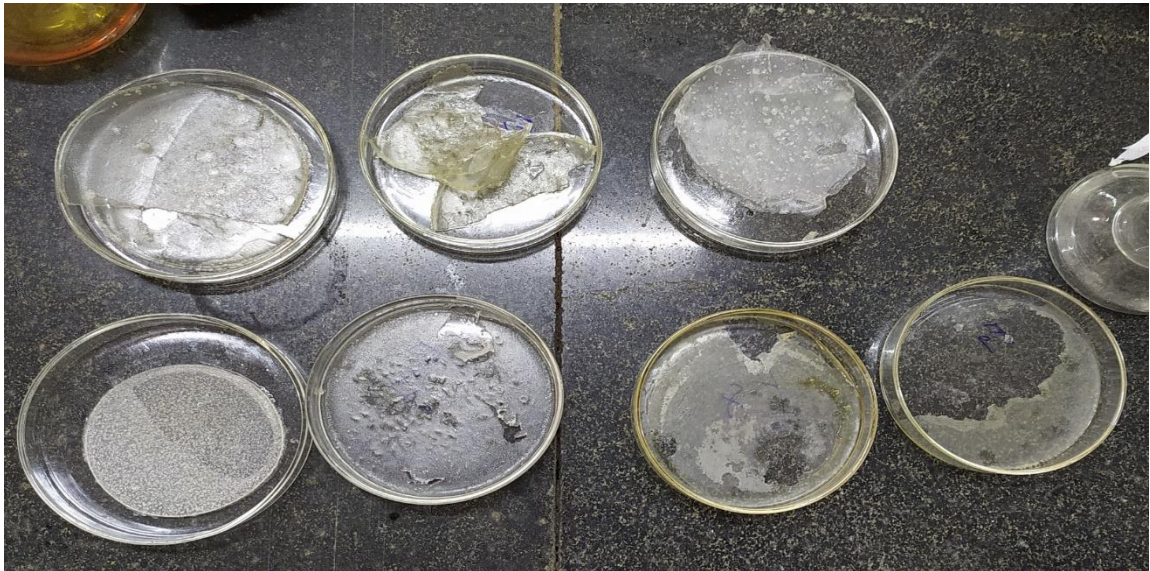


Figure 12 Batch 1



Figure 13 Batch 2 and 3

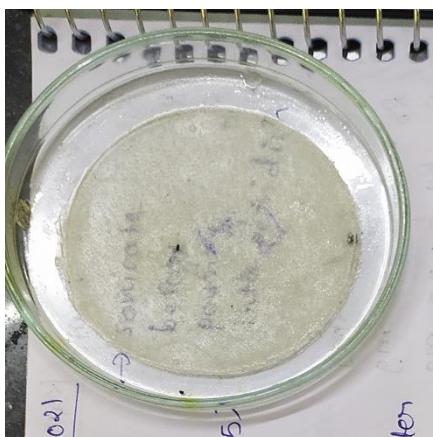


Figure 14 F1

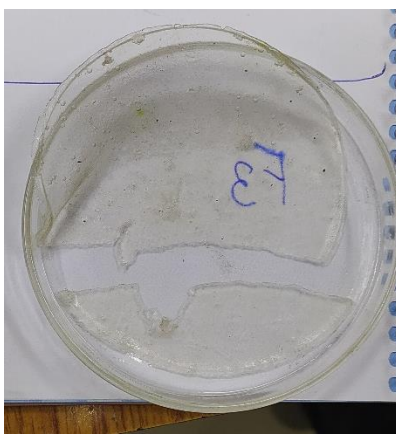


Figure 15 F2

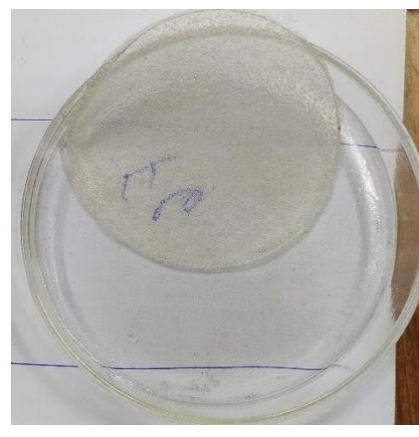


Figure 16 F3

5.6.3 Optimization of solvent ratio and Penetration enhancer concentration

Table 29 Results of Optimization of solvent ratio

Formulation number	Results
F1	Folding endurance 78
F2	Folding endurance 73
F3	Folding endurance 150
F4	Patch was not obtained
F5	Poor quality, upon removal breaks
F6	Gel like formulation
F7	Gel like formulation
F8	Folding endurance 95
F9	Gel like formulation
F10	Folding endurance 120
F11	Broke upon further handling
F12	Folding endurance 75
F13	Gel like formulation
F14	Broke upon handling
F15	Gel like formulation

