Development and Evaluation of Some Polyherbal Formulation for Hair and Skin-care

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CERTIFICATE

This is to certify that the thesis entitled, “Development and evaluation of some polyherbal formulation for hair and skin care”, submitted by Mrs. Bandana Biswas, is absolutely based upon her own work under my supervision and neither her thesis nor any part of the thesis has been submitted for any degree/diploma or any other academic award anywhere before.

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Declaration

I hereby declare that my work embodied in this Master degree thesis entitled **"**Development and evaluation of some polyherbal formulation for hair and skin care**"** have been carried out by me in the Department of Pharmaceutical Technology, Jadavpur University, West Bengal, Kolkata, India under the direct supervision of Prof. Pulok K. Mukherjee, Director, School of Natural Product Studies, Department of Pharmaceutical Technology, Jadavpur University, West Bengal, Kolkata, India. I also confirm that this work is original and has not been submitted partially or in full for any other degree or diploma to this or other University or Institute.

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Preface

The traditional knowledge with its holistic and systematic approach supported through experimental evidence may serve as an innovative and powerful discovery engine for newer, safer and affordable medicines. The work was performed and represented in this thesis deals with the Topical Herbal Formulations Development from Medicinal Plants. The work was based on the approach of exploration of medicinal herbs with multiple potentials ranging from anti-microbial to hair conditioning potential and others from traditional and modern scientific knowledge. The RP-HPLC and HPTLC standardization methods with respective bio-markers were adaptable owing to their good precision and excellent repeatability. Most analytical procedures were optimized in our laboratory. These methods can be used as potential cost-effective, simple and highly selective tools that can ensure both quality and batch-to-batch reproducibility of the products. Multiple topical surfactant based formulations were prepared for the hair, body, face and hands. The contents of heavy metals in the formulations were within the prescribed limit. Stability study supports its safer uses for long time without changes of any product qualities. The developed products were compared to marketed formulations. These formulations and the optimised procedures involved in their preparation may be utilized in future to give rise to better personal care products for the huge consumer base.

List of Abbreviations

Kg	Kilogram	**IC_{50}**	Inhibitory Concentration 50
G	Gram	**m/z**	Mass to Charge Ratio
Mg	Milligram		
µg	Microgram	**AAS**	Atomic Absorption Spectroscopy
L	Liter	**RT/ R_t**	Retention time
Ml	Milliliter	**Sl. No.**	Serial Number
µl	Microliter	**CAS#**	Chemical Abstracts Service Number
Qual#	Library Match Percentage	**REG#**	Registration Number
HPLC	High performancle liquid chromatography	**TIC**	Total Ion Current
HPTLC	High performancle Thin layer chromatography		
ESI	Electron Spray Ionisation	**C**	Celsius
TQD	Triple Quadrupole	**Fig**	Figure
ELSD	Evaporative Light Scattering Detector		
UV	Ultraviolet	**MIC**	Minimum Inhibitory Concentration
Vis	Visible	**MeOH**	Methanol
PDA	Photo Diode Array	**EtOH**	Ethanol
ACHE	*Acacia concinna* Hydroalcoholic Extract	**LIHE**	Lawsonia innermis Hydroalcoholic Extract
R_f	Retardation factor	**PPM**	Parts Per Million

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CHAPTER-1

Introduction

1.1. Morpho-anatomy of skin and hair

1.2. Topical drug delivery system

1.3. Polyherbal formulations and its significance

1.4. Herbs useful in skin and hair care

1.1. Morpho-anatomy of skin and hair

1.1.1. Morphological and anatomical features of skin

The skin is the largest organ of the body, accounting for about 15% of the total adult body weight. It performs many vital functions, including protection against external physical,chemical, and biologic assailants, as well as prevention of excess water loss from the body and a role in thermo regulation. The skin is continuous, with the mucous membranes lining the body's surface. The skin is composed of three layers: the epidermis, the dermis, and subcutaneous tissue (Kanitakis, 2002). The outermost level, the epidermis, consists of a specific constellation ofcells known as keratinocytes, which function to synthesize keratin, a long, thread like protein with a protective role. The middle layer, the dermis, is fundamentally made up of the fibrillar structural protein known as collagen. The dermis lies on the subcutaneous tissue, or panniculus, which contains small lobes of fat cells known as lipocytes. The thickness of these layers varies considerably, depending on the geographic location on the anatomy of the body. (James et al., 2006).The epidermis is a stratified, squamous epithelium layer that is composed primarily of two types of cells: keratinocytes and dendritic cells. The keratinocytes differ from the "clear" dendritic cells by possessing intercellular bridges and ample amounts of stainable cytoplasm (Murphy, 1997). The epidermis harbours a number of other cell populations, such as melanocytes, Langerhans cells, and Merkel cells, but the keratinocyte cell type comprises the majority of the cells by far. The epidermis commonly is divided into four layers according to keratinocyte morphology and position as they differentiate into horny cells, including the basal cell layer (stratum germinativum), the squamous cell layer (stratum spinosum), the granular cell layer (stratum granulosum), and the cornified or horny cell layer (stratum corneum) (James et al., 2006; Murphy, 1997). The lower three layers that constitute the living, nucleated cells of the epidermis are sometimes referred to as the stratum malpighii and rete malpighii (Murphy). At least 80% of cells in the epidermis are the keratinocytes (Chu, 2008).The basal layer, also known as the stratum germinativum, contains column-shaped keratinocytes that attach to the basement membrane zone with their long axis perpendicular to the dermis. These basal cells form a single layer and adhere to one another as well as to more superficial squamous cells through desmosomal junctions (Murphy, 1997). Overlying the basal cell layer is a layer of the epidermis that is 5–10 cells thick and known as the squamous cell layer or stratum spinosum (Murphy, 1997). The squamous layer is composed of a variety of cells that differ in shape, structure, and subcellular properties depending on their location. The most superficial layer of the epidermis containing living cells, the granular layer or stratum granulosum, is composed of flattened cells holding abundant keratohyaline granules in

their cytoplasm. These cells are responsible forfurther synthesis and modification of proteins involved inkeratinization (Chu, 2008).The granular layer varies in thickness in proportion to that of the overlying horny cell layer(Murphy, 1997).Horny cells (corneocytes) of the cornified layer providemechanical protection to the underlying epidermis and a barrier to prevent water loss and invasion by foreign substances(Jackson et al., 1993).

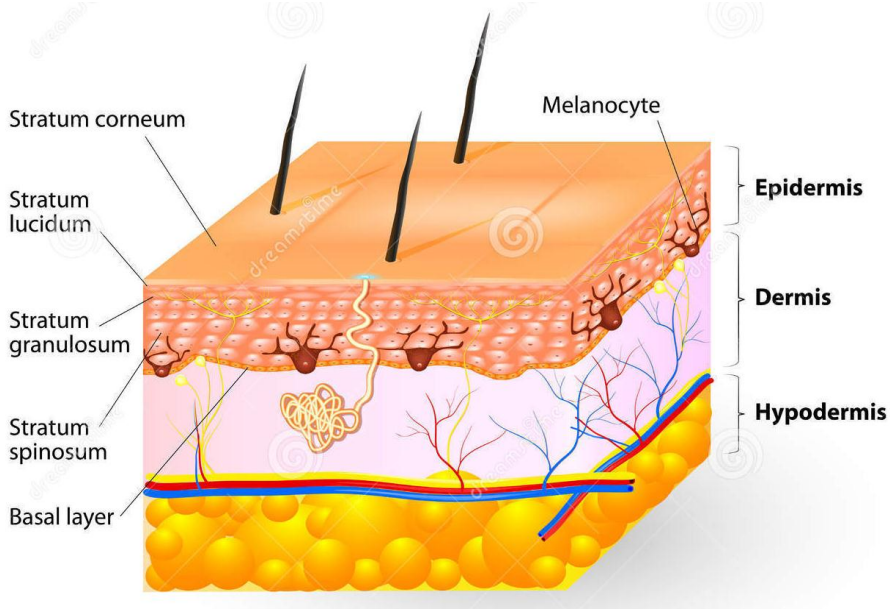


Fig. 1.A. Ultrastructure of skin

1.1.2. Morphological and anatomical features of hair

Hair is a unique character found on all mammals but noton other animals. In humans it is a special and cherishedfeature, especially in females, but its main functions arein protection of the skin from mechanical insults and to facilitate homeothermy (Harrison and Davis, 1999; Maderson, 2003), eyebrows and eyelashes, forexample, stop things entering the eyes, while scalp hairprevents sunlight, cold, and physical damage to the headand neck. (Randall and Botchkareva, 2009). It also has a sensory function, increasing theperception of the skin surface for tactile stimuli, and subserves important roles in sexual and social communication, considering the psychological impact on quality oflife seen in hair disorders, such as hirsutism, hair loss,etc. (Randall and Botchkareva, 2009; Randall 2001). Hair is a derivative of the epidermis. Externally, hair isthin, flexible tubes of dead, fully keratinized epithelialcells, whereas inside the skin, it is a

part of individual living hair follicles, cylindrical epithelial downgrowths into the dermis, and subcutaneous fat, which enlarge at thebase into the hair bulb surrounding the mesenchymal derived dermal papilla (Randall and Botchkareva, 2009).Hair has two separate structures: the follicle in the skinand the hair shaft, which is visible on the body surface.The hair shaft consists of a cortex and cuticle cells, andin some cases, a medulla in the central region. Themedulla is the central part of the hair, whereas the cortex, which represents the majority of the hair fiber composition and plays an important role in the physical andmechanical properties of hair, is the peripheral part andis made up of approximately 50–60% of macrofibrils,which consist of rods of microfibrils embedded into amatrix (Wolfram, 2003). The hair shaft cuticle covers the hair from theroot to the tip of the epidermis and is formed by flat overlapping cells (Gurdenet al., 2004).The follicle is the essential growth structure of hair.From the outermost aspect of the follicle, the histologicalstructures are:

1.The outer root sheath (ORS), which has been identifiedas a reservoir of multipotent stem cells, i.e., keratinocyte and melanocyte stem cells, and contains keratinocytes. The ORS forms a distinct bulge area between the insertion of the arrectorpili muscle and duct of the sebaceous gland (Randall and Botchkareva, 2009; Oshima et al., 2001). Adjoining the ORS on the dermalside is a basket-like arrangement of two orthogonallyarrayed layers of collagen fibers, the glassy layerknown as the dermal sheet.(Rogers, 2004)

2.The inner root sheath (IRS) consists of three layers: Henle's layer, Huxley's layer, and cuticle layer. The IRS cuticle layer adjoins the cuticle of the hair shaft, anchoringthe hair shaft to the follicle. IRS cells produce keratins and trichohyalin that serve as an intracellular cementgiving strength to the IRS to support and mold the growing hair shaft, as well as guide its upward movement.The IRS separates the hair shaft from the ORS.

3.The hair bulb is the portion of the follicle whichactively produces the hair. It encloses the follicular dermal papilla, dermal papilla cells, mucopolysaccharide-richstroma, nerve fibers, and a single capillary loop. The follicular papilla is believed to be one of the most importantdrivers to instruct the hair follicle to grow and form aparticularly sized and pigmented hair shaft; moreover, itis an essential source of growth factors (keratinocytegrowth factor, bone morphogenetic protein, hepatocytegrowth factor, insulin-like growth factor, stem cell factor), critical for hair growth and melanogenesis (Randall and Botchkareva, 2009; Peus et al., 1996).

The hair bulb can be further divided into two regions: a lowerregion of undifferentiated cells and an upper region inwhich the cells became differentiated. A line across thewidest part of the papilla separates the two regions at thecritical level (Auber's line). Below the Auber's line liesthe matrix, or germination center of the follicle, whereevery cell is mitotically active, and the dermal papilla.From the matrix, cells move to the upper part of thebulb, where they increase in volume and become elongated vertically.3The upper bulb can be divided into four parts: (i)above the critical level, in the wide portion of the bulb, isthe pre-elongation region, where the cells align themselvesvertically and become slightly larger; (ii) above thisregion, where the diameter of the bulb is narrower andthe cells become conspicuously elongated, is the cellularelongation region; (iii) immediately above, in the corticalpre-keratinization region, distinct fine fibers or fibrilsstainable with basic dyes can be seen; and (iv) further upis the keratogenous zone where the cells become hyalinized and the keratin of the hair is stabilized. Dependingupon the length of the follicle, the keratogenous zone terminates at approximately one-third of the way betweenthe tip of the papilla and the surface of the skin.Above the bulb, the upper hair follicle is composed oftwo anatomical parts: the infundibulum and the isthmus.The infundibulum is a funnel-shaped structure filled withsebum, a product of the sebaceous gland; it extends fromthe surface of the skin to the sebaceous duct, serves as areservoir, and provides an interface for interactions withhair follicle-associated cell populations. In detail, in theupper part, called the acroinfundibulum, the epithelium iscontinuous with the keratinized epidermis and is coveredby an intact, rather impermeable stratum corneum; thisbarrier is interrupted in the lower follicular infrainfundibulum, as the differentiation pattern switches from epidermal differentiation to a tricholemmal differentiation pattern. Only few differentiated corneocytes remain, andthe invagination of the epidermis in the infundibulummust be considered as highly permeable (Blume-Peytaviand Vogt, 2010).The isthmus completes the upper part of the hair follicle, and it extends from the duct of the sebaceous glandto the exertion of arrectorpili muscle (Randall and Botchkareva, 2009; Wosickaand Cal, 2010)

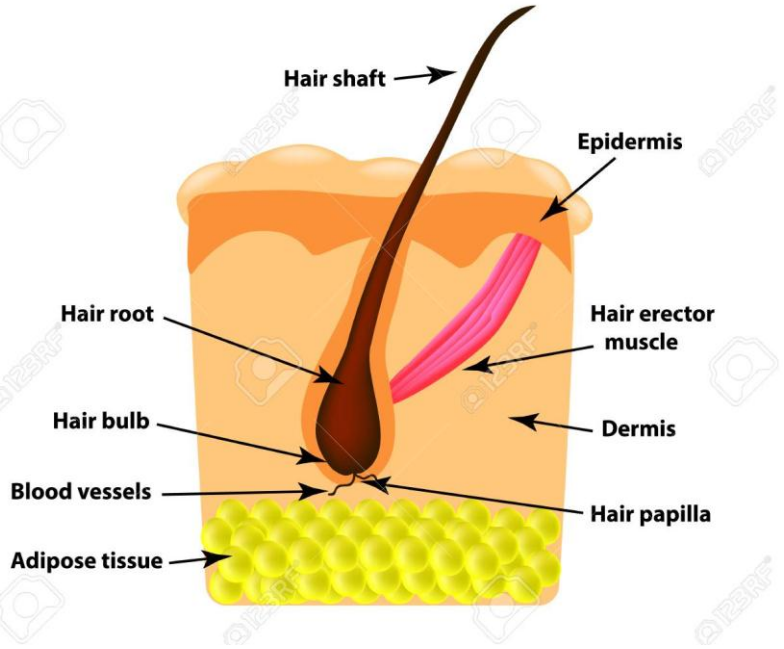


Fig. 1.B. Ultrastructure of hair

1.2. Topical drug delivery system (TDDS)

Topical drug delivery system (TDDS) is an attractive route for local and systemic treatment. The delivery of drugs onto the skin is recognized as an effective means of therapy for local dermatologic diseases. It can penetrate deeper into skin and hence give better absorption (Mikari et al., 2010). Topical application has many advantages over the conventional dosage forms. In general, they are deemed more effective less toxic than conventional formulations due to the bilayer composition and structure. In the formulation of topical dosage forms, attempts are being made to utilize drug carriers that ensure adequate localization or penetration of the drug within or through the skin in order to enhance the local and minimize the systemic effects, or to ensure adequate percutaneous absorption(Dodov et al., 2003).Topical formulation avoids the GI-irritation, prevent the metabolism of drug in the liver and increase the bioavailability of the drug. Topical preparations give its action directly at the site of action (Jani et al., 2010).

Advantages of TDDS (Chittodiya et al., 2013)

1. Thesystems are easy to apply and remove. It avoidsrisk and inconveniences associated withintravenous therapy.

2. They eliminate the variables, which influencesgastrointestinal absorption such as food intake,stomach emptying, intestinal motility and transittime.

3. Produces sustained and controlled level of drugin plasma thus reduces the chance of over or underdosing.

4. Reduces frequency of drug dosing.

5. Topical systems are easily retractable therebytermination of drug input, if toxic effects areobserved.

TDDS involve the introduction of a drug to the surface of the body, ina formulation which can be absorbed. When the topical formulationis applied onto the skin, it should interact with the skin environment,which could further influence the rate of release of the compound inorder to achieve adequate skin absorption (Sruthi et al., 2016). The TDDS aregenerally applied for the purpose as antiseptics, antifungal agents,skin emollients and protectants (Sah et al., 2017). Skin is the main route of topicaldrug delivery system (Kshirsagar, 2000). Skin is most accessible organ and ispotential to facilitate the delivery of several drugs with betterefficacy than rest of any other route of administration (Sah et al., 2017).In TDDS, drug reaches to the site of action via diffusion and theirabsorption takes place on the skin (Zi et al., 2008). This route is one of the bestoptions for the cutaneous purpose (Rathbone ta al., 2002). These systems are oftenvery easy for patients to use, which makes them appealing. In allcases, the goal of a drug delivery system is to get the right dosage tothe right place (Shaikh et al., 2011). Many topical preparations, however, containtherapeutically active ingredients which is dispersed or dissolved inthe base. The release rates of topical medications depend solely onthe physical and chemical, properties of the carrier and themedication used (Date et al., 2006; Foldvari, 2000; Elsayed et al., 2007).The topical formulations are known for its local dermatologicalaction and it should deliver the medication across the localized areaof the skin. To develop an ideal dosage form one must take intoaccount retention of the dosage form, the flux of drug across the skinand patient acceptability of the formulation (Dhote et al, 2012; Garg et al., 2014; Nair et al., 2013). As topicalformulations contain multiple components with active ingredients itshould be compatible and should provide stable chemicalenvironment for all the ingredients (Sruthi et al., 2016).

1.3. Polyherbal formulations and its significance

In the few decades, there has been exponentially growth in the field of herbal medicines. Nature always stands as a golden mark to exemplify the outstanding phenomena of symbiosis. Today about 80% of people in developing countries still relay on traditional medicine based largely on the different species of plants for their primary health care. About 500 of plants with medicinal uses are mentioned in ancient literature and 800 plants have been used in indigenous system of medicine. The various indigenous systems such as Ayurveda, siddha, unani use several plant species to treat different ailments (Sane et al., 2002).Herbal medicines are as crude drugs of vegetable origin utilized for the treatment of disease states, often of a chronic nature, or to attain or maintain a condition of improved health. Current demands for herbal medicines have resulted in an annual market of$1.5 billion and increasingly widespread availability. The treatment of injury or disease by plants or plant material, either in the crude or processed state, is known as traditional herbal medicine. The medicinal plants with ethno medicinal values are currently being screened for their therapeutic potential (Kunwaret al., 2010).Herbal product has been used abundantly over the years in curing several diseases. Natural products and related structures are essential sources of new pharmaceuticals, because of the immense variety of functionally relevant secondary metabolites of microbial and plant species5. Herb-herb combinations also known as polyherbal therapy have been used in Chinese medicine practice for thousands of years, yet scientific evidence of their therapeutic benefits is lacking (Che et al., 2013). Drug combination often produces a promising effect in treatment of diseases over a single drug. The concept of drug combination hasbeen well established in Western medicine and remarkable success has been achieved over the decades. In recent years, drug combination therapies in cancer and infectious diseases have offered new hope to patients (Risberget al., 2011). Naturally occurring herbs and herbal ingredients organized into certainformula have been shown to have potential interaction effects. These include mutual enhancement, mutual assistance, mutual restraint and mutual antagonism (Ramaiah et al., 2013). In the Ayurvedic system of medicine mainly polyherbal compounds are used for treatment of various infections (Reddy et al., 2010). Ayurvedic and herbal medicinal products contain a combination of botanicals; each ofthese contains a number of chemical compounds that may give the anticipated activity in combination. The increasing interest in the use of plant-based formulations is leading to a fast growing market for Ayurvedic medicines (Bhopeetal., 2011).Herbal medicines are in

widespread use and although many believe herbal medicines are safe, they are often used in combination and are drawn from plant sources with their own variability in species, growing conditions, and biologically active constituents. A major hypothetical advantage of botanicals over conventional single-component drugs is the presence of multiple active compounds that together can provide a potentiating effect that may not be achievable by any single compound. Polyherbal formulations have plant-based pharmacological agents which may exert synergistic, potentiative, agonistic antagonistic actions by virtue of its associated diverse active principles themselves. These pharmacological principles work together in a dynamic way to produce maximum therapeutic efficacy with minimum side effects (Benzie et al., 2012).