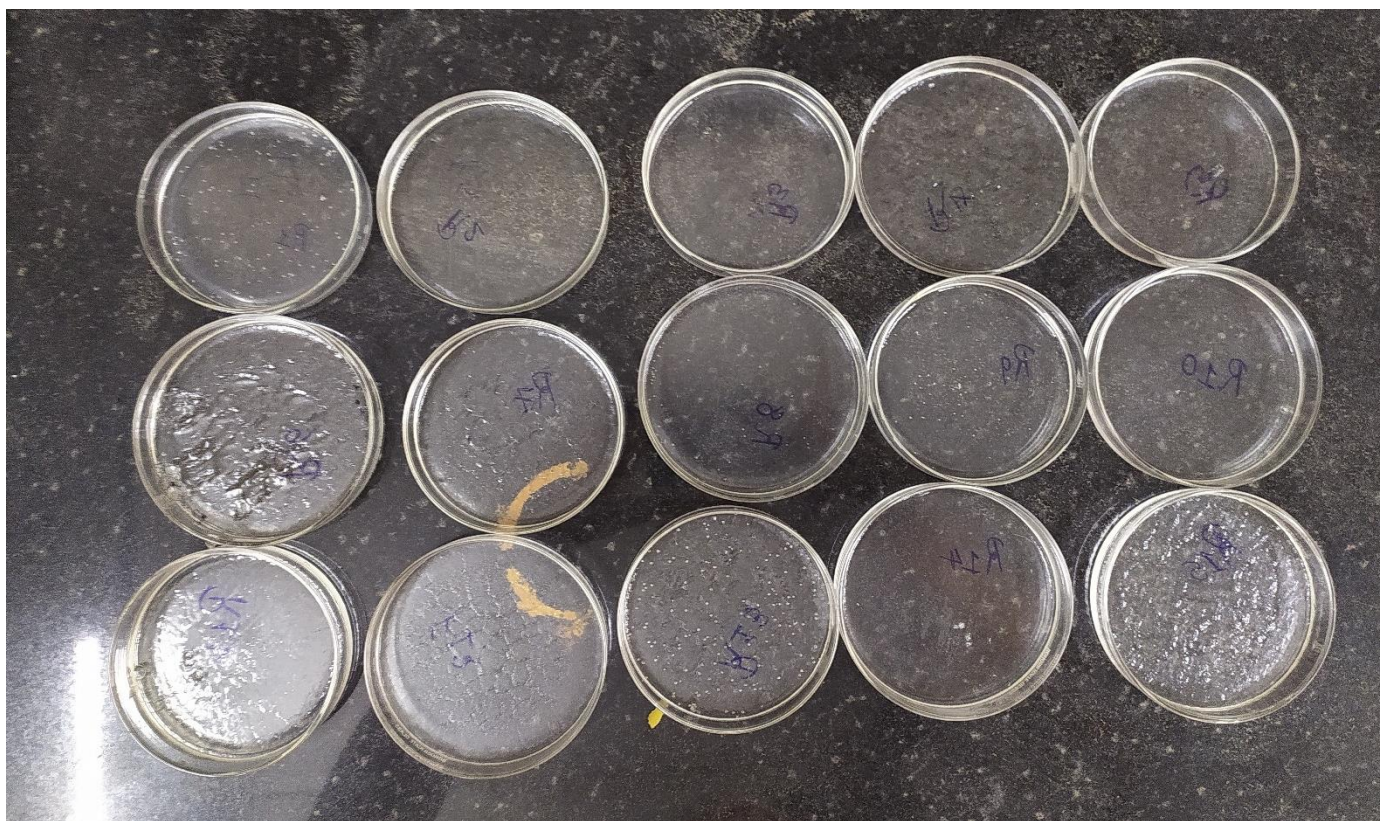


Figure 17 Formulation results from F1 to F15

5.6.4 Drug loaded patch formulation

Table 30 Drug loaded patch formulation

Sr. no	Ingredients	Optimized Quantity taken			
		F1	F2	F3	F4
1.	Boswellia serrata	250 mg	500 mg	750 mg	1 gm
2.	Capsicum annum	25 mg	50 mg	75 mg	100 mg
3.	Menthol	10 mg	50 mg	70 mg	100 mg
4.	HPMC	350 mg			
5.	PVP	350 mg			