1.4. Herbs used in skin and hair care

Various herbs have been used in skin and hair care. These are listed in table 1A and 1B

Table 1.A: Herbs used in skin care

Sl no	Botanical name with family	Common name	Use in skincare	References
1.	*Adhatoda vasica* (Acanthaceae)	Vasaca	Fresh leaves juice / extract is used for skin affection and control of scabies	Singh et al., 2011
2.	*Ailanthus excels* (Simaroubaceae)	Maharukh	Leaves extract checks skin eruption anduseful in skin creams and lotions	Kundu and lascar, 2010
3.	*Allium sativum* (Alliaceae)	Garlic	Garlic oil is useful to control sores, Pimples and acne. It may be used in skin lotions and creams	Lyantagaye, 2011
4.	*Aloe vera* (Liliaceae)	Ghikanwar	Leaves juice, its pulp or extracted material is applied on skin forsmoothness, healing	Gupta et al., 2006

			controlling skin burn, sun burn and injury etc	
5.	*Azadirachta indica* (Meliaceae)	Neem	useful incuring wounds, skin deseases, leprosy,ulcersetc	Siddiqui et al., 1987
6.	*Carica papaya* (Caricaceae)	Papaya	Milky juice of unripe fruit is a good ingredient for facial and face cream; fruit pulps make skin soft and remove blemishes	Quintal et al., 2011
7.	*Citrus limon* (Rutaceae)	Nimbu	Potential source of vitamin C; oil is used in various preparation to reduce skin itching and skin nourishment, pulpleft after extraction of juice is useful asa facial ingredients	Molina et al., 2010
8.	*Cocos nucifera* (Arecaceae)	Nariyal	Coconut oil is useful for skin itching and rashes	Yong et al., 2009
9.	*Cucumis sativus* (Cucurbitaceae)	Khira	Water extract of fruits and seeds protectskin from sunburn	Yan et al., 2011
10.	*Curcuma longa* (Zingiberaceae)	Haldi	Rhizome powder possessesantiinflammatory and anti-oxidant	Awasthi and Dixit, 2009

			properties; used in facial, face creams and ointments	
11.	*Eclipta alba* (Asteraceae)	Bhringraj	Paste of herb is useful to control skin diseases and eczema	Roy et al., 2008
12.	*Andropogon muricatus* (Poaceae)	Khas	Powdered root paste with red sandal wood is used to cure irritated skin and allergies	Shah and Gilani, 2012
13.	*Butea frondosa* (Fabaceae)	Dhak	Leaves extract is useful in pimples andseed extract for fungal infection andbruises	Londonkar and Ranirukmini, 2010
14.	*Juniperus communis* (Cupressaceae)	Aaraar	Whole plant extract is useful in skin creams to control skin rejuvenation	Melvina et al., 2005
15.	*Lavandula vera* (Lamiaceae)	Lavender	Essential oil is used in skin anti-acne	Zuzarte et al., 2010
16.	*Jasminum grandiflorum* (Oleaceae)	Chameli	Essential oil extracted from flowers is used in skin creams and lotions to control skin diseases. Essential oil extracted from plant is used in creams for the protection from sunburn	Chaturvedi and Tripathi, 2010
17.	*Leuca saspera* (Lamiaceae)	HulKhusa	Juice of leaves is applied to control scabies, skin psoriasis, chronic skin,	Srinivasan, 2011

			skin eruption and eczema	
18.	*Mallotus philippensis* (Euphorbiaceae)	Kamala	Flower powder is useful to control scabies ringworm, leprous eruption, etc	Oudhia, 2013
19.	*Ocimum sanctum* (Lamiaceae)	Tulsi	Leaves extract is useful to control skin infection and rejuvenation	Prakash and Gupta, 2005
20.	*Santalum album* (Santalaceae)	Chandan	Paste of hardwood is used in face pack;essential oil used in preparation ofcreams, ointments and lotions for skinbeautification and protection from sunburn; possesses anti-oxidant properties	Misra and Dey, 2013

Table 1.B: Herbs used in hair care

Sl no	Botanical name with family	Common name	Use in hair care	References
1.	*Acacia concinna* (Mimosaceae)	Shikakai	Pods extract is used as haircleanser and for control ofdandruff	Karanth, 2003
2.	*Arnica Montana* (Asteraceae)	Arnica	Flowers extract is used in hair oilas a tonic material. It stimulatesthe hair follicles	Knuesel et al., 2002
3.	*Betula pendula* (Betulaceae)	Birch	Extract of leaves is used as antidandruff	Rytter et al., 2003
4.	Brassica spp. (Brassicaceae)	Mustard	Seed oil is used as hair oil anduseful for hair nourishment	Kumar et al., 2005
5.	*Tagetes erecta* (Asteraceae)	Marigold	Flowers extract is used in haircreams for smoothening effect	Gopi et al., 2012

6.	*Cocos nucifera* (Arecaceae)	Nariyal	Kernel oil is well-established hairoil, which is used as such or as abasic raw material for preparinghair oils and tonics	Singla, 2012
7.	*Eclipta alba* (Asteraceae)	Bhangra	Whole plant extract is useful forhair‟s nourishment and dyeing	Roy et al., 2008
8.	*Juglan sregia* (Juglandaceae)	Akroot	Leaves and hull of fruits is usedfor hair dyeing	Gaur, 2008
9.	*Lawsonia inermis* (Lythraceae)	Henna	Leaves paste is used for hairdyeing and nourishment	Makhija, 2010
10.	*Nardostachys jatamansi* (Valerianaceae)	Jatamansi	Extract of rhizome is used in hairtonics for their growth	Amatya and Sthapit, 1994
11.	*Phyllanthus emblica* (Euphorbiaceae)	Amla	Fruit extract is used in oils forpromotion of hair growth	Zhang et al., 2003
12.	*Salvia officinalis* (Lamiaceae)	Sage	Aqueous extract is used as hairconditioner	Oniga et al., 2010
13.	*Sapindus mukorossi* (Sapindaceae)	Ritha	Extract of fruit coat works asnatural shampoo: used in herbalshampoo as hair cleanser	Sinha et al., 2000
14.	*Saussurea lappa*C.B. (Asteraceae)	Kuth	Roots extract is used in hairdyeing	Jia et al., 2005
15.	*Sesamum indicum* (Pedaliaceae)	Til	Seed oil is one of the majorsources of hair oils, which is usedas such or a base for preparingspecific hair oils	Suja et al., 2004
16.	*Terminalia bellerica* (Combretaceae)	Behera	Seed extract and oil is good forhair dyeing preparation	Misra, 1998
17.	*Terminalia chebula* (Combretaceae)	Harra	Seed extract is used in hair careformulations	Ali and Khan, 2009
18.	*Thymus serpyllum* (Lamiaceae)	Banajwain	Whole herb extract is useful forpreparing hair tonics	Paaver et al., 2008
19.	*Carthamus*	Safflower	Alcoholic extract is used in hairtonic	Bassil and

	tinctorius(Asteraceae)			Kaffka, 2002
20.	*Centella asiatica* (Apiaceae)	Mandukaparni	Whole plant extract is used forthe growth and maintenance ofhairs	Zheng and Qin, 2007

Chapter 2

Scope, objective and plan of work

2.1. Scope of the project

2.2. Objectives

2.3. Plan of work

2.1. Scope of the project

The present study deals with preparation of some topical herbal formulations using some medicinal plants having applications in skincare. The plants have been selected from the Ayurvedic system of medicine, based on proper literature search and ethno-pharmacological relevance. Three types of formulations will be prepared under this project- creams, lotion and shampoo. The plants selected for development of herbal shampoo are- *Hibiscus Rosa Sinensis, Cocos nucifera, Olea europea, Acacia coccinna* and *Lawsonia inermis*. The plants selected for the development of creams and lotions were *Tagetes erecta*, *Cucumis sativus*, *Carica papaya*, *Ocimum sanctum*. Within the scope of the study, at first we will perform the collection, authentication, extraction and standardization of the plants using suitable marker compounds. The standardization of the plant extracts will be performed using HPTLC and RP-HPLC analyses,. After this, some topical herbal formulations will be prepared using these plant extracts. First of all, suitable bases for the formulations will be prepared as per pharmacopoeial guidelines and literature reports. After this, the formulations will be evaluated for the physical properties, like spread-ability, wettability, grittiness, foaming index, rheological properties, pH, salinity etc.

2.2. Objectives

The present study deals with preparation of some topical herbal formulations using some medicinal plants having applications in skincare. The plants have been selected from the Ayurvedic system of medicine, based on proper literature search and ethno-pharmacological relevance. Three types of formulations have been prepared under this project- creams, lotion and shampoo. The plants selected for development of herbal shampoo are- *Hibiscus Rosa Sinensis, Cocos nucifera, Olea europea, Acacia coccinia* and *Lawsonia inermis*. The plants selected for the development of creams and lotions were *Tagetes erecta*, *Cucumis sativus*, *Carica papaya* and *Ocimum sanctum*.

The main objectives of the project have been presented in the following section-

i. Collection, authentication and extraction of the medicinal plants.

ii. Standardization of the medicinal plants using suitable marker compounds, with the help of HPTLC (High Performance Thin Layer Chromatography) and RP-HPLC (Reverse phase-High Performance Liquid Chromatography).

iii. Development of herbal shampoo, creams and lotions using the standardized plant extracts.

iv. Evaluation of the topical herbal formulations for the different physical propertiesfor the physical properties, like spread-ability, wettability, grittiness, foaming index, rheological properties, pH, salinity etc.

2.3: Plan of Work

The plan of work is represented in the schematic diagram (Fig 2.1).

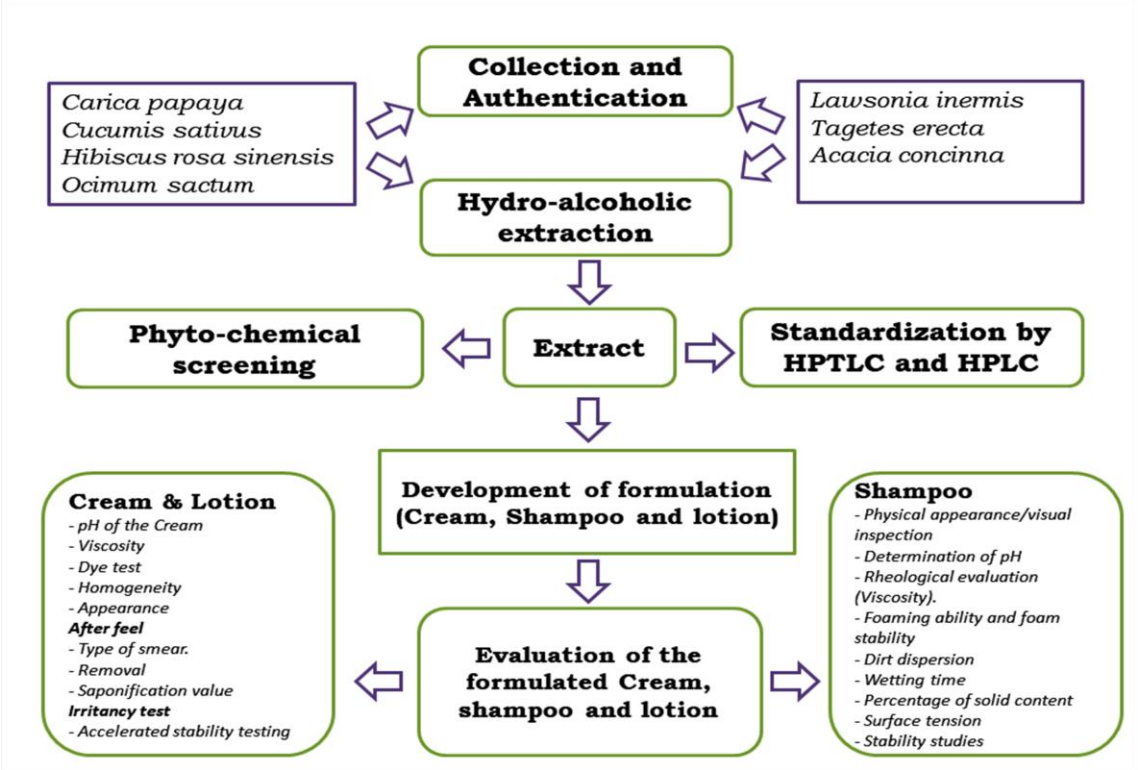


Fig 2.A: Plan of work

Chapter 3

Overview of topical formulations used in the study

3.1. Cream

3.2. Lotion

3.3. Shampoo

3.4. Plants selected for the study

3.1. Cream:

3.1.1. Formulation consideration:

I. **Drug selection**:

The simplest approach to choose a drug from a congeneric series or pharmacological class with the correct physicochemical properties to translocate accrose the barrier at an acceptable rate.

II. **Insoluble drug**:

Insoluble drug must be dispersed through the vehicle to ensure homogeneity of the product. The solid must be impalpable to the touch, otherwise grittiness results.

III. **Vehicles or semisolid bases**:

The vehicles used for a pharmaceutical semisolid are called as bases and they differ from that used for a cosmetics bases because for a cosmetics penetration into the skin is not required. Penetration or protection is desired in a pharmaceutical semisolid, and its cosmetics effect or appearance on the skin is less important.

A well formulated pharmaceutical semisolid should be both therapeutically effective and cosmetically appealing, with the major effort in the medical direction.

The USP recognizes four classes of semi-solid bases under the general classification of creams:

1. Oleaginous bases
2. Absorption bases (anhydrous form and emulsion form)
3. Water removal bases and
4. Water soluble bases

Oleaginous bases:

These bases are usually anhydrous and consist of substances such as hydrocarbons, vegetable oils, silicons, and certain synthetic esters, either alone or in combination. These bases are characterised by -

-Low capacity to absorb water (emollient effect)

-Exert high occlusiveness by forming water impermeable layer of skin

-Greasy and thus difficult to remove from skin

-Prolonged contact with skin and

-Poor aesthetic appeal

Absorption bases:

These bases are usually anhydrous and they do absorb aqueous solutions and can be considered water in oil emulsions. The bases do not absorb water on contact but with sufficient agitation the absorption bases are formed by the addition of substances miscible with

hydrocarbons and possessing polar groupings, such as the sulphates, sulfonate, carboxyl, hydroxyl or an ether linkage. Lanolin, lanolin isolates, cholesterol, lanosterol and other sterols, acetylated sterol, or partial esters of polyhydric alcohols (eg. Sorbitan mono stearate and mono oleate) may be added to make the hydrocarbon bases hydrophilic. Such hydrophilic mixtures have been known as absorption bases, although the tern absorption is a misnomer.(Liberman Lachman,Industrial pharmacy,2001)

The absorption bases are of two types:

-The anhydrous form

-The hyrdrous form

Anhydrous absorbtion bases:

Anhydrous lanolin (wool fat), bees wax, hydrophilic petrolatum and organo silicons are examples of anhydrous vehicles that absorbs water to form water in oil emulsions. Here the example of anhydrous base in Table 3.A (Liberman Lachman,Industrial pharmacy,2001).

Table: 3.A: Anhydrous absorbtion bases

Formulation 1	
Hydrophilic petroleum (USP XX)	**Weight taken (g)**
Cholesterol	30
Stearyl alcohol	30
White wax	80
White petroleum	860
Total	1000

Hydrous absorption bases:

These are w/o emulsions with ability to absorb additional water.Hydrous lanolin was the prototype or forerunner of the absorption bases because of ability to absorb water. Various absorption bases were developed as various lanolin isolates and derivatives became commercially available. Many of these lanolin fractions aid in the formation of water in oil emultions. Some examples of hydrous absorption base are as follows 3.B,3.C,3.D (Liberman Lachman,Industrial pharmacy,2001).

Table: 3.B: Hydrous absorption bases

Formulation 2	
Lanolin absorbtion base	**Weight (g)**
Lanolin alcohol	10
Lanolin	25
Mineral oil,low viscosity	30
Purified water	35

Table 3.C: Hydrous absorption bases

Formulation 3 & 4		
Oil phase	**Percentage (w/w) (Formula 3)**	**Percentage (w/w) (Formula 4)**
Lanolin,anhydrous USP	3.1	15.0
Petrolatam,white,USP	25.0	_
Mineral oil,heavy	25.0	8.0
Bees wax (white wax,USP)	10.0	7.0
Sorbitan sesquioleate	1.0	_
Propyl paraben	0.05	0.05
Amerchol	_	20.0
Aqueous phase		
Sodium borate,USP	0.7	_
Polyethylene glycol 1500	_	5
Methyl paraben	0.15	0.15
Purified water	35.0	49.8

Table -3.D: **Hydrous absorption bases**

Formulation 5	
Cold cream	%
A	
Purified water	34.0
Borax	1.0
Methyl paraben	0.25
B	
Light mineral oil	50.0
Synthetic bees wax	13.0
Glyceryl monostearate	1.00
Propyl paraben	0.15

Water –removable bases:

The water removable bases are oil in water emulsions and referred to as creams. The vanishing cream bases fall into this category. The following table 3.E is the example of water-removal bases (Liberman Lachman,Industrial pharmacy,2001).

Table-3. E: **Water –removable bases**:

Formulas for vanishing cream base				
	Anionic stearate Emulsifier % by wt	**Anionic Emulsifier % by wt**	**Nonionic Emulsifier % by wt**	**Cationic Emulsifier % by wt**
Stearic acid	13.0	7.0	14.0	-
Stearyl alcohol	1.0	5.0	-	-
Cetyl alcohol	1.0	2.0	1.0	-
Glyceryl monostearate	-	-	-	10.0
Isopropyl palmitate	-	-	1.0	-
Lanolin	-	-	-	2.0
Methylparaben	0.10	0.10	0.10	0.10
Propylparaben	0.05	0.05	0.05	-
Sorbitan monostearate	-	-	2.0	-
Glycerine	10.0	10.0	-	15.0
Sorbitan solution	-	-	3.0	-
Potassium hydroxide	-	-	1.5	-
Sodium lauryl sulphate	-	1.0	-	-
Polysorbate 60	-	-	1.5	-
Stearyl colamino formyl methyl pyridinium chloride	-	-	-	1.5
Purified water (Q.S)	100	100	100	100

Water soluble bases:

Water soluble vehicle are prepared from mixtures of high and low molecular weight polyethylene glycol or macrogol. The low molecular weight glycols in this category are liquids, those with a moderately high molecular weight are somewhat unctuous; and higher molecular weight polyethylene glycols are solids. Suitable combinations of high and low molecular weight polyethylene glycols yield products having ointment like consistency, which soften and melt when applied on skin.

Preservatives:

The preservatives are added to semisolids to prevent contamination, deterioration, and spoilage by bacteria and fungi, since many of the components in this preparations serve as substrates for these organisms. Chemical preservatives for semisolids must be carefully evaluated for their stability with regards to other components of the formulation as well as their containers. Plastic containers may absorbs the preservatives and thereby decrease the quantity available for inhibiting or destroying the microorganisms responsible for spoilage.Some preservatives may sting or irritate the mucous tissues of the eye or nasal passages (Liberman and Lachman, 2001).

Developement of formulation of cream and ointment:

1. Fusion method:

Anhydrous ointments manufactured by fusion process.The active substance is dissolved in melted fats and waxes,or in one of the component of the vehicle,and then mixed with the base.The melted mass must be mixed while cooling because the fatty alcohol,fatty acids,and waxes do not form true solution with petroleum and minral oil,but crystalline from the melt as the temperature failed.

2. Emulsification method:

Preparation of oil and aqueous phase:

The components of the oil or fat mixtures are placed into a beaker, melted and mixed. The flakes are preferable because of the convenience of handling. The powder may have occasional fine metal contaminants from the pulverising equipment.

Petroleum is inconvenience to handle unless it is melted and transferred by pouring from its drum. Transfer of large quantities of petroleum is expedited by heating the petroleum in the steel drum in which it is received from the suppliers by means of emersion heaters or by placing the drum in a hot room until the petroleum is fluid..

The oil phase is then strained through several layers of cheese cloth to remove any foreign matter. Alternatively the petroleum can be passed through a filter medium, particularly for an ophthalmic preparation. The oil phase is transferred to the emulsion mixing walls have been heated to the temperature of the oil phase to prevent some of its higher – melting components from congealing.

The components of the aqueous phase are dissolved in the purified water and filtered. A soluble drug may be added to the aqueous phase at this time, provided the high temperature does not degrade the active substance or the emulsion is not adversely affected; otherwise the soluble drug may be added in solution after the emulsion has formed and has cooled.

Mixing of phases:

The phases are usually mixed at a temperature of 70-72° C, because at this temperature intimate mixing of the liquid phases can occur.

The phase mixing temperature can be lowered at a few degrees if the melting point of the fat phase is low enough to prevent the premature crystallization or congealing of its components.

The simultaneous blending of the phases requires. The disperse or aqueous phase in an oil –in – water emulsion is added slowly to the inner phase with agitation. The initial low concentration of water in relation to the concentration of oil results in the formation of a water-in-oil emulsion.

Cooling the semi-solid emulsion:

Following the addition of the phases, the rate of cooling is generally slow to allow for adequate mixing while the emulsion is still liquid. The temperature of the cooling medium in, especially when the semisolid contains a large percentage of high-melting substances.

The drug added in the extracted solution form, if not already incorporated, or as crystals, provided it is soluble in the external phase.

Adjustment of the final water content of a water-in-oil emulsion is not easy once the emulsion has been formed. Several batch run help to determine the amount of water lost on heating in the particular process, and this water lost should be added to the required amount at the start of the manufacturer. The oil film surrounding each emulsified water droplet in a water –in-oil emulsion tends to retard evaporation, so that water loss is not excessive following this type of emulsion..(Liberman Luchman, Industrial pharmacy)

Homogenization:

The creams or ointments that require further treatment are then transferred or pumped to the proper homogenizer, the selection of which is governed by the degree and rate of shear stress required.

Herbal cream

Herbal cosmetics are the preparations used to enhance the human appearance. Herbal cosmetic are also known as “natural cosmetics”. With the beginning of the civilization, mankind had the magnetic dip towards impressing others with their looks. At the time, there were no fancy fairness creams or any cosmetic surgeries. The only thing they had was the knowledge of nature, compiled in the ayurveda. With the science of ayurveda, several herbs and floras were used to make ayurvedic cosmetics that really worked Ayurvedic cosmetics not only beautified the skin but acted as the shield against any kind of external affects for the body. [5] Herbal cosmetics are the products in which herbs are used in crude or extract form. The basic idea of skin care cosmetic lies deep in the Rigveda, Yajurveda, Ayurveda, Unani and Homeopathic system of medicine.

Herbal Skin Care Products: Lavender Silk Soaps, Lotions creams, Body powder**,** Lavender Herbal body powder, Skin Care Creams.

Materials and methods

Preparation of extracts: Air dried and coarsely powdered (100gm) of crude drug placed in Soxhlet extractor separately and run for 6 hours at 500c using distilled water. The extracts were as a solvent filtered and concentrated to dryness. Some materials and amounts are given in table 3.F.(Liberman and Lachman,Industrial pharmacy,2001).

Table-3.F: Materials and amount of extract

Sl. No	Herbal extract	Quantity (%)
1.	Neem extract	1
2.	Aloe juice	2
3.	Haldi extract	0.5
4.	Amba haldi extract	0.5
5.	Khas	1
6.	Rose water	0.5

Preparation of cream base: Oil in water (O/W) emulsion-based cream (semisolid formulation) was formulated. The emulsifier (stearic acid) and other oil soluble components (Cetyl alcohol, almond oil) were dissolved in the oil phase (Part A) and heated to 75° C. The preservatives and other water soluble components (Methyl paraban, Propyl paraban, Triethanolamine, Propylene glycol) were dissolved in the aqueous phase (Part B) and heated to 75° C. After heating, the aqueous phase was added in portions to the oil phase with continuous stirring until cooling of emulsifier took place.

Drug formulation

The appropriate base was selected and two different creams were formulated. The emulsifier (stearic acid) and other oil soluble components (Cetyl alcohol, almond oil) were dissolved in the oil phase (Part A) and heated to 75° C. The preservatives and other water soluble components (Methyl paraban, Propyl paraban, Triethanolamine, Propylene glycol, all extracts) were dissolved in the aqueous phase (Part B) and heated to 75° C. After heating, the aqueous phase was added in portions to the oil phase with continuous stirring until cooling of emulsifier took place..(Liberman Luchman,Industrial pharmacy)

Evaluation parameter

• *pH of the Cream*

• *Viscosity*

• *Dye test*

• *Homogeneity*

• *Appearance*

After feel

• *Type of smear*

• *Removal*

• *Saponification value*

Irritancy test

• *Accelerated stability testing*

Marketed formulation:

Table-3.G: Some examples of Marketed formulation

Manufacturers	**Products**
Anti Acne Cream Yash Medicare Private Limited, Ahmedabad, Gujarat	Anti acne cream, topical acne cream. Also supply Medicare products such as topical anti-fungal, anti-parasitic, dermatitis, anti-burns, topical steroids, and topical antibiotics, anti scabitic and anti seborrheics.
Roy & Company, Mumbai, Maharashtra	Anti-acne cream, skin care creams, aloe Vera, aloe Vera gel, anti-wrinkle cream anti migraine drug, hair oil, hair shampoo, aloe Vera skin gel, aloe Vera cream and herbal anti marks cream.
Surya Herbal Ltd. Noida, Uttar Pradesh	Anti-acne cream, natural fairness cream, blueberry face cleanser, essential moisturiser normal dry, nourishing day cream, nourishing night cream.
APCOS NATURALS, , Mohali, Punjab	Ayurvedic acne treatment kit, ayurvedic blemish treatment kit, ayurvedic every day care kit, ayurvedic fairness kits, ayurvedic age defying wrinkle treatment kit
Amrut Pharmaceuticals, , Belgaum, Karnataka	Anti-acne creams, anti-acne face creams and ayurvedic herbal anti acne creams. Also offering creams for ailments such as dryness of skin, itching, fungal infections, crack foots, burns for fire and chapped lips.
Bio Life Technologies, New Delhi, Delhi	Anti-acne creams, herbal anti acne creams, natural anti acne creams and skin anti acne creams. Also deals in anti-pimple creams, anti-marks cream, fairness creams and anti-septic creams.

3.2. Lotion

The appearance and function of the skin are maintained by an important balance between the water content of the stratum corneum and skin surface lipids. The skin represents the most superficial layer of the body and so it is constantly exposed to different environmental stimuli.

Exposure to external factors as well as endogenous factors may disrupt this balance. In addition, frequent use of soaps, detergents and topical irritants such as alcohol and hot water can remove the skin surface lipids. Disruption of skin barrier led to various type of skin problems most common condition is loss of water content which lead to dryness of skin such as roughness, scaling, cracks, redness and an uncomfortable feeling of tightness, sometimes with itching and stinging. Treatment with moisturizers aims at maintaining skin integrity and the well-being by providing a healthy appearance of the individual.

Evaluation of lotion formulation:

In vitro characterization of lotion formulation:

- *pH and rheological measurements*
- *Accelerated stability studies:*
- *Permeation studies*

In vivo characterization:

- *Sensatory perception test*

Materials and methods

Materials used:

Propylene glycol (Merck, Germany), ethanol (Merck, Germany), sodium acetate (Merck, Germany), isopropyl alcohol (Fluka, Switzerland), Carbomer 980 (Fisher, Germany); ɣ-carrageenan No. 2249

(Fluka Biochemika, Switzerland), turpentine oil (MS Traders, China), diclofenac diethylamine (Novartis, Pakistan) were used as received with minimum purity of 99%. Polydimethyl siloxane membrane with 400 µm-thicknesses was purchased from Samco, USA.

Preparation of lotion formulations

All lotion formulations were prepared by mixing the ingredients.Essentially, 2 g of DDA was dissolved in 20 mL of ethanol and this solution was added to the 20 mL of phosphate buffered saline containing 980 mg of carbomer. These were mixed for 30 minutes until a clear solution was obtained. To these solutions, permeation enhancers, PG and TO, were added in varying concentration. Finally, the volume was made up to 100 mL by adding ethanol. An enhancer free

lotion was also prepared as a control.The following table 3.H contains some synthetic ingredients (Liberman Lachman,Industrial pharmacy,2001)

Table-3.H: Synthetic ingredients used in moisturizers

Sl.no	Ingredients	Side effects	Used as/in
1	Propylene Glycol	Allergic reactions Eczema	hives and Humectant
2	Petrolatum and occlusive agent	Dryness and chapping	Emollient
3	Dimethicone	Harsh to skin or cause tumor	Adhesives& Emollient
4	Paraben	Allergic reactions and skin rashes	Antimicrobial agent
5	Diethanolamine (DEA), Triethanolamine (TEA)	Allergic reactions, eye irritation, dryness of hair and skin	Emulsifiers
6	Diazolidinyl Urea, Imidazolidinyl Urea, enzalkonium chloride	Contact dermatitis	Preservatives
7	Synthetic Colors	Carcinogenic	Colouring agent
8	Synthetic Fragrances	Headaches, dizziness, rash, hyperpigmentation, violet coughing	For fragrance

3.3. Shampoo

Developing a detergent mixture that is not selective, not too detersive, and well adapted to scalp and hair is a delicate compromise. The variability of the substrate and the duality of the desired aim need to be taken into consideration. Thus, multiple problems of compatibility, dosing, and balance have to be solved. To cleanse without causing dryness, to lubricate dry hair while cleansing, to impart a clean beauty or a specific treatment, to ensure a delicate sequential replacement of soil by conditioning are a multiparameter equation. This multiparameter equaequation necessarily leads to multiple solutions, according to the prevalence given to one or another criterion. It also suggests that the simplest shampoo formulation will be composed of a variety of ingredients.

Raw materials used in the manufacture of shampoo:

Table-3.I: Raw materials used in the manufacture of shampoo

The raw material used	Examples
Surfactants: • Anionic • Cationic • Nonionic • Amphoteric	 Sodium lauryl sulphate Polyquarternium-10 Laureth-3 or 4,cocamide dea Cocamido propyl betaine
Foaming agent	Sarcosinate,lactylates
Conditioning agent	Quaternium 80 and 87
Viscosifying agent • Electrolytes • Natural gums • Cellulose derivatives • Carboxy vinyl polymer • Others	 NH4Cl,NaCl Gum Karaya,tragacanth,alginates Hydroxyl ethyl cellulose,methyl cellulose Carbopol 934 PVP,Phosphate esters
Opacifying agents	Glycol stearate,glycoldistearate
Stabilizing agents/additives • Clarifying agents • Preservatives • Buffers • Sun-screen agents • Colours • Pearlescence agents • Antioxidants • Perfumes	 EDTA,citric acid Phenyl mercuric salts Citic and tartaric acid Benzophenons Green ,blue,yellowetc Ethylene glycol monostearate BHT,BHA Sandle,musk,rose,etc.

3. Evaluation of shampoos

- *Physical appearance/visual inspection*
- *Determination of pH*
- *Rheological evaluation (Viscosity).*
- *Foaming ability and foam stability*
- *Dirt dispersion*
- *Wetting time*
- *Percentage of solid content*
- *Surface tension*
- *Stability studies*

3.4. Plants selected for the study

3.J. Plants Selected for developing the formulation:

Sr. no.	Plant name	Used in
1.	*Hibiscus rosa sinensis*	Shampoo
2.	*Acacia coccinia*	Shampoo
3.	*Lawsonia inermis*	Shampoo
4.	*Ocimum sanctum*	Shampoo, cream
5.	*Tagetes erecta*	Cream, lotion
6.	*Cucumis sativus*	Cream, lotion
7.	*Carica papaya*	Cream, lotion

Chapter 4

Literature review, extraction, phytochemical screening and HPTLC and RP-HPLC standardization of the selected plants:

4.1.*Carica papaya* Linn

4.2.*Cucumis sativus*

4.3.*Hibiscus rosa sinensis*

4.4. *Ocimum sanctum*

4.5.*Lawsonia inermis*

4.6.*Tagetes erecta*

4.7.*Acacia concinna*

4.1.1: Literature review of *Carica papaya*

Taxonomy:

Kingdom: Plantae

Sub Kingdom: Tracheobionta

Class: Magnoliopsida

Subclass: Dilleniidae

Superdivision: Spermatophyta

Phyllum: Steptophyta

Order: Brassicales

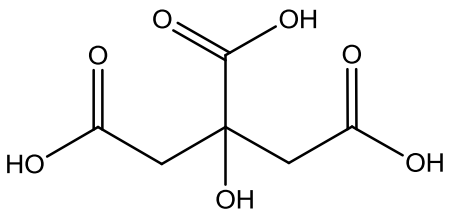
Family: Caricaceae

Genus: Carica

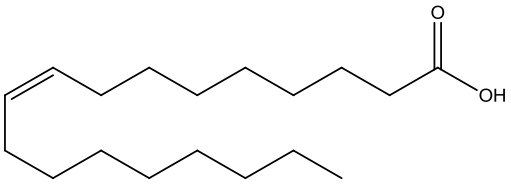
Botanical Name: *Carica papaya* Linn

4.1.2: Phytochemistry

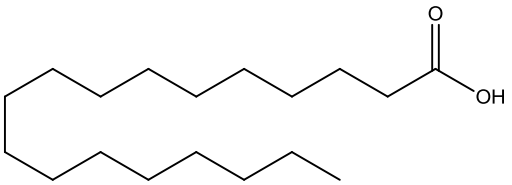
Raw papaya pulp contains 88% water, 11% carbohydrates, and negligible fat and protein. In a 100 gram amount, papaya fruit provides 43 kilocalories and is a significant source of vitamin C of the Daily Value, DV) and a moderate source of folate (10% DV), but otherwise has low content of nutrients.(Halim et al., 2011). Papaya skin, pulp and seeds contain a variety of phytochemicals, including carotenoids and polyphenols, as well as benzyl isothiocyanates (5) and benzyl glucosinates, with skin and pulp levels that increase during ripening.Papaya seeds also contain the cyanogenic substance prunasin alongwith citric acid (1), oleic acid (2), stearic acid (3) , linoleic acid (4) and ascorbic acid (6) (Mahmood et al.,2005).



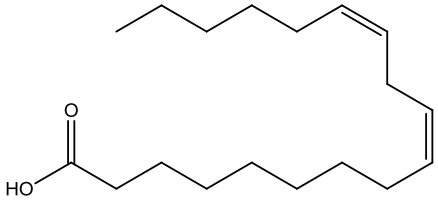
Citric acid (1)



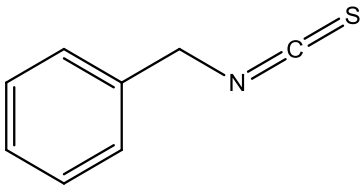
Oleic acid (2)



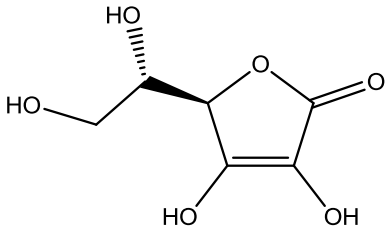
Stearic acid (3)



Linoleic acid (4)



Benzylisothiocyanate (5)



Ascorbic acid (6)

4.1.3: Pharmacology

The different parts of the *Carica papaya* Linn including leaves, seeds, latex and fruit exhibited to have medicinal value. Different properties of papaya such as antioxidant and free radical scavenging activity, anticancer activity, anti-inflammatory activity, treatment for dengue fever, anti-diabetic activity, wound healing activity and antifertility effects have been reported (Yogiraj et al., 2014).

4.1.4:Collection, authentication and extraction of *Carica papaya fruits:*

4.1.4.1.Collection and authentication

The ripe fruits of *Carica papaya* were purchased from local vendor present in Jadavpur Kolkata and authenticated. The voucher specimens (Specimen no: SNPS/JU/2018/1099)was deposited at School of Natural Product Studies. Jadavpur University,Kolkata for future references.

Fig 4.1.a: Picture and herbarium specimen of *Carica papaya*

4.1.4.2. Extraction of Carica papaya fruit

Preparation of hydro-alcoholic solution: 1kg of fresh ripe papaya fruit was cut and chopped into small pieces. Then it was cold macerated using 80:20 ratio of ethanol:water for 48 hrs with occasional shaking. The material was filtered using a Whatman no-1 filter paper. The liquid extract obtained was concentrated under pressure in rotary evaporator, IKA-HB10(IKA India Private Limited, India). The concentrated extract was further freeze dried in a lyophilizer (INST IND, Kolkata, India). Then it was transferred to a glass screw cap container, labeled and stored in a desiccator for future use. Yield obtained was 28.3 % w/w

4.1.4.3. Qualitative analysis of *Carica papaya:* (Mukherjee et al, 2002)

4.1.4.3.1: Preparation of reagents

4.1.4.3.1.1: Mayer's reagent

1.36 gm. of mercuric iodide in 60 ml of water mixed with a solution which contains 5gm of potassium iodide in 20 ml of water.

4.1.4.3.1.2: Libermann-Burchard reagent

5 gm. of acetic anhydride was carefully mixed under cooling with 5 ml concentrated sulphuric acid; this mixture was added continuously to 50 ml of absolute ethanol with cooling.

4.1.4.3.1.3: Dragendorff's reagent

1.7 gm. basic bismuth nitrate and 20 gm tartaric acid are dissolved in 80 ml of water. This solution was mixed with a solution containing 16 gm potassium iodide and 40 ml of water.

4.1.4.3.1.4: Fehling's solution A

34.64 gm. copper sulphate was dissolved in a mixture of 0.5 ml of sulphuric acid and sufficient water to produce 500 ml.

4.1.4.3.1.5: Fehling's solution B

176 gm. of sodium potassium tartarate and 77gm of NaOH are dissolved in sufficient water to produce 500ml. Equal volumes of solution A & B are mixed at the time of use.

4.1.4.3.1.6: Benedict's reagent

1.73 gm. of cupric sulphate, 1.73 gm of sodium citrate and 10 gm anhydrous sodium carbonate are dissolved in water and the volume was made up to 100 ml with water.