6.	Methanol	13 ml			
7.	Water	7 ml			
8.	PEG 400	3 ml			

9.	Propylene glycol	0.5 ml
----	------------------	--------

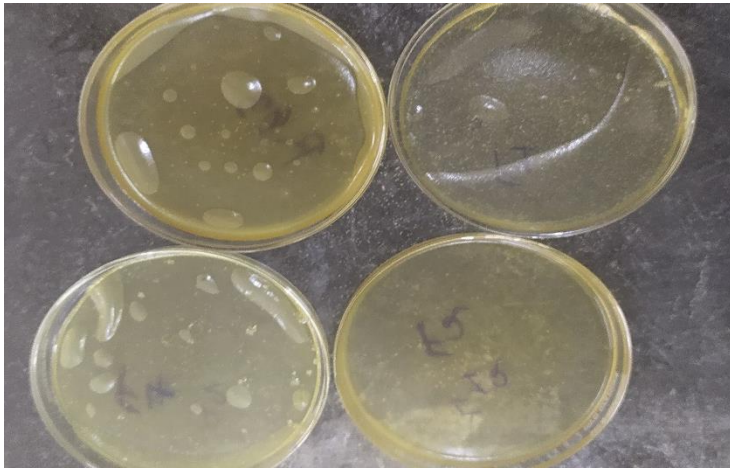


Figure 18 drug loaded patches F1 to F4



Figure 19 F4



Figure 20 F3



Figure 21 F1



Figure 22 Formulation 3



Figure 23 F2

5.6.5 Tensile strength

Three optimized polyherbal patch formulation were selected for testing tensile strength.

Table 31 Tensile strength

Formulation number	Tensile strength Mean load (gm)
F1	90
F2	151
F3	52

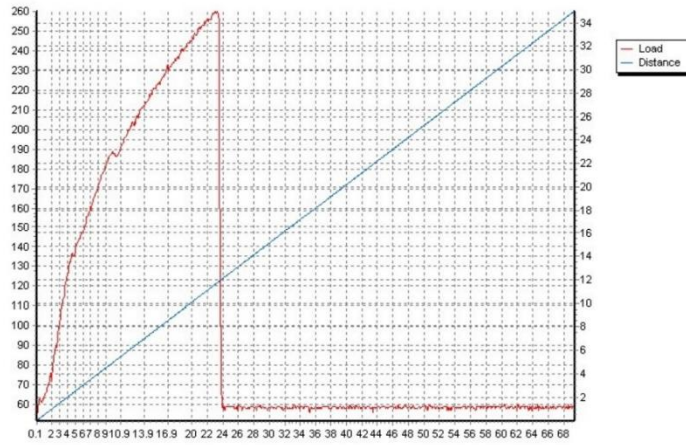


Figure 24 Tensile strength graph of Formulation

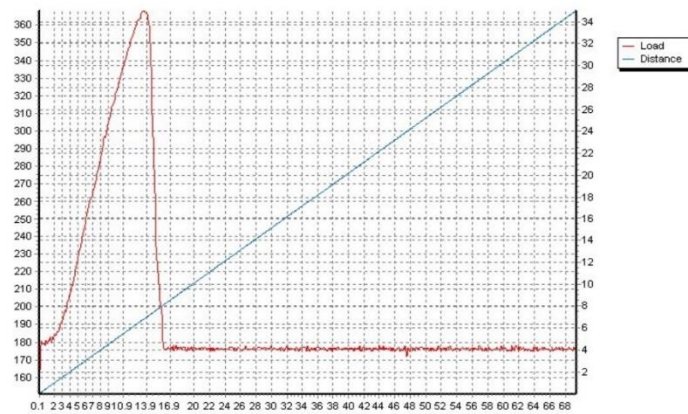


Figure 25 Tensile strength graph of formulation 2

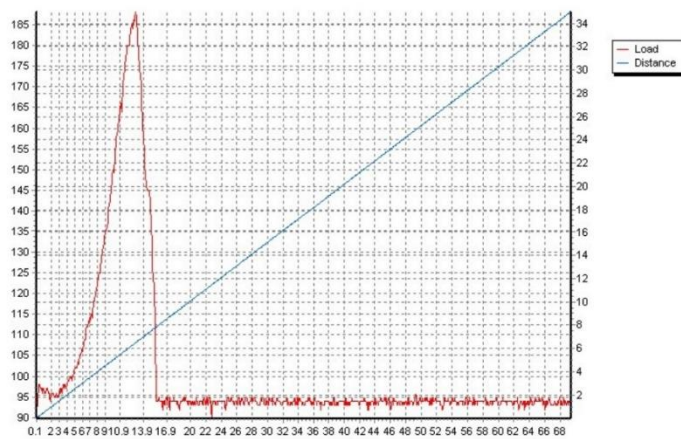


Figure 26 Tensile strength graph of formulation 1

5.6.6 Weight variation

Formulation number	Weight of patch (gm)
F1	2.38
F2	2.42
F3	2.76
F4	3.10

Average weight = 2.66 ± 0.5 gm

5.6.7 Folding endurance

Formulation number	Folding endurance
F1	157
F2	188
F3	175
F4	115

5.6.8 pH

Formulation number	Ph
F1	5.8
F2	5.7
F3	5.8
F4	6.0

5.6.9 Thickness

Formulation number	Thickkness (mm)
F1	0.32 ± 0.0577
F2	0.37 ± 0.0287
F3	0.43 ± 0.0155
F4	0.48 ± 0.01732

6.6.10 Percentage moisture content and percentage moisture uptake

Formulation number	% Moisture content	% Moisture uptake
F1	12.26 %	1.0 %
F2	5.21 %	2.34 %
F3	8.23 %	0.94 %
F4	7.26 %	0.30 %

5.7 TLC Fingerprinting for estimation of Phytoconstituents present in prepared polyherbal formulation

Visualization after derivatization with Anisaldehyde Sulphuric acid reagent

Capsaicin



Figure 27 TLC of formulation after derivatization (1)