4.1.4.3.1.7: Molish's reagent

2.5 gm. of pure α-naphthol was dissolved in 25 ml of ethanol.

4.1.4.3.2: Methodology

4.1.4.3.2.1: Detection of alkaloid

4.1.4.3.2.1.1:. Mayer's test

1.2 ml of extract was taken in a test tube. 0.2 ml of dilute hydrochloric acid and 0.1 ml of Mayer's reagent were added. Formation of yellowish buff colored precipitate gives positive test for alkaloid.

4.1.4.3.2.1.2: Dragendroff's test

0.1 ml of dilute hydrochloric acid and 0.1 ml of Dragendroff's reagent were added in 2 ml solution of extract in a test tube. Development of orange brown colored precipitate suggested the presence of alkaloid.

4.1.4.3.2.1.3: Wagner's test

2 ml of extract solution was treated with dilute hydrochloric acid and 0.1 ml of Wagner's reagent. Formation of reddish brown precipitate indicated the positive response for alkaloid.

4.1.4.3.2.2: Detection of glycosides

4.1.4.3.2.2.1: Legal test

Extract was dissolved in pyridine, sodium nitroprusside solution was added to it and made alkaline. Pink red color was produced.

4.1.4.3.2.2.2: Baljet test

To a drug extract, sodium picrate solution was added. Yellow to orange color was produced.

4.1.4.3.2.2.3: Borntrager's test

Few ml of dil. sulphuric acid added to the test solution. Boiled, filtered and extracted the filtrate with ether or chloroform. Then organic layer was separated to which ammonia was added, pink red color was produced in organic layer.

4.1.4.3.2.2.4: Keller Killiani test

Sample was dissolved in acetic acid containing trace of ferric chloride and transferred to the surface of conc. sulphuric acid. At the junction of liquid reddish brown color was produced which gradually becomes blue. Detection of phenolic compounds and tannins

4.1.4.3.2.2.5: Ferric chloride test

5 ml of extract solution was allowed to react with 1 ml of 5% ferric chloride solution. Greenish black coloration indicated the presence of tannins.

4.1.4.3.2.3: Detection of saponins

4.1.4.3.2.3.1: Foam test

1 ml solution of the extract was diluted with distilled water to 20 ml and shaken in a graduated cylinder for 15 min. Development of stable foam suggested the presence of saponins.

4.1.4.3.2.3.2: Potassium dichromate test

1 ml extract was treated with 1% lead acetate solution. Formation of white precipitate indicated the presence of saponins.

4.1.4.3.2.4: Detection of phytosterols

4.1.4.3.2.4.1: Libermann-Burchard Test

10 mg of extract was dissolved in 1ml of chloroform. 1 ml of acetic anhydride was added following the addition of 2ml of concentrated sulphuric acid, a reddish violet color developed, indicating the presence of steroids.

4.1.4.3.2.4.2: Salkowski Test

1 ml of concentrated sulphuric acid was added to 10 mg of extract dissolved in 1 ml of chloroform. A reddish-blue color exhibited by chloroform layer and green fluorescence by the acid layer suggested the presence of steroids.

4.1.4.3.2.5: Detection of triterpenoids

4.1.4.3.2.5.1: Nollar's test

In the test tube 2 ml of 0.01% anhydrous stannous chloride in thionyl chloride solution and test solution was added. Purple colour formed changed to deep red colour after few minutes indicates the presence of triterpenoids. Detection of flavonoids

4.1.4.3.2.5.2: Shinoda test

To the extract magnesium turnings and then conc. hydrochloric acid was added. Red color was produced.

4.1.4.3.2.6: Detection of protein and amino acids

4.2.3.2.6.1:Ninhydrin test

Extract solution was treated with ninhydrin (Triketohydrindene hydrate) at the pH range of 4-8. Development of purple color indicated the positive response for amino acids.

4.1.4.3.2.6.2: Biuret test

1 ml of 40% NaOH mixed with 2 drops of 1% copper sulphate was added to the extract, a violet color indicated the presence of proteins. Detection of deoxy sugars

4.1.4.3.2.6.3: Keller Kiliani test

To 1gm of the sample, 10 ml of 70% ethanol were added and boiled for 2-3 min. it was filtered and to the 5 ml of the filtrate, 5 ml of distilled water and 0.5 ml strong lead acetate solution were added. It was filtered and 5 ml of chloroform were added to the filtrate. Excess chloroform was pipetted off and gentle evaporation of chloroform was done on a porcelain dish. It was cooled and to the residue, 3 ml

of glacial acetic acid and 2 drops of 5% ferric chloride were added. The solution was transferred to the surface of 2 ml concentrated sulphuric acid. Reddish brown color (which changed to bluish green to dark on standing) at the junction confirmed the presence of deoxy sugars in the sample.

4.1.4.3.2.7: Detection of reducing sugars

4.1.4.3.2.7.1: Fehling's test

5 ml of the extract solution, mixed with 5 ml of Fehling's solution was boiled for 5 minutes. Formation of brick red colored precipitate demonstrated the positive test for reducing sugars.

4.1.4.3.2.7.2: Benedict's test

To 5 ml of the extract solution, 5 ml of Benedict's solution was added in a test tube and boiled for few min. Development of brick red precipitate confirmed the presence of reducing sugar.

Result of 4.1.A.phytochemical screening

Alkaloids	Glycosides	Saponin	Flavoinds	Carbohydrates	Tannins
-	+	+	+	+	-

4.1.4: HPTLC Standardization of *Carica papaya:*

4.1.4.1. Equipments and reagents: The CAMAG HPTLC system consisting of WINCATS software, LINOMAT V automatic sample applicator,scanning densitometer CAMAG scanner 3and photo documentation apparatus CAMAG reprostar 3. All the solvents were used of analytical grade. 100µl syringe (HAMILTON, Switzerland) was used for sample application on HPTLC plates. Stationary phase used was aluminium based silica gel plate 60 F254 (Merck,Mumbai) with 10cm×10cm. Ethanol : glacial acetic acid of analytical grade were purchased from Merck(Mumbai,India). Whatman's syringe filter (NYL 0.45µ) was used for the filtration of the sample and standard.

4.1.4.2.Chromatographic condition:

HPTLC analysis of *Carica papaya* was performed with biomarker ascorbic acid using isocratic technique by external methods. Mobile phase was optimized with Ethanol:glacial acetic acid =9.5:0.5 (v/v). The temperature was kept at 25°C and mobile phase was developed in a twin trough glass chamber. Standard stock solution was applied consequently in the range of 2-10 µl with 2 µl gradual increment.All total 8 tracks in HPTLC plates were used for standardization including standard and sample solution respectively in a band wise fashion. After development, plate was dried and scanned at 254 nm.

4.1.4.3: Preparation of Standard and Sample solution:

Standard solution: About 1 mg of ascorbic acid standard was weighed and put into 2 ml ependorff tube. The standard was dissolved in methanol and volume was made up to 1ml with methanol. It was then mixed with vortex mixture and put to ultrasonication bath till the material completely dissolved. It was then filtered through 0.45µ syringe filter and kept for further studies.

Preparation of sample solution: About 10mg of *Carica papaya* extract was dissolved in 1ml methanol in eppendorff tubes. Then the extract was dissolved in methanol and subjected to ultra-sonication till the extract was completely dissolved. It was then filtered through 0.45 µ syringe filter and kept for further study.

Optimization of mobile phase: The mobile phase was optimized for the HPTLC analysis. Out of the many mobile phases tried the following mobile phase gave the better separation.

Ethanol : glacial acetic acid=9.5:0.5 (v/v).

Thus optimized mobile phase was ethanol:glacial acetic acid=9.5:0.5 (v/v).

Application of standard and extract: The external standard calibration curve for ascorbic acid was prepared with calibration solutions in a concentration range of 200 to 1000ng/ml. Then 50 µl of standard solution was drawn into Hamilton syringe and put to CAMAG LINOMAT V applicator to give the concentrations of standard required. The same method was applied for the extract.

$$Y = m X + C$$

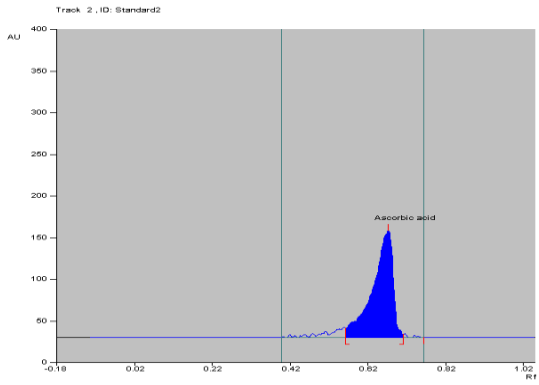
Where,

X = Concentration of Analyte/ metabolite in ng/ml

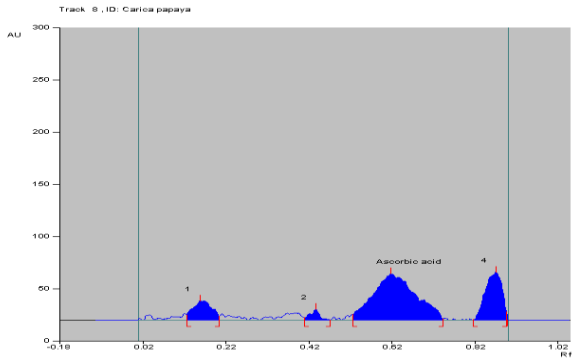
Y = Peak area

m = Slope of calibration Curve

C = Intercept.



4.1.a: HPTLC chromatogram of ascorbic acid



4.1.b: HPTLC chromatogram of *Carica papaya*

4.1.4.4. Method validation for HPTLC study:

Validation of HPTLC method was done as recommended by the International conference on Harmonisation (ICH) guidelines (ICH,1996,2005) and FDA (1994) Guidelines defining the Linearity ,Specificity, Limits of Quantification and detection, precision, accuracy, and robustness.

Specificity:

The results for HPTLC standardization profiles were checked in terms of specificity according to the ICH guidelines to minimize errors due to the contamination of the sample. The specificity of the method was determined by analyzing the standard and test sample. The identity was confirmed by comparison of retardation factor and absorbance of standard compound with extracts.

Limits of Detection (LOD) and Limit of Quantification (LOD):

The LOD and LOQ were calculated by the method based on standard deviation (σ) and the slope (S) of the calibration plot, using the formula LOD=3:1 σ/S and LOQ= 10:1 σ/s (ICH, 1996, 2005 and FDA, 1994); where, σ=standard deviation of the response from a number of blank run and S=slope of calibration plot.

Accuracy

Accuracy of the method was determined by percentage recovery of marker in the plant extract and fractions. The method was studied by performing standard addition technique and it is expressed in terms of percentage relative standard deviations (%RSD) from mean recovery of the theoretical concentrations. Prior to injection, the tests were spiked with two different known amounts of standard compounds in doublets. Analyses were done under the sent ambient condition to calculate the overall

average recovery. The mean amounts of the markers achieved were taken as real values to calculate the spike recoveries.

Precision*:*

The precision of the method was assessed by injecting four replicates at two different concentrations for the reference compound, the extract and fractions. Values were represented as % RSD of intraday and inter-day runs. The mean amount and RSD values were calculated. The intra-day precision of the assay was determined by analyzing two concentrations in one day. Also, the intra-day precision was resolute over two successive days by analyzing the same concentrations. Injections were done in four replicates to determine the repeatability of the process.

Robustness

The robustness of the proposed method was investigated by an analysis of samples under different experimental conditions. The test solutions were analyzed with variation of flow rate, mobile phase composition, detection wave length and column temperature and using different columns of same configuration to determine their influence effect on the retention time.

Statistical Analysis

Statistical analysis was performed using the graph pad prism Version 5.0. The results were represented as the mean ± SD.

Results and Discussion

The R_f value of ascorbic was found to be 0.38. The percentage content of ascorbic acid in *Carica papaya* extract was found to be as mentioned 1.28% w/w. This was determined by a calibration curve with the equation of $Y = 745.6 + 4.985X$ (correlation coefficient = 0.99261 and standard deviation = ± 0.9%) as shown in fig. where X represents amount of ascorbic acid and Y represents area under the curve.

4.1.5: RP-HPLC standardization of *Carica papaya* extract

4.1.5. 1: Equipments and reagents

4.1.5.1.Chemicals and reagents

Solvents used for chromatography were HPLC grade, viz., Methanol:Water = 85:15 (v/v). Membrane filters 0.45 µm pore size from Millipore and Whatman NYL 0.45 µm syringe filter was used for the filtration of samples. Ascorbic acidstandard marker compound (Sigma Aldrich, USA),

4.1.5. 2: HPLC instrument and chromatographic conditions

HPLC system consisted of a Shimadzu (Japan) 600 quaternary HPLC pump, a Rheodyne-7725i injection valve (Japan) with a sample loop of 20 µL, a Shimadzu PDA detector and the max-plot containing the peaks were obtained using LC solutions software. A Waters Spherisorb (Ireland) C18 column (250 × 4.6 mm, 5 µm particle size) was used as stationary phase. Mobile phase composition was optimized as (channel A) methanol: (channel B) water acidified with 1% glacial acetic acid (60:40 v/v) by means of isocratic elution (1 mL/min) and detection at 280 nm. Analysis of the carica papaya, methanol extracts and fractions were performed using the above protocol and the chromatograms were recorded. Peaks were identified by comparison of retention times and peak area (response) of standard compounds and extract.

4.1.5.3: Standard and sample solution

A primary stock solution (1 mg/mL) containing Ascorbic acid was prepared by dissolved in methanol and subsequently diluted to 10-500 µg/mL and was used for mobile phase optimization. Methanol extract of *Carica papaya* was re-dissolved (1 mg/mL) using mobile phase and was filtered through 0.45 µm syringe filter prior to injection into the HPLC column. Amount of Ascorbic acid present in the methanol extract was calculated after triplicate injection (n=3) by comparing and interpolating the extract peak area (response) with respect to the standard marker.

4.1.5.4: Calibration curve (linearity)

The linear calibration plot of *Carica papaya* was constructed by means of linear regression analysis between peak areas and concentrations in the range of 10-500 µg/mL of standard solution. The amount of standard compound in the solution was obtained by constructing the calibration curve of standard.

4.1.5. 5: Specificity

Chromatograms of *Carica papaya* and ascorbic acid were verified in terms of specificity; according to the ICH recommendation to minimize the errors possibly happens during the experiment or the contamination of the sample with impurity. The purity of the peaks was checked by multivariate analysis by comparison of retention times and peak area of standard compounds and extract.

4.1.5. 6: Limits of detection and quantification (LOD and LOQ)

The LOD and LOQ were calculated by the method based on the standard deviation (σ) and the slope (S) of the calibration plot, using the formula LOD = 3:1σ/S and LOQ = 10:1σ/S (ICH, 2005 and FDA, 1994); where, σ = standard deviation of the response from a number of blank run and S = slope of the calibration plot.

4.1.5.7: Robustness

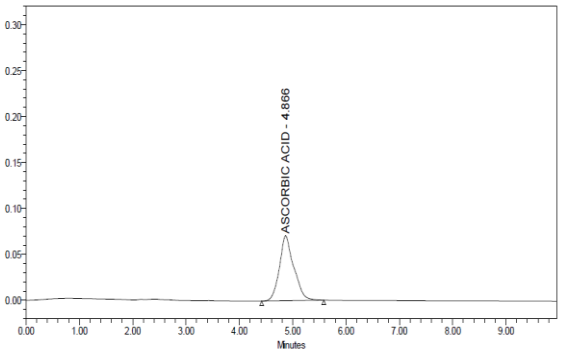
The robustness of the proposed method was investigated by analyzing the samples under different experimental conditions such as variation of flow rate, mobile phase composition, detection wavelength, column temperature and using different column of same configuration to determine their influence on the retention time.

4.1..5.8: Results

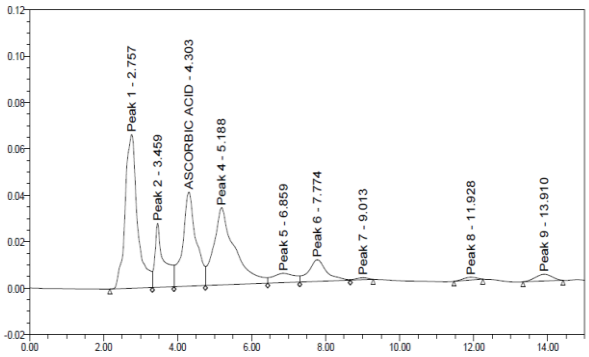
The retention time (R_t) of *Carica papaya* was found to be 4.3min. The calibration curves were obtained by plotting peak areas against concentrations. Standard compound exhibited good linearity in the concentration range of 10 - 100 µg/mL. The correlation coefficient (r^2) was estimated to be 0.997. Instrumental precision was noted account by repetitive injection of the same sample of three concentrations.

The relative standard deviation (R.S.D.) was estimated to assess instrumental precision. Intra-assay precision was performed by making five standard solutions of three different concentrations and measuring the R.S.D. values. The R.S.D. values of both instrumental precision and intra-assay precision of *Carica papaya* were estimated to be <2.5%, indicating the RP-HPLC analytical method for these two standard compounds were precise. Intermediate precision of the method was optimized by calculating intra-day and inter-day repeatability. The R.S.D. values in all tested groups were found to be <3%, which is acceptable. The sensitivity of RP-HPLC method was determined by limit of detection (LOD) and limit of quantification (LOQ). LOD is the lowest concentration of the sample, the method can detect and LOQ is the lowest concentration that can be quantified accurately by the method. The LOD and LOQ of standard compound was estimated to be 0.3 and 0.8 µg/mL for *Carica papaya* resulted that the method was sensitive to detect the compounds in sample.

The recovery measures the closeness between the theoretically added amount and the practically obtained amount and was performed by spiking with a known amount of standard compounds in sample. The recovery of *Carica papaya* at different concentrations was found to be 90 to 105% with R.S.D. values <2%. It indicates that standard compound can be fully recovered in the crude extract. The amount of *Carica papaya* present in extract was estimated to be about 1.3% (w/w), respectively. The percentage amounts of standard constituents present in the crude extract were expressed by its weight (µg/mg).



4.1: d: HPLC Chromatogram of Ascorbic acid



4.1.e: HPLC Chromatogram of *Carica papaya*

4.2.1: Literature review of *Cucumis sativus:*

Botanical classification:

Taxonomy:

Botanical name: *Cucumis sativus*

Family: *Cucurbitaceae* (Pumpkin family)

Kingdom: Plantae

Sub Kingdom: Tracheobionta

Class: Magnoliopsida

Subclass: Dilleniidae

Superdivision: Spermatophyta

Phyllum: Steptophyta

Order: Brassicales

Vernacular name:

Common name: Cucumber

- Hindi: Khira
- Manipuri: Thabi
- Marathi: Kankri
- Tamil: Vellarikkay
- Malayalam: Vellari
- Telugu: Dosakaya
- Kannada: Soutekayi
- Sanskrit: Trapushpa, Kantakilata

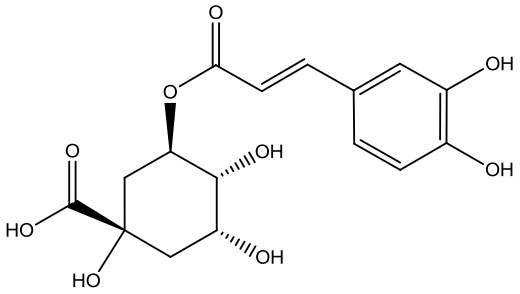
Plant description:

Cucumbers (*Cucumis sativus*) are botanically categorized as berries, which are available in many different sizes shapes and colors. They range from thick, stubby little fruits (10 - 12 cm long) to Dutch greenhouse varieties (of up to 50 cm long). The most popular variety is the long smooth salad cucumber which has a smooth, dark-green skin. Its little brother, the "gherkin" is actually a cucumber that has been harvested when little and pickled in brine (Grosch et al., 1991)

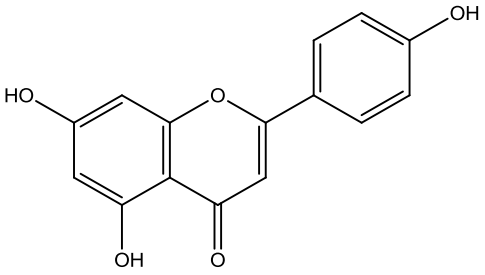
Phytochemistry:

Cucumber fruit is composed mostly of water,more than 96% of edible unpeeled fruit is water. Other constituents of *Cucumis sativus* are vitamins, minerals, amino acids, pectins, stars, sugar, vitamin c, cucurbitacin are found in cucumbers.According to another source, traces of essential oil, amino acids, pectins, starch, sugars, vitamin C, and curcurbitacin are found in cucumbers.5 Glycosides, steroids, flavonoids, carbohydrates, terpenoids, and tannins were identified in an aqueous extract of the cucumber fruit (Osman et al.,1994).The presence of cucurbitacins (triterpenoid substances-well-known for their

bitterness and toxicity), is the characteristic property of the family Cucurbitaceae. Besides apigenin (7) and chlorogenic acid (8) are also present.