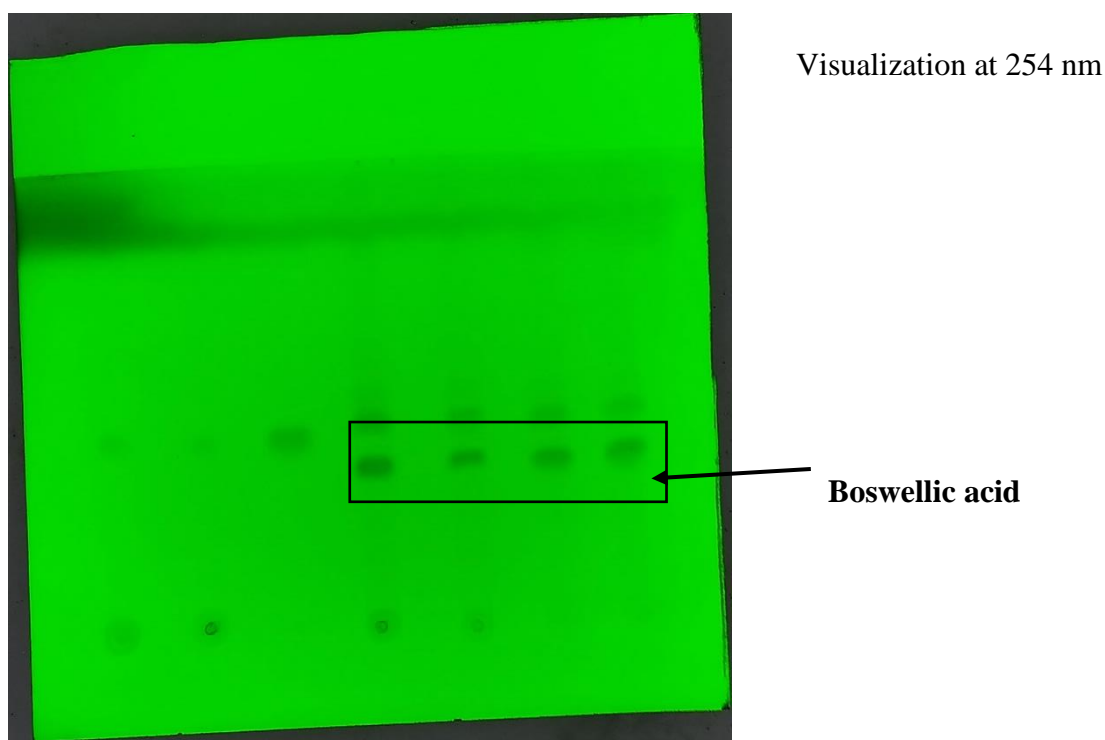
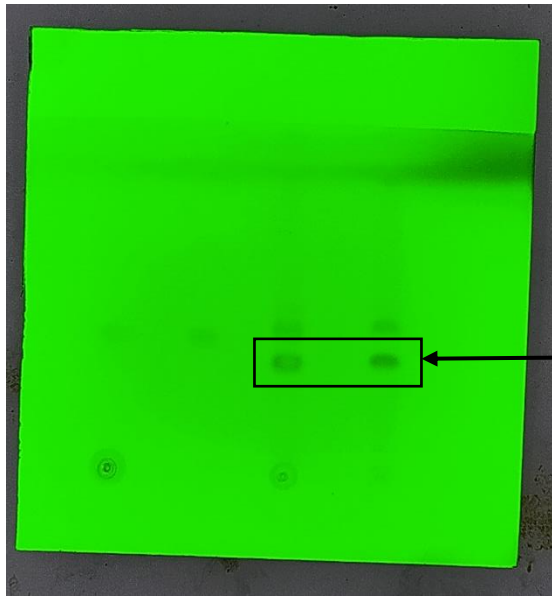


Figure 28 TLC of formulation at 254 nm (1)

Number of spot (Left to right)

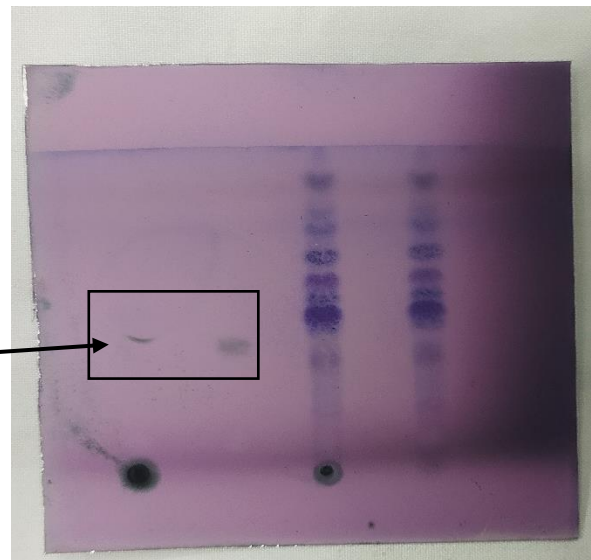
Spot	Rf Value
1. <i>Capsicum annuum extract</i>	0.4
2. <i>Capsicum annuum extract</i>	0.38
3. Capsaicin	0.4
4. Formulation	Capsaicin 0.4 Boswellic acid 0.54
5. Formulation	Capsaicin 0.4 Boswellic acid 0.55
6. <i>Boswellia serrata extract</i>	0.54
7. <i>Boswellia serrata extract</i>	0.55



Visualization in UV 254 nm

Boswellic acid (Rf 0.54)

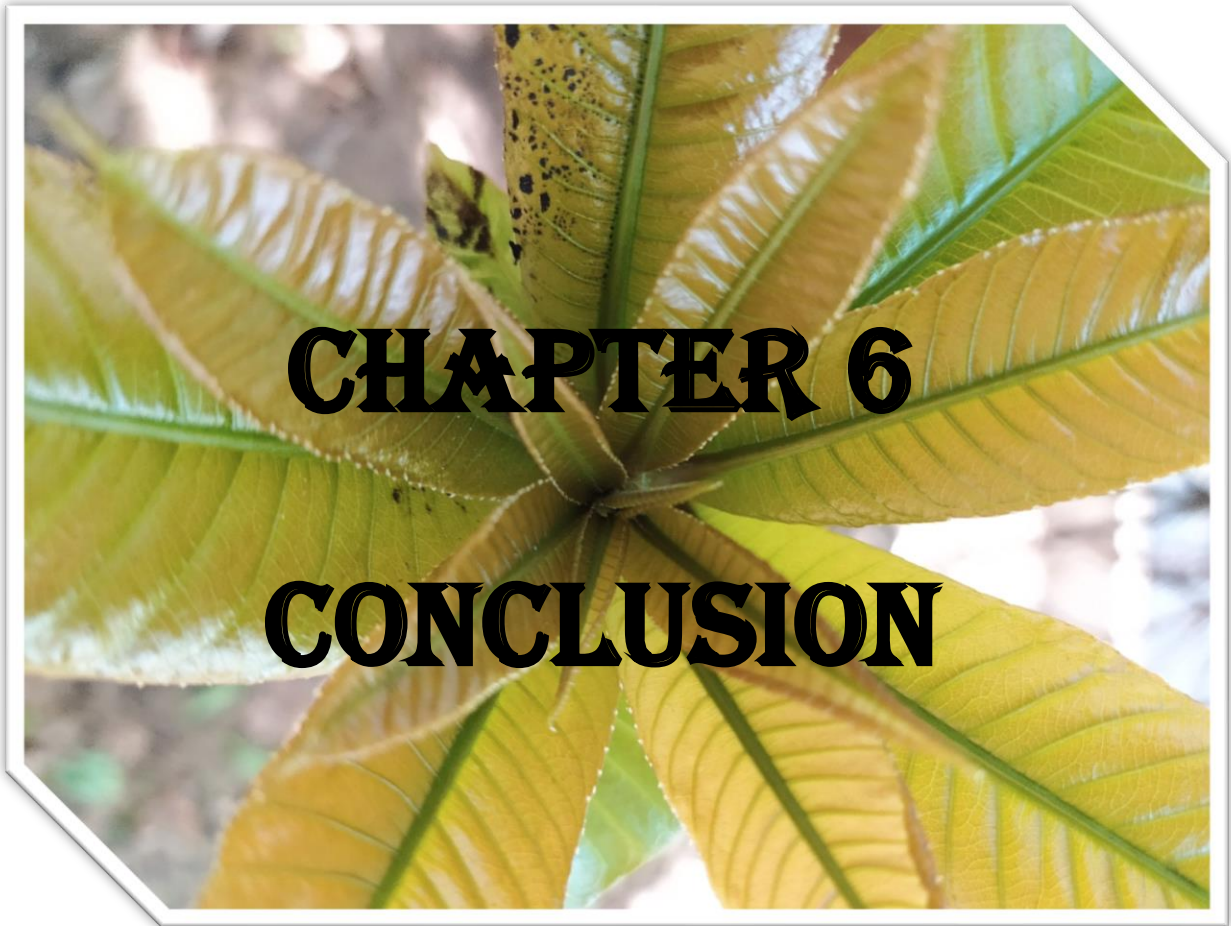
Visualization after derivatization with Anisaldehyde sulphuric acid reagent