Chlorogenic acid (7)



Apigenin (8)

Pharmacology

Cucurbitacins present in C. sativus exhibited cytotoxicity and anti-cancer activity. Besides this, cucurbitacins also exhibited wide ranges of in-vitro or even in-vivo pharmacological effects and in used as purgative, anti-inflammatory and anti-fertility agent. The seeds are useful for quitting burning

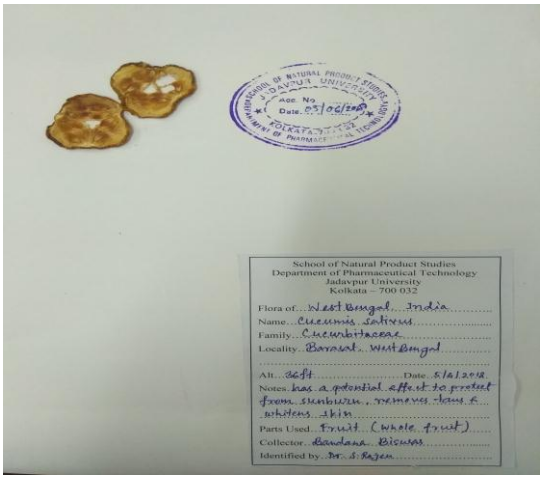
sensation,constipation, tonic and intermittent fevers . Gill et al.,(2009) suggested that the methanolic extract of *C. sativus* seeds possessed significant ulcer potential which could be due to the antioxidant activity at a dose of 300 mg/kg in pyloric ligationand water immersion stress induced rat models . The induction effect of UV-B on the antioxidant enzymes such as superoxide dismutase (SOD), guaiacol peroxidase (POD),

ascorbic acid peroxidase (APX) and glutathione reductase(GR) and its suppressed modulation to detoxify excess ROS via external application of α-tocopherol and benzoquinone incucumber cotyledons have also been examined. Boiled leaves with cumin seeds are useful for throat infection.Powdered leaf with sugar acts as a diuretic and it is also given to treat remitted and inflammatory fevers (Mukherjee et al., 2013).

4.2.2: Collection, authentication and extraction of *Cucumis sativus*

4.2.2.1: Collection and authentication

The fruits of *Cucumis sativus* were purchased from local vendor present in Jadavpur Kolkata. The voucher specimens(specimen no. SNPS/JU/2016/1100) was deposited at School of Natural Product Studies, Jadavpur University, Kolkata, India for future reference.



4.2.a:Picture and herbarium specimen of *Cucumus sativus*

4.2.2.2: Extraction of Cucumis sativus

4.2.2.2.1: Preparation of aquous extract of Cucumis sativus:

900gm Cucumis fruits were thoroughly washed under running water. Then it was smashed with grinder and filtered with muslin cloth. The material was filtered again using a Whatman No. – 45 filter paper. The liquid extract obtained was concentrated on a water bath.The concentrated extract was further freeze dried under pressure in a lyophilizer. (INST IND, Kolkata, India) at -36 ºC for 1 hr. Then it was transferred to a glass screw cap container, labeled and stored in a desiccator for future use.

Yield: 15% of freeze dried extract was obtained.

4.2.3: Phytochemical Screening of *Cucumis sativus*

The methodology is described in section 4.1.

Table 4.2.A. Results of Phytochemical Screening of Cucumis sativus above mentioned tests; (+ present; - : absent)

Carbohydrate	Phytosterol	Alkaloids	Terpenoids	Flavonoids	Tannins &Phenolics	Saponins	Volatile Oils
+	+	+	+	+	+	+	-

4.2.4: HPTLC standardization of *Cucumis sativus:*

4.2.4.1: Equipments and reagents

The CAMAG HPTLC system consisting of WINCATS software, LINOMAT V automatic sample applicator, and automatic development chamber, scanning densitometer CAMAG scanner 3 and photo documentation apparatus CAMAG reprostar 3 were used. All the solvents were used of analytical grade.100 µl syringe (HAMILTON, Switzerland) was used for sample application on HPTLC plates. Stationary phase used was aluminium based silica gel plate 60 F254 (Merck, Mumbai) with 20 cm x 10 cm in a particle size of 5-10 µm. chloroform ,glacial acetic acid,methanol of analytical grade were purchased from Merck (Mumbai, India). Whatman's syringe filter (NYL 0.45 µ) was used for the filtration of samples and standard.

4.2.4.2: Chromatographic conditions

HPTLC analysis of *Cucumis sativus* was performed with biomarker chlorogenic acid using isocratic technique by external methods. Mobile phase was optimized with Chloroform: Glacial acetic acid: methanol: water=6.4:3.2:1:2:0.8 (v/v/v/v). The temperature was kept at 25°c and mobile phase was developed in a twin trough glass chamber. Standard stock solution was applied consequently in the range of 2-10 µl with 2 µl gradual increment. All total 25 tracks in HPTLC plates were used for standardization including standard and sample solution respectively in a band wise fashion. After development, plates were dried by hand dryer. The coloured bands were observed at 254nm.

4.2.4.3: Preparation of Standard/Sample Solutions

4.2.4.3.1: Standard Solution

Chlorogenic acid 1.0 mg/ml

About 1 mg of Chlorogenic acid standard was weighed and put in to 2 mL eppendorf tube. The standard was dissolved in methanol and volume was made upto 1 ml with methanol. It was then mixed in vortex mixture and put to ultrasonication bath till the material completely dissolved. It was then filtered through 0.45µ syringe filter and kept for further study.

4.2.4.3.2: Preparation of sample solution

About 10 mg of cucumis extract of each variety was dissolved in 1 ml methanol in two different eppendorf tubes. Then the extract was dissolved in methanol and subjected to ultrasonication till the extracts were completely dissolved. It was then filtered through 0.45µ syringe filter and kept for further study.

4.2.4.4: Optimization of Mobile Phase

The mobile phase was optimized for the HPTLC analysis. Out of the many mobile phases had been tried and following mobile phase gave the better separation thus optimized mobile phase was Chloroform: Glacial acetic acid: methanol: water =6. 4:3.2:1:2:0.8 (v/v/v/v)

4.2.4.5: Application of standard and extracts

The external standard calibration curve for chlorogenic acid was prepared with calibration solutions in a concentration range of 200 to 1000 ng/ml. Then 50 µl of standard solution was drawn into CAMAG LINOMAT syringe and put to linomat applicator to give the concentrations of standard required. The same method applied for the extract.

4.2.4.6: Development

The plate was then dried and developed in a CAMAG twin trough glass chamber with the mobile phase. After development, the plate was dried and scanned in camag TLC scanner 3 at a wavelength of 254 nm

Software used : WINCATS

Analysis mode : Peak area versus concentration of standard and extracts

Calculation : By using following equation by WINCATS.

$$Y = m X + C$$

Where,

X = Concentration of Analyte/ metabolite in ng/ml

Y = Peak area

m = Slope of calibration Curve

C = Intercept.

4.2.4.7: Method validation for HPTLC study

Validation of the HPTLC method was done as recommended by the International Conference on Harmonisation (ICH) guidelines (ICH, 1996, 2005) and FDA (1994) Guidelines defining the Linearity, Specificity, Limits of Quantification and detection, precision, accuracy and robustness.

4.2.4.7.1: Specificity

The results for HPTLC standardization profiles were checked in terms of specificity according to the ICH guidelines to minimize errors due to the contamination of the sample. The specificity of the method was determined by analyzing the standard and test samples. The purity of peaks was checked using multivariate analysis by comparison of retention times and peak area of standard compound with extract and fractions.

4.2.4.7.2: Limits of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ were calculated by the method based on standard deviation (σ) and the slope (S) of the calibration plot, using the formula LOD=3:1 σ/S and LOQ= 10:1 σ/s (ICH, 1996, 2005 and FDA, 1994); where, σ =standard deviation of the response from a number of blank run and S = slope of calibration plot.

4.2.4.7.3: Accuracy

Accuracy of the method was determined by percentage recovery of marker in the plant extract and fractions. The method was studied by performing standard addition technique and it is expressed in terms of percentage relative standard deviations (%RSD) from mean recovery of the theoretical concentrations. Prior to injection, the tests were spiked with two different known amounts of standard compounds in doublets. Analyses were done under the sent ambient condition to calculate the overall average recovery. The mean amounts of the markers achieved were taken as real values to calculate the spike recoveries.

4.2.4.7.4: Precision

The precision of the method was assessed by injecting four replicates at two different concentrations for the reference compound, the extract and fractions. Values were represented as % RSD of intraday and inter-day runs. The mean amount and RSD values were calculated. The intra-day precision of the assay was determined by analyzing two concentrations in one day. Also, the intra-day precision was resolute over two successive days by analyzing the same concentrations. Injections were done in four replicates to determine the repeatability of the process.

4.2.4.7.5: Robustness

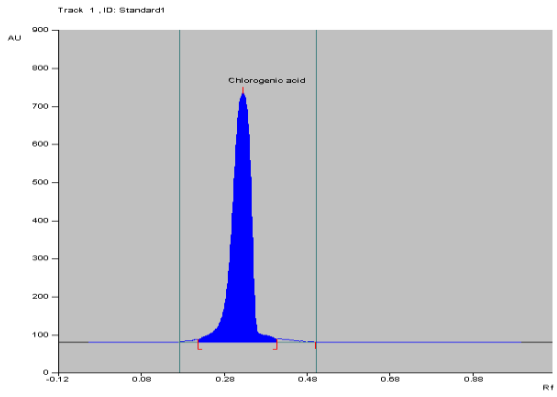
The robustness of the proposed method was investigated by an analysis of samples under different experimental conditions. The test solutions were analyzed with variation of flow rate, mobile phase composition, detection wave length and column temperature and using different columns of same configuration to determine their influence effect on the retention time.

4.2.4.8 Statistical Analysis

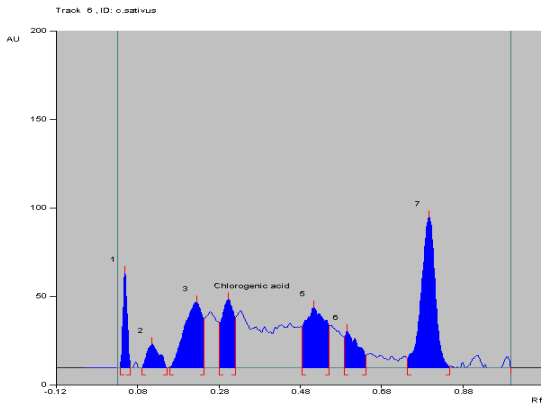
Statistical analysis was performed using the graph pad prism Version 5.0. The results were represented as the mean ± SD.

4.2.4.9. Results and Discussion

The $R_f$value of chlorogenic acid was found to be 0.61. The percentage content of chlorogenic acid in *Cucumis sativus aquous* extract was found to be as mentioned 3.21 %. This was determined by a calibration curve with the equation of Y = Y = 745.6 + 4.985X (correlation coefficient = 0.95405 and standard deviation = ± 10.9%) as shown in fig. where X represents amount of chlorogenic acid and Y represents area under the curve.



4.2.b: HPTLC Chromatogram of chlorogenic acid



4.2.c:HPTLC chromatogram of *Cucumis sativus*

4.2.5: RP-HPLC standardization of *Cucumis sativus* extract

4.2.5.1: Chemicals and reagents

Solvents used for chromatography were HPLC grade, viz., Methanol: Water (1% acetic acid) = 60:40 (v/v). Membrane filters 0.45 µm pore size from Millipore and Whatman NYL 0.45 µm syringe filter was used for the filtration of samples. Chlorogenic acid standard marker compound (Sigma Aldrich, USA),

4.2.5.2: HPLC instrument and chromatographic conditions

HPLC system consisted of a Shimadzu (Japan) 600 quaternary HPLC pump, a Rheodyne-7725i injection valve (Japan) with a sample loop of 20 µL, a Shimadzu PDA detector and the max-plot containing the peaks were obtained using LC solutions software. A Waters Spherisorb (Ireland) C18 column (250 × 4.6 mm, 5 µm particle size) was used as stationary phase. Mobile phase composition was optimized as (channel A) methanol: (channel B) water acidified with 1% glacial acetic acid (60:40 v/v) by means of isocratic elution (1 mL/min) and detection at 280 nm. Analysis of the trigonelline, methanol extracts and fractions were performed using the above protocol and the chromatograms were recorded. Peaks were identified by comparison of retention times and peak area (response) of standard compounds and extract.

4.2.5.3: Standard and sample solution

A primary stock solution (1 mg/mL) containing Chlorogenic acid was prepared by dissolved in methanol and subsequently diluted to 10-500 µg/mL and was used for mobile phase optimization. aquous extract of *cucumis sativus* was re-dissolved (1 mg/mL) using mobile phase and was filtered

through 0.45 µm syringe filter prior to injection into the HPLC column. Amount of chlorogenic acid present in the aquous extract was calculated after triplicate injection (n=3) by comparing and interpolating the extract peak area (response) with respect to the standard marker.

4.2.5.4: Calibration curve (linearity)

The linear calibration plot of chlorogenic acid was constructed by means of linear regression analysis between peak areas and concentrations in the range of 10-500 µg/mL of standard solution. The amount of standard compound in the solution was obtained by constructing the calibration curve of standard.

4.2.5. 5: Specificity

Chromatograms of chlorogenic acid and aquous extract were verified in terms of specificity; according to the ICH recommendation to minimize the errors possibly happens during the experiment or the contamination of the sample with impurity. The purity of the peaks was checked by multivariate analysis by comparison of retention times and peak area of standard compounds and extract.

4.2.5. 6: Limits of detection and quantification (LOD and LOQ)

The LOD and LOQ were calculated by the method based on the standard deviation (σ) and the slope (S) of the calibration plot, using the formula LOD = 3:1σ/S and LOQ = 10:1σ/S (ICH, 2005 and FDA, 1994); where, σ = standard deviation of the response from a number of blank run and S = slope of the calibration plot.

4.2.5.7: Robustness

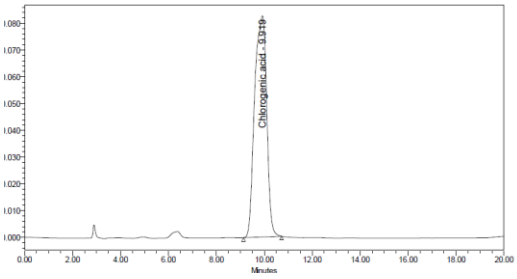
The robustness of the proposed method was investigated by analyzing the samples under different experimental conditions such as variation of flow rate, mobile phase composition, detection wavelength, column temperature and using different column of same configuration to determine their influence on the retention time.

4.2.5.8: Results

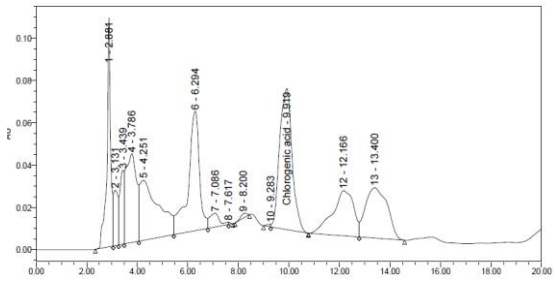
The retention time (R_t) of chlorogenic acid was found to be 9.93 min. The calibration curves were obtained by plotting peak areas against concentrations. Standard compound exhibited good linearity in the concentration range of 10 - 100 µg/mL. The correlation coefficient (r^2) was estimated to be 0.993. Instrumental precision was noted account by repetitive injection of the same sample of three concentrations.

The relative standard deviation (R.S.D.) was estimated to assess instrumental precision. Intra-assay precision was performed by making five standard solutions of three different concentrations and measuring the R.S.D. values. The R.S.D. values of both instrumental precision and intra-assay precision of chorogenic acid were estimated to be <2.5%, indicating the RP-HPLC analytical method for these two standard compounds were precise. Intermediate precision of the method was optimized by calculating intra-day and inter-day repeatability. The R.S.D. values in all tested groups were found to be <3%, which is acceptable. The sensitivity of RP-HPLC method was determined by limit of detection (LOD) and limit of quantification (LOQ). LOD is the lowest concentration of the sample, the method can detect and LOQ is the lowest concentration that can be quantified accurately by the method. The LOD and LOQ of standard compound was estimated to be 0.3 and 0.8 µg/mL for chlorogenic acid resulted that the method was sensitive to detect the compounds in sample.

The recovery measures the closeness between the theoretically added amount and the practically obtained amount and was performed by spiking with a known amount of standard compounds in sample. The recovery of chlorogenic acid at different concentrations was found to be 90 to 105% with R.S.D. values <2%. It indicates that standard compound can be fully recovered in the crude extract. The amount of chlorogenic acid present in extract was estimated to be about 4.41% (w/w), respectively. The percentage amounts of standard constituents present in the crude extract were expressed by its weight (µg/mg).



4.2.d: HPLC Chromatogram of Chlorogenic acid



4.2.e: **: HPLC Chromatogram of *Cucumis sativus***

4.3.1: Literature review of *Hibiscus rosa sinensis*

Botanical identity

Taxonomical description:

Botanical name: *Hibiscus rosasinensis*

Family: Malvaceae (mallow family)

Kingdom: Plantae

Order: Malvaceae

Vernacular name:

Common name: China Rose, Chinese hibiscus

• Hindi: Gurhal

• Manipuri: Juba kusum athonba

• Kannada: Dasavala

• Malayalam: Chemparati

•Tamil: cembarutti

• Marathi: Jaswand

• Konkani: Dosni Phool

Plant description:

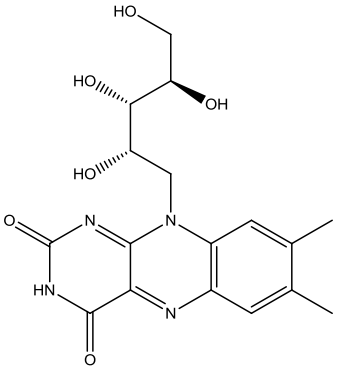
1. Large shrub or small tree grows to 15 feet high

2. Toothed leaves are arranged alternately dark green, and shiny

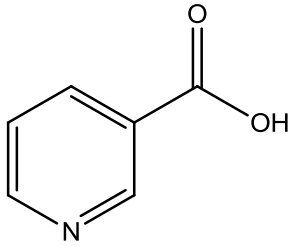
3. Flowers are glorious and huge at their best.

Phytochemistry:

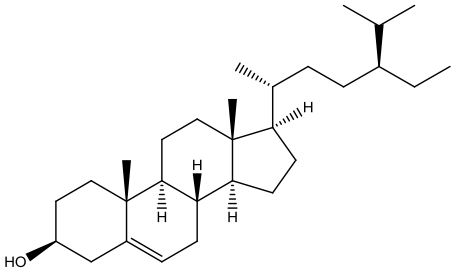
Flowers contain cyaniding diglucoside, flavonoids and vitamins, thiamine, riboflavin (9), niacin (10) and ascorbic acid (Ghani, 2003). Quercetin-3-diglucoside, 3,7-diglucoside, cyanidin (11),β –sitosterol (12), quercetin (13) and anthocyanin (14) have been isolated from deep yellow flowers all above compounds and kaempferol-3-xylosylglucoside have been isolated from ovary white flowers (Nakamura,1990).



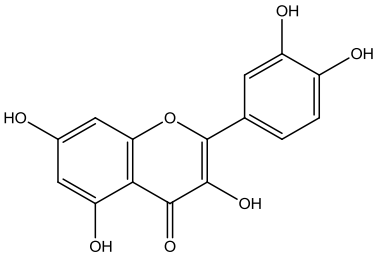
Riboflavin (9)



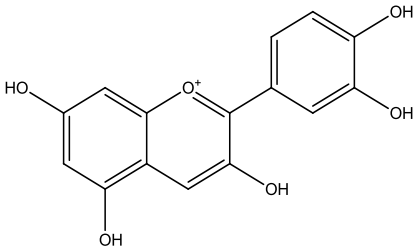
Niacin (10)



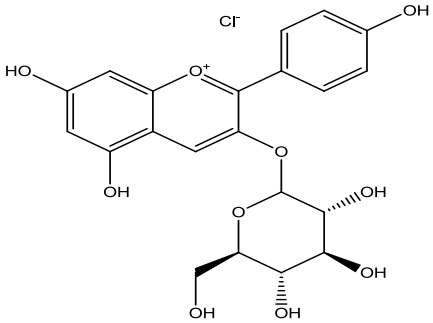
β –sitosterol (11)



Quercetin (12)



Cyanidin (13)



Anthocyanin (14)

Pharmacological uses

Abortifacient effect, Acid phosphatase stimulation, Alkaline phosphatase inhibition, Analgesic activity, Androgenic effect, Anticonvulsive activity, Anti-FSH activity, Antiestrogenic effect, Antifertility effect, Antifungal activity, Antigonadotropin effect, Antihypertensive activity, Antiimplantation effect, Anti-inflammatory activity, Antiovulatory effect, Antipyretic activity, Antispasmodic activity,

Antispermatogenic effect, Antiviral activity, Barbiturate potentiation, Beta-glucuronidase inhibition, Beta-glucuronidase stimulation, CNS depressant activity, contragestative agent, Embryo toxic effect., Estrogenic effect, Estrous cycle disruption effect, Gonadotropin synthesis inhibition, Hypoglycemic effect, Hypotensive activity, Hypothermic activity, Inotropic effect positive. Juvenile hormone activity, Plant germination inhibition, Lactate-dehydrogenase-X inhibition, Luteotropic effect, Menstruation induction effect, Radical scavenging effect, teratogenic effect, toxicity assessment (quantitative)(Vidyapati,1979)

4.3.2: Collection, authentication and extraction of *Hibiscus rosasinensis*

The flower of *Hibiscus rosa sinensis* were purchased from local vendor Jadavpur,Kolkata and authenticated. The voucher specimens (specimen no. SNPS/JU/2018/1101) was deposited at School of Natural Product Studies, Jadavpur University, Kolkata, India for future references.

4.3.a: Picture and herbarium specimen *Hibiscus rosa sinensis*

Extraction of *Hibiscus Rosa Sinensis*:

Preparation of hydroalcoholic solution: 1kg of fresh hibiscus flower petals were shade dried. Then it was cold macerated using 80:20 in ration ethanol: water mixture for 48 hrs with occasional shaking. The material was filtered by using a Whatman no-45 filter paper. The liquid extract obtained was concentrated under pressure in rotary evaporator,IKA-HB10 (IKA India Private Limited, India),for 40 minutes at 44^0C,700mm Hg(pressure) and 30 revolution per minute. The concentrated extract was further freeze dried under pressure in a lyophiliser (INST IND, Kolkata, India) at -36^0C for 1 hr. Then it was transferred to a glass screw cap container, labelled and stored in a desiccator for future use. Yield: 15.2 % (w/w) of freeze died hibiscus was obtained.

4.3.3.Qualitative evaluation: Results of Phytochemical screening of *Hibiscus Rosa Sinensis*

Methodology is described in section 4.1

Alkaloids	Alkaloids	Triterpenoids	Coumarins	Steroids	Tannins	Saponins	Flavones	Glycosides
+	-	+	+	+	+	-	+	-

4.3.4: HPTLC Standardization of *Hibiscus Rosa Sinensis*

4.3.4.1. Equipments and reagents: The CAMAG HPTLC system consisting of WINCATS software, LINOMAT V automatic sample applicator ,and automatic development chamber,scanning densitometer CAMAG scanner 3and photo documentation apparatus CAMAG reproeter 3 were used. All the solvents were used of analytical grade. 100µl syringe (HAMILTON, Switzerland) was used for sample application on HPTLC plates. Stationary phase used was aluminium based silica gel plate 60 F254 (Merck,Mumbai) with 20cm×10cm in a particle size of 5-10 µm. Toluene and ethyle acate ,of analytical grade were purchased from Merck(Mumbai,India). Whatman's syringe filter (NYL 0.45µ) was used for the filtration of the sample and standered.

4.3.4.2. Chromatoghrapic condition:

HPTLC analysis of *Hibiscus rosa sinensis* was performed with biomarker quercetin -3 using isocratic technique by external methods. Mobile phase was optimised with Ehyl acetate: toluene: methanol: glacial acetic acid=7.5:2:0.5:0.2 (v/v/v/v). The temperature was kept at 25^0C and mobile phase was developed in a twin through glass chamber. Standard stock solution was applied consequently in the range of 2-10 µl with 2 µl gradual increment. All total 25 tracks in HPTLC plates were used for standardization including standard and sample solution respectively in a band wise fashion. After development, plated were dried by hand dryer. The colored bands were observed at 254 nm.

4.3.4.3: Preparation of Standard and Sample solution:

Standard solution: About 1 mg of quercetin standard was weighed and put into 2 ml ependorff tube. The standard was dissolved was methanol and volume was made uo to 1ml with methanol. It was then mixed with vortex mixture and put to ultrasonication bath till the material completely dissolved. It was then filtered through 0.45µ syringe filter and kept for further studies.

Preparation of sample solution: About 10 mg of *Hibiscus rosa sinensis* extract was dissolved in 1ml methanol in eppendorf tubes. Then the extract was dissolved in methanol and subjected to

ultrasonication till the extract was completely dissolved.Il was then filtered through 0.45 µ syringe filter and kept for further study.

Optimization of mobile phase: The mobile phase was optimized for the HPTLC analysis. Out of the many mobile phases have been tried and following mobile phase gave the better separation. Thus optimized mobile phase was Ehyl acetate: toluene: Methanol: Glacial acetic acid=7.5:2:0.5:0.2 (v/v/v/v).

Application of standard and extract: The external standard calibration curve for quercetin was prepared with calibration solutions in a concentration range of 200 to 1000ng/ml. Then 50 µl of standard solution was drawn into CAMAG LINOMAT syringe and put to linomat applicator to give the concentrations of standard required. The same method is applied for the extract.

Development: The plate was then dried and developed in a CAMAG twin through glass chamber with the mobile phase. After development, the plate was dried and scanned in Camag TLC scanner 3 at a wavelength of 254 nm.

Software used : WINCATS

Analysis mode: Peak area versus concentration of standard and extracts

Calculation: By using the following equation by WINCATS.

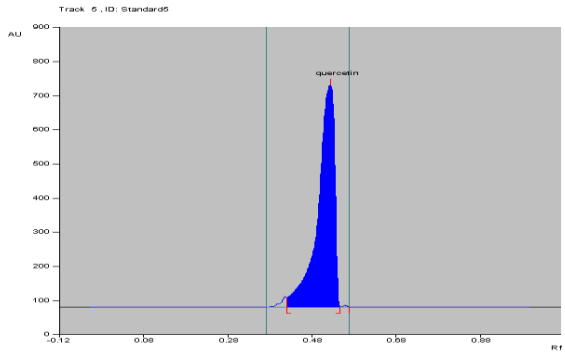
$$Y = m X + C$$

Where , X= concentrate of analyte/ metabolite in ng/ml

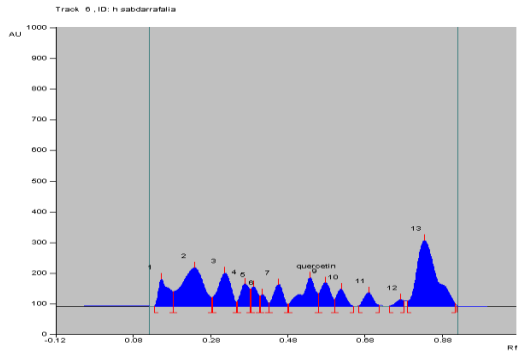
Y= Peak area

m = Slope of calibration curve

C=Intercept



4.3.b.HPTLC chomatogram of quercetin



4.3.c.HPTLC chromatogram of hibiscus rosa sinensis

4.3.4.7: Method validation for HPTLC study

Validation of the HPTLC method was done as recommended by the International Conference on Harmonisation (ICH) guidelines (ICH, 1996, 2005) and FDA (1994) Guidelines defining the Linearity, Specificity, Limits of Quantification and detection, precision, accuracy and robustness.

4.3.4.7.1: Specificity

The results for HPTLC standardization profiles were checked in terms of specificity according to the ICH guidelines to minimize errors due to the contamination of the sample. The specificity of the method was determined by analyzing the standard and test samples. The purity of peaks was checked using multivariate analysis by comparison of retention times and peak area of standard compound with extract and fractions.

4.3.4.7.2: Limits of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ were calculated by the method based on standard deviation (σ) and the slope (S) of the calibration plot, using the formula LOD=3:1 σ/S and LOQ= 10:1 σ/s (ICH, 1996, 2005 and FDA, 1994); where, σ =standard deviation of the response from a number of blank run and S = slope of calibration plot.

4.3.4.7.3: Accuracy

Accuracy of the method was determined by percentage recovery of marker in the plant extract and fractions. The method was studied by performing standard addition technique and it is expressed in terms of percentage relative standard deviations (%RSD) from mean recovery of the theoretical concentrations. Prior to injection, the tests were spiked with two different known amounts of standard compounds in doublets. Analyses were done under the sent ambient condition to calculate the overall average recovery. The mean amounts of the markers achieved were taken as real values to calculate the spike recoveries.

4.3.4.7.4: Precision

The precision of the method was assessed by injecting four replicates at two different concentrations for the reference compound, the extract and fractions. Values were represented as % RSD of intraday and inter-day runs. The mean amount and RSD values were calculated. The intra-day

precision of the assay was determined by analyzing two concentrations in one day. Also, the intra-day precision was resolute over two successive days by analyzing the same concentrations. Injections were done in four replicates to determine the repeatability of the process.

4.3.4.7.5: Robustness

The robustness of the proposed method was investigated by an analysis of samples under different experimental conditions. The test solutions were analyzed with variation of flow rate, mobile phase composition, detection wave length and column temperature and using different columns of same configuration to determine their influence effect on the retention time.

4.3.4.8 Statistical Analysis

Statistical analysis was performed using the graph pad prism Version 5.0. The results were represented as the mean ± SD.

4.3.4.9. Results and Discussion

The $R_f$value of quercetin was found to be 0.44. The percentage content of quercetin in *hibiscus* methanolic extract was found to be as mentioned 1.02 %. This was determined by a calibration curve with the equation of Y = -3788.573+26.061*X (correlation coefficient = 0.9929 and standard deviation = ± 3.08%) as shown in fig. where X represents amount of quercetine and Y represents area under the curve.

4.3.5: RP-HPLC standardization of *Hibiscus rosa sinensis* extract

4.3.5.1: Chemicals and reagents

Solvents used for chromatography were HPLC grade, viz., toluene, acetic acid and methanol at ratio of 5:3:2(v/v/v). Membrane filters 0.45 µm pore size from Millipore and Whatman NYL 0.45 µm syringe filter was used for the filtration of samples. quercetin standard marker compound (Sigma Aldrich, USA),

4.3.5.2: HPLC instrument and chromatographic conditions

A rapid, specific, reversed phase, HPLC-UV method with an isocratic elution of acetonitrile and 2% v/v acetic acid (40% : 60% v/v) (pH 2.6) at a flow rate of 1.3 mL/minutes, a column temperature of 35°C, and ultraviolet (UV) detection at 370 nm was developed. The method was validated and

applied to the quantification of different types of market available Chinese medicine extracts, pills and tablets.

4.3.5.3: Standard and sample solution

A primary stock solution (1 mg/mL) containing asiaticoside was prepared by dissolved in methanol and subsequently diluted to 10-500 µg/mL and was used for mobile phase optimization. Methanol extract of *hibiscus* was re-dissolved (1 mg/mL) using mobile phase and was filtered through 0.45 µm syringe filter prior to injection into the HPLC column. Amount of quercetin present in the methanol extract was calculated after triplicate injection (n=3) by comparing and interpolating the extract peak area (response) with respect to the standard marker.

4.3.5.4: Calibration curve (linearity)

The linear calibration plot of asiaticoside was constructed by means of linear regression analysis between peak areas and concentrations in the range of 10-500 µg/mL of standard solution. The amount of standard compound in the solution was obtained by constructing the calibration curve of standard.

4.3.5.5: Specificity

Chromatograms of quercetin and methanol extract were verified in terms of specificity; according to the ICH recommendation to minimize the errors possibly happens during the experiment or the contamination of the sample with impurity. The purity of the peaks was checked by multivariate analysis by comparison of retention times and peak area of standard compounds and extract.

4.3.5.6: Limits of detection and quantification (LOD and LOQ)

The LOD and LOQ were calculated by the method based on the standard deviation (σ) and the slope (S) of the calibration plot, using the formula LOD = 3:1σ/S and LOQ = 10:1σ/S (ICH, 2005 and FDA, 1994); where, σ = standard deviation of the response from a number of blank run and S = slope of the calibration plot.

4.3.5.7: Robustness

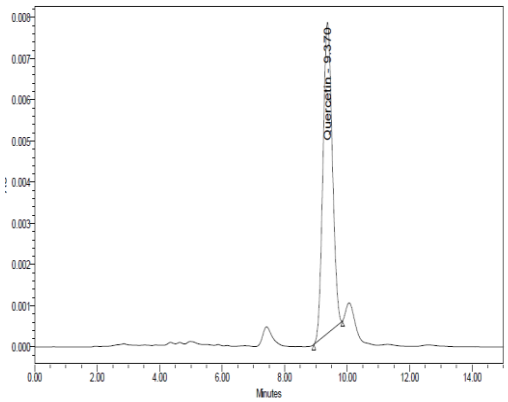
The robustness of the proposed method was investigated by analyzing the samples under different experimental conditions such as variation of flow rate, mobile phase composition, detection wavelength, column temperature and using different column of same configuration to determine their influence on the retention time.

4.3.5.8: Results

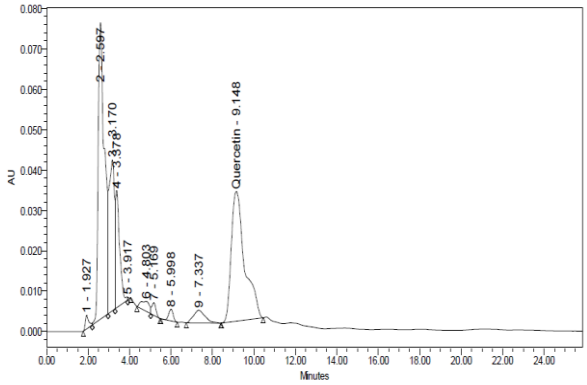
The retention time (RT) of quercetin was found to be 9.14min. The calibration curves were obtained by plotting peak areas against concentrations. Standard compound exhibited good linearity in the concentration range of 10 - 100 µg/mL. The correlation coefficient (r^2) was estimated to be 0.9929. Instrumental precision was noted account by repetitive injection of the same sample of three concentrations.

The relative standard deviation (R.S.D.) was estimated to assess instrumental precision. Intra-assay precision was performed by making five standard solutions of three different concentrations and measuring the R.S.D. values. The R.S.D. values of both instrumental precision and intra-assay precision of quercetin was estimated to be <2.5%, indicating the RP-HPLC analytical method for these two standard compounds were precise. Intermediate precision of the method was optimised by calculating intra-day and inter-day repeatability. The R.S.D. values in all tested groups were found to be <3%, which is acceptable. The sensitivity of RP-HPLC method was determined by limit of detection (LOD) and limit of quantification (LOQ). LOD is the lowest concentration of the sample, the method can detect and LOQ is the lowest concentration that can be quantified accurately by the method. The LOD and LOQ of standard compound was estimated to be 0.3 and 0.8 µg/mL for quercetin resulted that the method was sensitive to detect the compounds in sample.

The recovery measures the closeness between the theoretically added amount and the practically obtained amount and was performed by spiking with a known amount of standard compounds in sample. The recovery of quercetin at different concentrations was found to be 90 to 105% with R.S.D. values <2% (data not shown). It indicates that standard compound can be fully recovered in the crude extract. The amount of quercetin present in extract was estimated to be about 1.7% (w/w), respectively. The percentage amounts of standard constituents present in the crude extract were expressed by its weight (µg/mg).



4.3.d: : HPLC Chromatogram of Quercetin



4.3.e: : HPLC Chromatogram of *Hibiscus rosa sinensis*

4.4.1: Literature review of *Ocimum sanctum*

Taxonomy:

- Kingdom: Plantae
- Division: Magnoliophyta
- Class : Magnoliopsida
- Order : Lamiales
- Family : Lamiaceae
- Genus : Ocimum
- Species : O.sanctum
- Binomial name: *Ocimum sanctum* L.