Capsaicin
Rf (0.4)

Number of spot and Rf Value (from left to right)

Spot	Rf value
1. <i>Capsicum annuum extract</i>	0.4
2. Capsaicin	0.4
3. Formulation	Capsaicin 0.4 Boswellic acid 0.54
4. <i>Boswellia serrata extract</i>	0.54



CHAPTER 6

CONCLUSION

6 CONCLUSION

In the present study, two Medicinal plant extracts and one topical cooling agent were selected based on literature survey and effectiveness. Natural remedies are more acceptable in the belief that they are safe with less/no side effects as compared to synthetic ones.

The present study is focused on formulation development, standardization of extracts and evaluation of developed polyherbal patch formulation.

Developing an analytical method by selecting suitable marker quantifying them in the developed patch formulation.

Various topical preparation for pain relief purpose are available in the market, but developing patch formulation will enhance patient compliance and gives faster action by incorporating herbal extracts into formulation is goal.

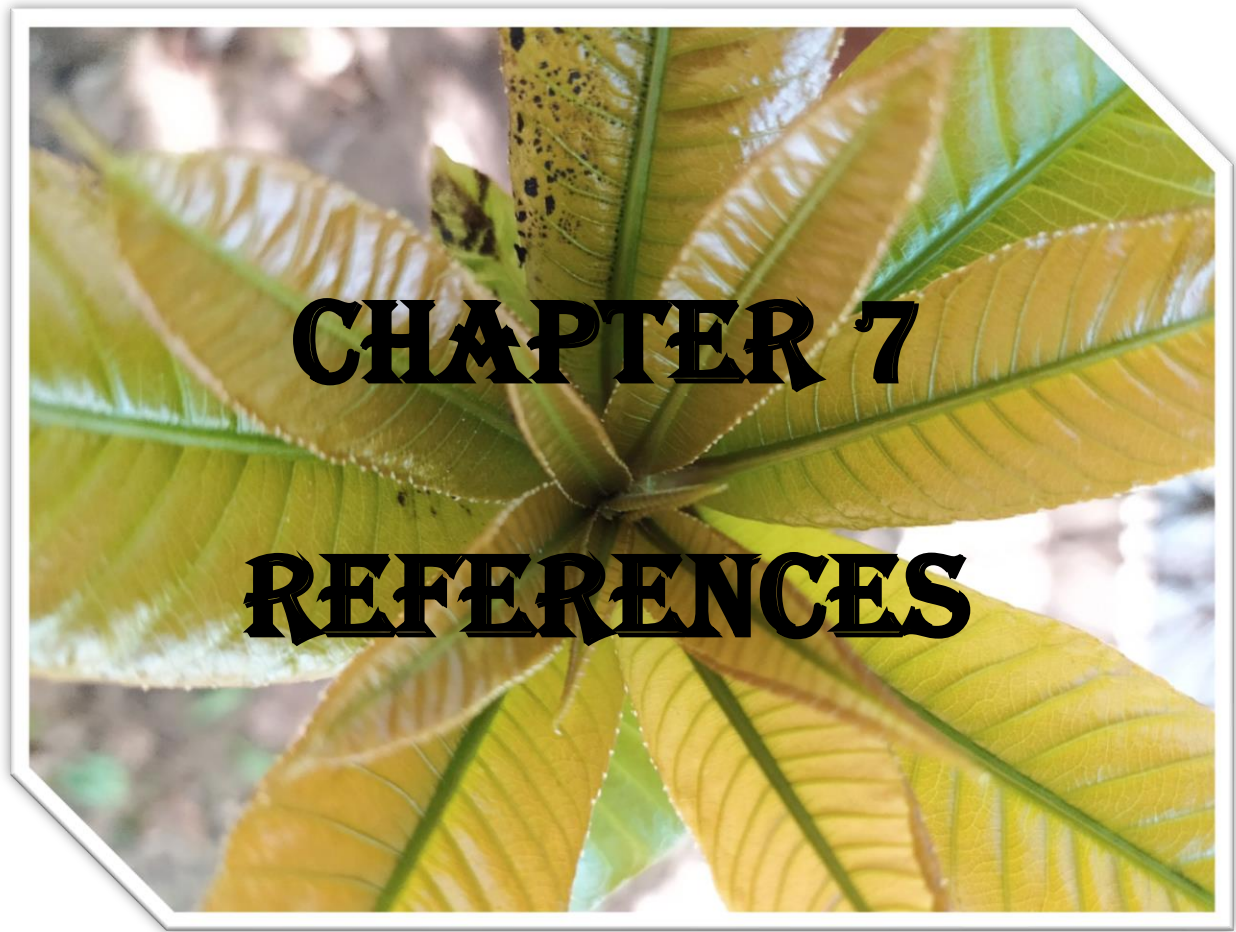
The finalised formulation contains 750 mg of *Boswellia serrata* extract, 0.05 % Capsaicin in *Capsicum annuum* extract and 7 % Methanol. While using PEG 400 used as Plasticizer and Propylene Glycol as Penetration enhancer was appropriate for the patch formulation.