Synonyms:

Sanskrit Surasa, Apetrakshasi

Assamese: Tulasi

Bengali : Tulasi

English : Holy Basil

Gujrati : Tulasi, Tulsi

Hindi : Tulasi

Kannada : Tulasi, Shree Tulasi

The Ocimum (Basil) comprises some of the most popular herbs in the world. It belongs to the family Lamiaceae and includes more than 150 different species and varieties distributed in the tropical regions of Asia, Africa, Central and South Africa (Sharma et al., 2011)

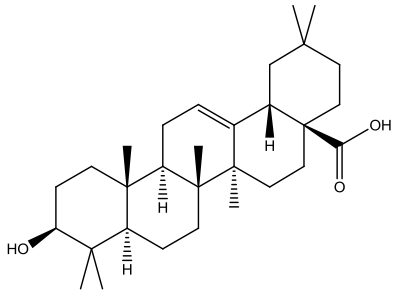
Plant Description:

a) Macroscopic

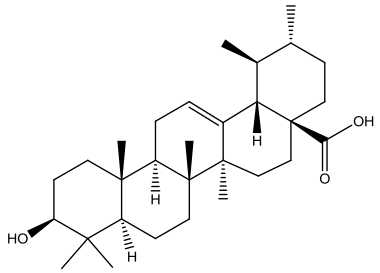
Roots are thin, wiry, branched, hairy, soft, blackish-brown externally and pale, violet internally. Stem iserect, herbaceous, woody, branched; hairy, sub quadrangular, externally purplish-brown to black, internally cream, coloured; fracture, fibrous in bark and short in xylem, odour faintly aromatic. Leaf is 2.5-5 cm long and 1.6 - 3.2 cm wide, elliptic oblong, obtuse or acute, entire or serrate, pubescent on both sides, petiole thin, about 1.5-3 cm long hairy; odour, aromatic; taste, characteristic.Flower ispurplish or crimson coloured, small in close whorls; bracts about 3 mm long and broad, pedicels longer than calyx, slender, pubescent, calyx ovoid or campanulate 3-4 mm bilipped, upper lip broadly obovate or suborbicular, shortly apiculate, lower lip longer than upper having four mucronate teeth, lateral two short and central two largest, corolla about 4 mm long, pubescent; odour, aromatic; taste, pungent.Seed is rounded to oval; brown,mucilaginous when soaked in water, 0.1 cm long, slightly notched at the base; no odour; taste, pungent, slightly mucilaginous (Sethi et al.,2003)

Phytochemistry:

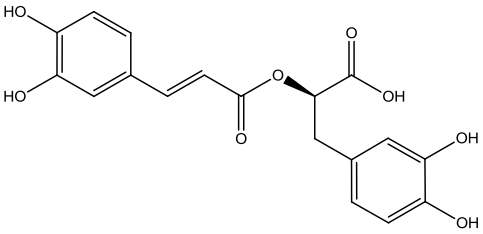
Some of the phytochemical constituents of tulsi are oleanolic acid (15), ursolic acid (16), rosmarinic acid (17), eugenol (18), carvacrol (19), linalool (20), β-caryophyllene (21) (about 8%).Tulsi essential oil consists mostly of eugenol (~70%) β-elemene (22) (~11.0%), β-caryophyllene (~8%) and germacrene (~2%), with the balance being made up of various trace compounds, mostly terpenes(Gupta et al.,2006)



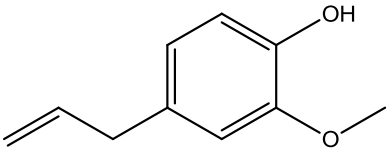
Oleanolic acid (15)



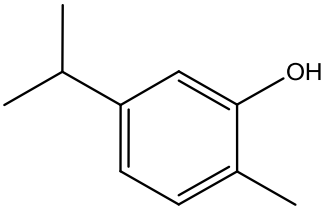
Ursolic acid (16)



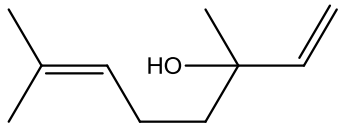
Rosmarinic acid (17)



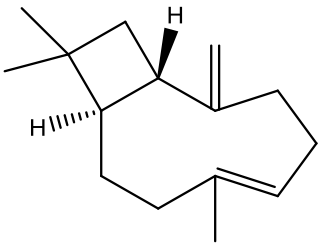
Eugenol (18)



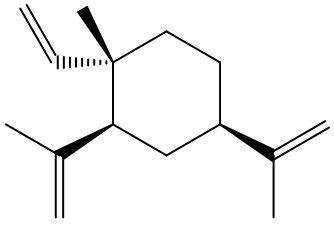
Carvacrol (19)



Linalool (20)



β-caryophyllene (21)



B-elemene (22)

***Pharmacology**:*

Health Benefits of Basil (Tulsi)

The health benefits of holy basil or tulsi include oral care, relief from respiratory disorders, fever,asthma, lung disorders, heart diseases and stress.Holy Basil (scientific name is Ocimum Sanctum) or Tulsi is undoubtedly the best medicinal herb ever known. It has endless miraculous and medicinal values and is being worshipped in India since thousands of years. Even going closer to a Tulsi plant alone can protect you from many infections.

A few leaves dropped in drinking water or foodstuff can purify it and can kill germs in it. Even

smelling it or keeping it planted in a pot indoors can protect the whole family from infections, cough and cold and other viral infections. Holy Basil is so good for boosting up the immune system that cannot be explained in words. It protects from nearly all sorts of infections from viruses, bacteria, fungi and protozoa. Recent studies show that it is also helpful in inhibiting growth HIV and carcinogenic cells.(Talwar et al,2003)

1. Healing Power

The tulsi plant has many medicinal properties.The leaves are a nerve tonic and also sharpen.memory. They promote the removal of the catarrhal matter and phlegm from the bronchial tube. The leaves strengthen the stomach and induce copious perspiration. The seed of the plant are mucilaginous.

2. Fever & Common Cold

The leaves of basil are specific for many fevers. During the rainy season, when malariaand dengue fever are widely prevalent, tender leaves, boiled with tea, act as preventiveagainst these diseases. In case of acute fevers, a decoction of the leaves boiled with powdered cardamom in half a litre of water and mixed with sugar and milk brings down thetemperature. The juice of tulsi leaves can be used to bring down fever. Extract of tulsi leaves in fresh water should be given every 2 to 3 hours. In between one can keep giving sips of cold water. In children, it is every effective in bringing down the temperature.

3. Coughs

Tulsi is an important constituent of many Ayurvedic cough syrups and expectorants. Ithelps to mobilize mucus in bronchitis and asthma. Chewing tulsi leaves relieves cold and flu.

4. Sore Throat

Water boiled with basil leaves can be taken as drink in case of sore throat. This water can also be used as a gargle.

5. Respiratory Disorder

The herb is useful in the treatment of respiratory system disorder. A decoction of the leaves, with honey and ginger is an effective remedy for bronchitis, asthma, influenza, cough and cold. A decoction of the leaves, cloves and common salt also gives immediate relief in case of influenza. They should be boiled in half a litre of water till only half the water is left and add then taken.

4.4.2: Collection, authentication and extraction of *Ocimum sanctum* leaves

4.4.2.1: Collection and authentication

The fresh leaves of *Ocimum sanctum* were purchased from local vendor present in Jadavpur supermarket near Jadavpur University, Kolkata Voucher specimens (specimen no. SNPS/JU/2016/1475) was deposited at School of Natural Product Studies, Jadavpur University, and Kolkata, India for future references.

4.4.a: Picture and herbarium specimen of *Ocimum sanctum*

4.4.2.2: Extraction of Ocimum sanctum leaves:

4.4.2.2.1: Preparation of hydro-alcoholic extract of Ocimum sanctum

100gm fresh plant materials were thoroughly washed under running water and shade dried. The material was cold macerated using a hydro alcoholic mixture of methanol and water (70:30) for 4 days with occasional stirring. The material was filtered using a Whatman No. – 45 filter paper. The liquid extract obtained was concentrated under pressure in rotary evaporator, IKA – HB 10 (IKA® India Private Limited, India), for 40 mins at 44ºC, 700mm Hg (pressure) and 30 revolutions per minute. The concentrated extract was further freeze dried under pressure in a lyophilliser (INST IND, Kolkata, India) at -36 ºC for 1 hr. Then it was transferred to a glass screw cap container, labeled and stored in a desiccator for future use.

Yield: 16% of freeze dried product was obtained.

4.4.3: Phytochemical Screening of *Oscimum sanctum* Hydro-alcoholic extract

Table 7.A. Results of Phytochemical Screening of Oscimum sanctum By different above mentioned tests; (+ : present; - : absent)

The methodology is described in section 4.1

Alkaloid	Tannin	Saponin	Steroid	Flvatannin	Terpenoid	Flavonoid	Cardiac glucoside
+	+	+	+	-	+	+	+

4.4.4: HPTLC standardization of *Osimum sanctum*

4.4.4.1: Equipments and reagents

The CAMAG HPTLC system consisting of WINCATS software, LINOMAT V automatic sample applicator, and automatic development chamber, scanning densitometer CAMAG scanner 3 and photo documentation apparatus CAMAG reprostar 3 were used. All the solvents were used of analytical grade.100 µl syringe (HAMILTON, Switzerland) was used for sample application on HPTLC plates. Stationary phase used was aluminium based silica gel plate 60 F254 (Merck, Mumbai) with 20 cm x 10 cm in a particle size of 5-10 µm. Tolune and ethyl acetate, of analytical grade were purchased from Merck (Mumbai, India). Whatman's syringe filter (NYL 0.45 µ) was used for the filtration of samples and standard.

4.4.4.2: Chromatographic conditions

HPTLC analysis of *Ocimum sanctum* cultivars was performed with biomarker eugenol using isocratic technique by external methods. Mobile phase was optimized with Toluene: ethyl acetate = 7:3(v/v). The temperature was kept at 25°c and mobile phase was developed in a twin trough glass chamber. Standard stock solution was applied consequently in the range of 2-10 µl with 2 µl gradual increment. All total 8 tracks in HPTLC plates were used for standardization including standard and sample solution respectively in a band wise fashion. After development, plates were dried by hand dryer. The coloured bands were observed at 254 nm.

4.4.4.3: Preparation of Standard/Sample Solutions

4.4.4.3.1: Standard Solution

About 1 ml of eugenol standard was weighed and put in to 2 mL eppendorf tube. The standard was dissolved in methanol and volume was made up to 1 ml with methanol. It was then mixed in vortex mixture and put to ultrasonication bath till the material completely dissolved. It was then filtered through 0.45 µm syringe filter and kept for further study.

4.4.4.3.2: Preparation of sample solution

About 10 mg of *Ocimum sanctum* extract of each variety was dissolved in 1 ml methanol in two different eppendorf tubes. Then the extract was dissolved in methanol and subjected to ultrasonication till the extracts were completely dissolved. It was then filtered through 0.45µ syringe filter and kept for further study.

4.4.4.4: Optimization of Mobile Phase

The mobile phase was optimized for the HPTLC analysis. Out of the many mobile phases had been tried and following mobile phase gave the better separation thus optimized mobile phase was toluene: ethyl acetate = 7:3 (v/v)

4.4.4.5: Application of standard and extracts

The external standard calibration curve for eugenol was prepared with calibration solutions in a concentration range of 200 to 1000 ng/ml. Then 50 µl of standard solution was drawn into CAMAG LINOMAT syringe and put to linomat applicator to give the concentrations of standard required. The same method applied for the extract.

4.4.4.6: Development

The plate was then dried and developed in a CAMAG twin trough glass chamber with the mobile phase. After development, the plate was dried and scanned in Camag TLC scanner 3 at a wavelength of 254 nm

Software used : WINCATS

Analysis mode : Peak area versus concentration of standard and extracts

Calculation : By using following equation by WINCATS.

$Y = m X + C$

Where,

X = Concentration of Analyte/ metabolite in ng/ml

Y = Peak area

m = Slope of calibration Curve

C = Intercept.

4.4.4.7: Method validation for HPTLC study

Validation of the HPTLC method was done as recommended by the International Conference on Harmonisation (ICH) guidelines (ICH, 1996, 2005) and FDA (1994) Guidelines defining the Linearity, Specificity, Limits of Quantification and detection, precision, accuracy and robustness.

4.4.4.7.1: Specificity

The results for HPTLC standardization profiles were checked in terms of specificity according to the ICH guidelines to minimize errors due to the contamination of the sample. The specificity of the method was determined by analysing the standard and test samples. The purity of peaks was checked using multivariate analysis by comparison of retention times and peak area of standard compound with extract and fractions.

4.4.4.7.2: Limits of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ were calculated by the method based on standard deviation (σ) and the slope (S) of the calibration plot, using the formula LOD=3:1 σ/S and LOQ= 10:1 σ/s (ICH, 1996, 2005 and FDA,

1994); where, σ =standard deviation of the response from a number of blank run and S = slope of calibration plot.

7.4.7.3: Accuracy

Accuracy of the method was determined by percentage recovery of marker in the plant extract and fractions. The method was studied by performing standard addition technique and it is expressed in terms of percentage relative standard deviations (%RSD) from mean recovery of the theoretical concentrations. Prior to injection, the tests were spiked with two different known amounts of standard compounds in doublets. Analyses were done under the sent ambient condition to calculate the overall average recovery. The mean amounts of the markers achieved were taken as real values to calculate the spike recoveries.

4.4.4.7.4: Precision

The precision of the method was assessed by injecting four replicates at two different concentrations for the reference compound, the extract and fractions. Values were represented as % RSD of intraday and inter-day runs. The mean amount and RSD values were calculated. The intra-day precision of the assay was determined by analyzing two concentrations in one day. Also, the intra-day precision was resolute over two successive days by analyzing the same concentrations. Injections were done in four replicates to determine the repeatability of the process.

4.4.4.7.5: Robustness

The robustness of the proposed method was investigated by an analysis of samples under different experimental conditions. The test solutions were analyzed with variation of flow rate, mobile phase composition, detection wave length and column temperature and using different columns of same configuration to determine their influence effect on the retention time.

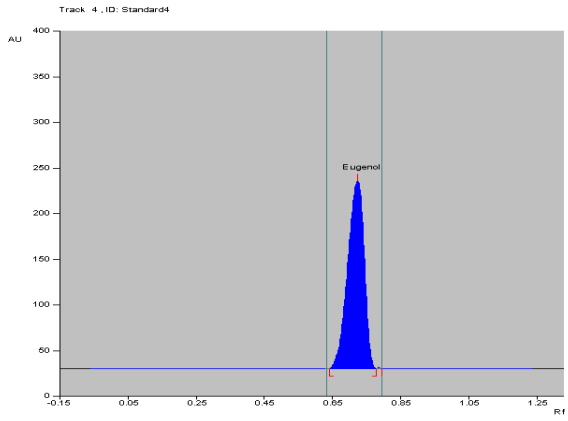
4.4.4.8 Statistical Analysis

Statistical analysis was performed using the graph pad prism Version 5.0. The results were represented as the mean ± SD.

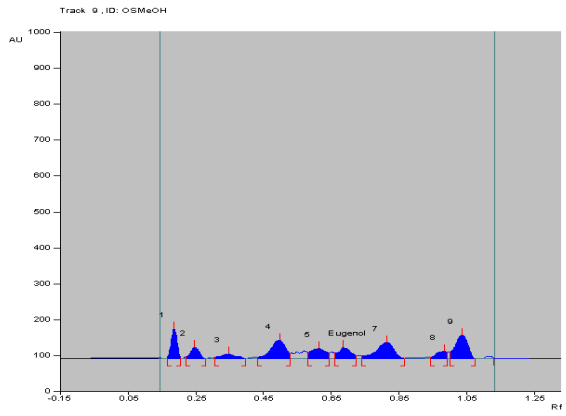
4.4.4.9. Results and Discussion

The R_f value of eugenol was found to be 0.61. The percentage content of eugenol in *Ocimum sanctum* hydro-alcoholic extract was found to be as mentioned 4.41% w/w. This was determined by a

calibration curve with the equation of $Y = 8.795 * X + 42.615$ (correlation coefficient = 0.95405 and standard deviation = ± 10.9%) as shown in fig. where X represents amount of eugenol and Y represents area under the curve.



4.4.b.HPTLC Chromatogram of eugenol



4.4.c.HPTLC chromatogram of *Ocimum sanctum*

4.4.5: RP-HPLC standardization of *Ocimum sanctum* extract

4.4.5.1: Equipments and reagents

4.4.5.1: Chemicals and reagents

Solvents used for chromatography were HPLC grade, viz. acetonitrile: water (40:60) (v/v). Membrane filters 0.45 µm pore size from Millipore and Whatman NYL 0.45 µm syringe filter was used for the filtration of samples. gallic acidstandard marker compound (Sigma Aldrich, USA),

4.4.5.2: HPLC instrument and chromatographic conditions

HPLC system consisted of a Waters (Milford, MA, USA) 600 quaternary HPLC pump, a Rheodyne-7725i injection valve (USA) with a sample loop of 20 µL, a Waters 2489 UV-Vis dual wavelength detector and the max-plot containing the peaks were obtained using Empower™2 software. A Waters Spherisorb (Ireland) C18 column (250 × 4.6 mm, 5 µm particle size) was used as stationary phase. Mobile phase composition was optimized as (channel A) water: (channel B) acetonitrile by means of isocratic elution (1mL/min) and detection at 272nm. Analyses of the eugenol, methanol extracts were

performed using the above protocol and the chromatograms were recorded. Peaks were identified by comparison of retention times and peak area (response) of standard compounds and extract.

4.4.5.3: Standard and sample solution

A primary stock solution (1 mg/mL) containing eugenol was prepared by dissolved in methanol and subsequently diluted to 10-500 µg/mL and was used for mobile phase optimization. Methanol extract of *ocimum sanctum* was re-dissolved (1 mg/mL) using mobile phase and was filtered through 0.45 µm syringe filter prior to injection into the HPLC column. Amount of eugenol present in the methanol extract was calculated after triplicate injection (n=3) by comparing and interpolating the extract peak area (response) with respect to the standard marker.

4.4.5.4: Calibration curve (linearity)

The linear calibration plot of eugenol was constructed by means of linear regression analysis between peak areas and concentrations in the range of 10-500 µg/mL of standard solution. The amount of standard compound in the solution was obtained by constructing the calibration curve of standard.

4.4.5.5: Specificity

Chromatograms of eugenol and methanol extract were verified in terms of specificity; according to the ICH recommendation to minimize the errors possibly happens during the experiment or the contamination of the sample with impurity. The purity of the peaks was checked by multivariate analysis by comparison of retention times and peak area of standard compounds and extract.

4.4.5.6: Limits of detection and quantification (LOD and LOQ)

The LOD and LOQ were calculated by the method based on the standard deviation (σ) and the slope (S) of the calibration plot, using the formula LOD = 3:1σ/S and LOQ = 10:1σ/S (ICH, 2005 and FDA, 1994); where, σ = standard deviation of the response from a number of blank run and S = slope of the calibration plot.

4.4.5.7: Robustness

The robustness of the proposed method was investigated by analyzing the samples under different experimental conditions such as variation of flow rate, mobile phase composition, detection

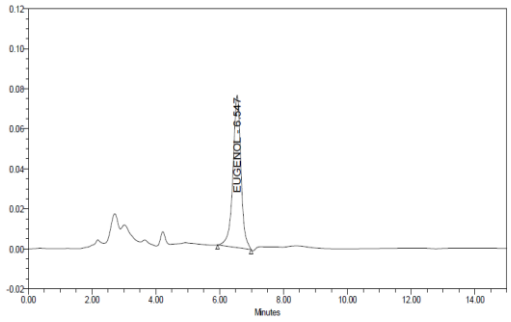
wavelength, column temperature and using different column of same configuration to determine their influence on the retention time.

4.4.5.8: Results

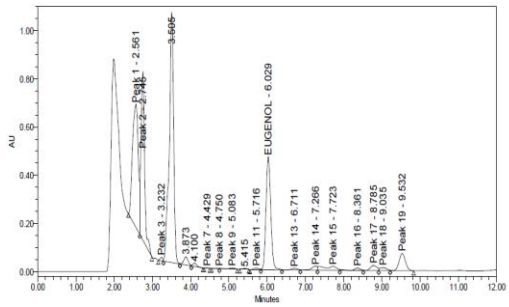
The retention time (R_t) of eugenol was found to be 6.029 min. The calibration curves were obtained by plotting peak areas against concentrations. Standard compound exhibited good linearity in the concentration range of 10-100 µg/ml. The correlation coefficient (r^2) was estimated to be 0.993. Instrumental precision was noted account by repetitive injection of the same sample of three concentrations.