This was suitable for skin pH. The developed polyherbal patch formulation showed less/no irritation on skin, when applied.

It is important for the formulation to be stable for the longer period of time and maintain its competence. This was determined by accelerated stress studies as per ICH guidelines. The selected formulation was stable at room temperatures i.e. at 25⁰ c /60 ± 5 RH and 35⁰ c /60 ± 5 RH throughout the stress studies.

The analytical method development and its validation of selected marker compound was also performed as per ICH guidelines. A novel HPTLC method for the simultaneous estimation of Capsaicin in polyherbal patch was developed. The method proposed was found to be easier, less time consuming and have all parameters within limits according to the ICH guidelines. Results from TLC shows that it was successfully incorporated all herbal drugs into the developed formulation.

From the entire study, it can be concluded that the formulated polyherbal patch has better effectiveness for the anti-inflammatory activity.



CHAPTER 7

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7 References

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GSTIN- 07CIRPD7 50H1ZV

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Certificate Of Analysis

Product Name: Capsicum Annum Extract Powder
Batch Number: VH/ CAE /CAE854
Quantity: 500Gm
Manufacture Date: Jan 20
Exp Date: Dec 23

Analysis	Specification	Results
Appearance	Reddish Brown Fine Powder	Complied
Odor	Characteristic	Complied
Taste	Characteristic	Complied
Assay/Extract Ratio	10:1	Complied

Loss on Drying	≤5.0%	2.89 %
Sieve Analysis	Pass 80 mesh	Complied
Bulk Density	45-55g/100mL	49.2g/100mL
Heavy Metal	NMT 20ppm	Complied
As	NMT 2ppm	Complied
Total Plate Count	NMT 5000cfu/g	Complied
Yeast & Mold	NMT 1000cfu/g	Complied
E.Coli	Negative	Complied
Salmonella	Negative	Complied

Conclusion Conform with specification

Storage Store in cool & dry place. Keep away from strong light and heat. Shelf life 3 years when properly stored

The information herein is correct based on our knowledge; please evaluate the raw material prior to use in a finished product.
Computer generated certificate, valid without signature.