The relative standard deviation (R.S.D.) was estimated to assess instrumental precision. Intra-assay precision was performed by making five standard solutions of three different concentrations and measuring the R.S.D. values. The R.S.D. values of both instrumental precision and intra-assay precision of eugenol were estimated to be <2.5%, indicating the RP-HPLC analytical method for these two standard compounds were precise. Intermediate precision of the method was optimized by calculating intra-day and inter-day repeatability. The R.S.D. values in all tested groups were found to be <3%, which is acceptable. The sensitivity of RP-HPLC method was determined by limit of detection (LOD) and limit of quantification (LOQ). LOD is the lowest concentration of the sample, the method can detect and LOQ is the lowest concentration that can be quantified accurately by the method. The LOD and LOQ of standard compound was estimated to be 0.3 and 0.8 µg/mL for eugenol resulted that the method was sensitive to detect the compounds in sample.

The recovery measures the closeness between the theoretically added amount and the practically obtained amount and was performed by spiking with a known amount of standard compounds in sample. The recovery of eugenol at different concentrations was found to be 90 to 105% with R.S.D. values <2% (data not shown). It indicates that standard compound can be fully recovered in the crude extract. The amount of eugenol present in extract was estimated to be about 4.41% (w/w), respectively. The percentage amounts of standard constituents present in the crude extract were expressed by its weight (µg/mg).



4.4.d:HPLC Chromatogram of Eugenol



4.4.e: HPLC Chromatogram of *Ocimum sanctum*

4.5.1: Literature reviewof *Lawsonia inermis*

Taxonomical classification

Family: Lythraceae

Species: L.inermis

Genus: Lawsonia

Kingdom: Plantae

Vernacular names

Hindi: Henna

Malayalam: Mailanchi

Marathi: Mehndi

Sanskrit: Mendika

Tamil: Henna

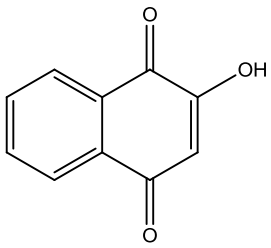
Lawsonia inermis belongs to the family *Lythraceae.* It is a biennial dicotyledonous herbaceous shrub. A native of North Africa and South-West Asia, the plant is now widely cultivated throughout the tropics as an ornamental and dye plant. A much branched glabrous shrub or small tree (2 to 6 m in height). Leaves are small, opposite in arrangement along the branches, sub-sessile, about 1.5to 5 cm long, 0.5 to 2 cm wide, greenish brown to dull green, elliptic to broadly lanceolate with entire margin, petiole short and glabrous and acute or obtuse apex with tapering base. Young branches are green in colour and quadrangular which turn red with age. Bark is greyish brown, unarmed when young but branches

of older trees are spine tipped. Inflorescence is a large pyramid shaped cyme. Flowers are small, about 1 cm across, numerous, fragrant, white or rose coloured with four crumbled petals. Calyx is with a 0.2 cm tube and 0.3 cm spread lobes. Fruit is a small brown coloured round capsule. Fruit opens irregularly and splits into four sections at maturity and is many seeded. Seeds are about 3 mm across, numerous, smooth, pyramidal, hard and thick seed coat with brownish coloration (Sastri, 1962).*L. innermis* has been used cosmetically and medicinally for over 9,000 years. Traditionally in India, mehndi is applied to hands and feet. It symbolizes fertility. Its use became popular in India owing to its cooling effect in the hot Indian summers. Its leaves, flowers, seeds, stem bark and roots are used in traditional medicine to treat a variety of problems like rheumatoid arthritis, headache, ulcers, diarrheoa, leprosy, fever, leucorrhoea, diabetes, cardiac disease, hepatoprotective and colouring agent (Chetty 2008 and Chopra, 1956). *L. innermis* leaf has an orange-red dye and leaf paste or powder is widely used for decorating hands, nails and feet with patterns. It is also used as a hair dye. It is used for alleviating jaundice, skin diseases, venereal diseases, smallpox and spermatorrhoea. Flowers are very fragrant and used to extract a perfume, which is used as base for local scents. An infusion of the flowers is a valuable application to bruises. Decoction of the flowers is describes as an emmenagogue. Seeds are deodorant. Powered seeds with real ghee (clarified butter) are effective against dysentery. Seeds in powered form are good medicine for liver disorders and associated problems. The bark is applied in the form of a decoction to burns and scalds. It is given internally in a variety of affections, such as jaundice, enlargement of the spleen, calculus, as an alternative in leprosy and obstinate skin affections. Root is considered as a potent medicine for gonorrhoea and herpes infection. Root is astringent may be pulped and used for sore eyes. Pulped root may also be applied to the heads of children for boils. Cambodians drink a decoction as a diuretic. Decoction of the root generally in combination with prepared indigo as a powerful abortifacient. The root is supposed to be useful in treatment of hysteria and nervous disorders (Reddy,1988).

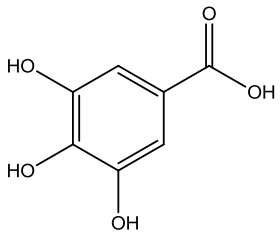
Phytochemistry

The principal colouring matter of *L. innermis* is lawsone, 2- hydroxy-1:4 napthaquinone besides lawsone other constituents present are gallic acid, glucose, mannitol, fats, resin (2 %), mucilage and traces of an alkaloid. Leaves have tannic acid and a green resin, soluble in ether and alcohol. Flowers possess essential oil with brown, strong fragrance and consist mainly of α- and β- ionones. Seeds possess proteins, carbohydrates, and fibers, oils having behenic acid, arachidic acid, stearic

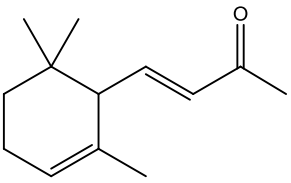
acid, palmitic acid, oleic acid and linoleic acid. The unsaponified portion contains waxes and colouring matter. The root has a red colouring agent.



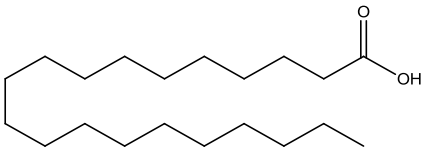
Lawsone (23)



Gallic acid (24)



β-ionone (25)



Arachidic acid (26)

Pharmacology

Antidiabetic activity ethanolic extract of *L. inermis* showed significant hypoglycaemic and hypolipidaemic activities in alloxan induced diabetic mice after oral administration (Syansuddin et al., 2008 and Arayne et al., 2007). Immunomodulatory effect methanolic extract at 1 mg/ml concentration had displayed immunostimulant action as indicated by promotion of T-lymphocyte proliferative responses. Naphthoquinone fraction obtained from leaves L. inermis showed significant immunomodulatory effect (Dikshit et al., 2000). Alcoholic extract of the bark showed hepatoprotective effect against the carbon tetrachloride induced elevation in serum marker enzymes (GOT and GPT), serum bilirubin, liver lipid peroxidation and reduction in total serum protein, liver glutathione, glutathione peroxidase, glutathione-s-transferase, glycogen, superoxide dismutase and catalase activity. The results suggest hepatoprotective and antioxidant activity of extract of L. alba bark (Ahmed et al., 2000 and Anand et al., 1992). Extracts used by traditional healers to treat infectious diseases were screened for their antibacterial activity against both gram positive and gram negative bacteria. The ethyl acetate extract of *L. inermis* L. was found to be the most active against all the bacteria in the test system (ali et al., 2001). Studies on lawsone suggested that it is a weak bacterial mutagen for *Salmonella typhimurium*. It was suggested that *L. innermis* and hydroxy napthaquinone

possess no genotoxic risk to the patient (Dama et al., 1999). Aqueous, methanol and chloroform crude extracts of leaf showed the in-vitro antimicrobial activity to inhibit the growth of six human pathogenic fungi and four types of bacteria in dose dependent manner (Sadabi, 2007). The leaves of *L. inermis* L. were also found to exhibit strong fungi toxicity and non-phytotoxicity. The minimum effective dose against test organism was found to be 1000ppm (Tripathi et al., 1978). The ethanolic fraction of the leaves displayed highly potent activity against Sembiki forest virus (SFV) in swiss mice and chick embryo models (Ahgel et al., 2005). During an ethnopharmacological survey of antiparasitic medicinal plants used in Ivory Coast, seventeen plants were identified and collected. Polar, non-polar and alkaloidal extracts of various parts of these species were evaluated invitro in an antiparasitic drug screening. Antimalarial, leishmanicidal, trypanocidal, antihelminthiasis and antiscabies activities were determined. Among the selected plants, L. inermis L. showed interesting trypanocidal activities (Okpekon et al., 2004). The antidermatophytic activity of ethanol, ethyl acetate and hexane fractions of *L. inermis* were tested on strains of *Tinea rubrum* and *Tinea mentagrophytes*. All these extracts showed significant antidermatophytic properties in-vitro (Natarajan, 2000). Ethanolic extract of the plant was used to demonstrate the wound healing activity on rats. When compared with the control and reference standard animals: a high rate of wound contraction, a decrease in the period of epithelialization, high skin breaking strength, a significant increase in the granulation tissue weight and hydroxyproline content. Histological studies of the tissue showed increased well organized bands of collagen, more fibroblasts and few inflammatory cells when compared with the controls which showed inflammatory cells, scanty collagen fibers and fibroblasts (Nayak et al., 2007).

4.5.2: Collection, authentication and extraction of *Lawsonia innermis* leaves

4.5.2.1: Collection and authentication

The fresh leaves of *Lawsonia innermis* were purchased from local vendor present in Jadavpur supermarket near Jadavpur University Kolkata. Voucher specimens (specimen no. SNPS/JU/2016/ 1102) was deposited at School of Natural Product Studies, Jadavpur University, and Kolkata, India for future references. The plant material was authenticated by Dr. S. Rajan, Msc, DPIM, DCA, PhD, Field Botanist and the voucher specimen number was issued.

Fig. 4.5.a: Picture and herbarium specimen of *Lawsonia inermis*

4.5.2.2: Extraction of L. innermis leaves

4.5.2.2.1: Preparation of hydro-alcoholic extract of L. innermis (EOHE)

100gm fresh plant materials were thoroughly washed under running water. Then it was freeze dried under pressure in a lyophilliser (INST IND, Kolkata, India) at -36 ºC for 2hrs. 100gm of the freeze dried product was cold macerated using a hydro alcoholic mixture of methanol and water (70:30) for 4 days with occasional stirring. The material was filtered using a Whatman No. – 45 filter paper. The liquid extract obtained was concentrated under pressure in rotary evaporator, IKA – HB 10 (IKA® India Private Limited, India), for 40 mins at 44ºC, 700mm Hg (pressure) and 30 revolutions per minute. The concentrated extract was further freeze dried under pressure in a lyophilliser (INST IND, Kolkata, India) at -36 ºC for 1 hr. Then it was transferred to a glass screw cap container, labeled and stored in a desiccator for future use.

Yield: 18.2% of freeze dried LIHE was obtained.

4.5.3: Phytochemical Screening of *L. innermis* Hydro-alcoholic extract

Table 4.5.A. Results of Phytochemical Screening of LIHE By different tests; (+: present; - : absent)

Carbohydrate	Phytosterol	Alkaloids	Terpenoids	Flavonoids	Tannins & Phenolics	Saponins	Volatile Oils
+	+	+	+	+	+	+	-

4.5.4: HPTLC standardization of *Lawsonia innermis*

4.5.4.1: Equipments and reagents

The CAMAG HPTLC system consisting of WINCATS software, LINOMAT V automatic sample applicator, and automatic development chamber, scanning densitometer CAMAG scanner 3 and photo documentation apparatus CAMAG reprostar 3 were used. All the solvents were used of analytical grade.100 µl syringe (HAMILTON, Switzerland) was used for sample application on HPTLC plates. Stationary phase used was aluminium based silica gel plate 60 F254 (Merck, Mumbai) with 20 cm x 10 cm in a particle size of 5-10 µm. Tolune and ethyl acetate, of analytical grade were purchased from Merck (Mumbai, India). Whatman's syringe filter (NYL 0.45 µ) was used for the filtration of samples and standard.

4.5.4.2: Chromatographic conditions

HPTLC analysis of *Lawsonia innermis* cultivars was performed with biomarker gallic acid using isocratic technique by external methods. Mobile phase was optimized with Ethyl acetate : Toluene : Methanol : Glacial acetic acid = 7.5 : 2 : 0.5 : 0.2 (v/v/v/v). The temperature was kept at 25°c and mobile phase was developed in a twin trough glass chamber. Standard stock solution was applied consequently in the range of 2-10 µl with 2 µl gradual increment. All total 25 tracks in HPTLC plates were used for standardization including standard and sample solution respectively in a band wise fashion. After development, plates were dried by hand dryer. The coloured bands were observed at 254nm.

4.5.4.3: Preparation of Standard/Sample Solutions

4.5.4.3.1: Standard Solution

Gallic acid, 1.0 mg/ml

About 1 mg of gallic acid standard was weighed and put in to 2 mL eppendorf tube. The standard was dissolved in methanol and volume was made up to 1 ml with methanol. It was then mixed in vortex mixture and put to ultrasonication bath till the material completely dissolved. It was then filtered through 0.45µ syringe filter and kept for further study.

4.5.4.3.2: Preparation of sample solution

About 10 mg of *Lawsonia innermis* extract of each variety was dissolved in 1 ml methanol in two different eppendorf tubes. Then the extract was dissolved in methanol and subjected to

ultrasonication till the extracts were completely dissolved. It was then filtered through 0.45µ syringe filter and kept for further study.

4.5.4.4: Optimization of Mobile Phase

The mobile phase was optimized for the HPTLC analysis. Out of the many mobile phases had been tried and following mobile phase gave the better separation thus optimized mobile phase was Ethyl acetate : Toluene : Methanol : Glacial acetic acid = 7.5 : 2 : 0.5 : 0.2 (v/v/v/v)

4.5.4.5: Application of standard and extracts

The external standard calibration curve for gallic acid was prepared with calibration solutions in a concentration range of 200 to 1000 ng/ml. Then 50 µl of standard solution was drawn into CAMAG LINOMAT syringe and put to linomat applicator to give the concentrations of standard required. The same method applied for the extract.

4.5.4.6: Development

The plate was then dried and developed in a CAMAG twin trough glass chamber with the mobile phase. After development, the plate was dried and scanned in Camag TLC scanner 3 at a wavelength of 254 nm

Software used : WINCATS

Analysis mode : Peak area versus concentration of standard and extracts

Calculation : By using following equation by WINCATS.

$$Y = m X + C$$

Where,

X = Concentration of Analyte/ metabolite in ng/ml

Y = Peak area

m = Slope of calibration Curve

C = Intercept.

4.5.4.7: Method validation for HPTLC study

Validation of the HPTLC method was done as recommended by the International Conference on Harmonisation (ICH) guidelines (ICH, 1996, 2005) and FDA (1994) Guidelines defining the Linearity, Specificity, Limits of Quantification and detection, precision, accuracy and robustness.

4.5.4.7.1: Specificity

The results for HPTLC standardization profiles were checked in terms of specificity according to the ICH guidelines to minimise errors due to the contamination of the sample. The specificity of the method was determined by analysing the standard and test samples. The purity of peaks was checked using multivariate analysis by comparison of retention times and peak area of standard compound with extract and fractions.

4.5.4.7.2: Limits of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ were calculated by the method based on standard deviation (σ) and the slope (S) of the calibration plot, using the formula LOD=3:1 σ/S and LOQ= 10:1 σ/s (ICH, 1996, 2005 and FDA, 1994); where, σ =standard deviation of the response from a number of blank run and S = slope of calibration plot.

4.5.4.7.3: Accuracy

Accuracy of the method was determined by percentage recovery of marker in the plant extract and fractions. The method was studied by performing standard addition technique and it is expressed in terms of percentage relative standard deviations (%RSD) from mean recovery of the theoretical concentrations. Prior to injection, the tests were spiked with two different known amounts of standard compounds in doublets. Analyses were done under the sent ambient condition to calculate the overall average recovery. The mean amounts of the markers achieved were taken as real values to calculate the spike recoveries.

4.5.4.7.4: Precision

The precision of the method was assessed by injecting four replicates at two different concentrations for the reference compound, the extract and fractions. Values were represented as % RSD of intraday and inter-day runs. The mean amount and RSD values were calculated. The intra-day precision of the assay was determined by analysing two concentrations in one day. Also, the intra-day

precision was resolute over two successive days by analysing the same concentrations. Injections were done in four replicates to determine the repeatability of the process.

4.5.4.7.5: Robustness

The robustness of the proposed method was investigated by an analysis of samples under different experimental conditions. The test solutions were analysed with variation of flow rate, mobile phase composition, detection wave length and column temperature and using different columns of same configuration to determine their influence effect on the retention time.

4.5..4.8 Statistical Analysis

Statistical analysis was performed using the graph pad prism Version 5.0. The results were represented as the mean ± SD.

4.5.4.9. Results and Discussion

The R_f value of gallic acid was found to be 0.52. The percentage content of gallic acid in *Lawsonia innermis* hydro-alcoholic extract was found to be as mentioned 1.64%. This was determined by a calibration curve with the equation of Y = -2372.845+ 22.521*X (correlation coefficient = 0.98308 and standard deviation = ± 2.93%) as shown in fig. where X represents amount of gallic acid and Y represents area under the curve.

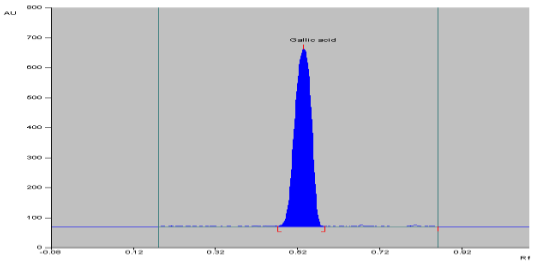


Fig. 4.5.a: HPTLC Chromatogram of Gallic acid

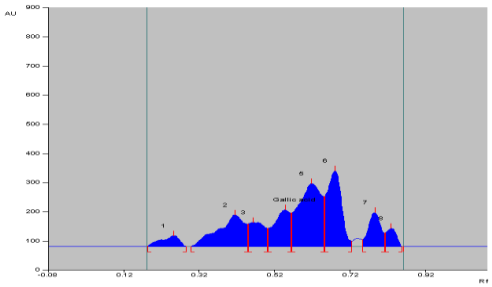


Fig. 4.5.b: HPTLC Chromatogram of *Lawsonia inermis*

4.5.5: RP-HPLC standardization of *L. innermis* extract

4.5.5.1: Chemicals and reagents

Solvents used for chromatography were HPLC grade, viz., water: acetonitrile 80:20 (v/v). Membrane filters 0.45 µm pore size from Millipore and Whatman NYL 0.45 µm syringe filter was used for the filtration of samples. gallic acidstandard marker compound (Sigma Aldrich, USA),

4.5.5.2: HPLC instrument and chromatographic conditions

HPLC system consisted of a Waters (Milford, MA, USA) 600 quaternary HPLC pump, a Rheodyne-7725i injection valve (USA) with a sample loop of 20 µL, a Waters 2489 UV-Vis dual wavelength detector and the max-plot containing the peaks were obtained using Empower™2 software. A Waters Spherisorb (Ireland) C18 column (250 × 4.6 mm, 5 µm particle size) was used as stationary phase. Mobile phase composition was optimized as (channel A) water: (channel B) acetonitrile by means of isocratic elution (1mL/min) and detection at 272nm. Analysis of the gallic acid, methanol extracts and fractions were performed using the above protocol and the chromatograms were recorded. Peaks were identified by comparison of retention times and peak area (response) of standard compounds and extract.

4.5.5.3: Standard and sample solution

A primary stock solution (1 mg/mL) containing gallic acid was prepared by dissolved in methanol and subsequently diluted to 10-500 µg/mL and was used for mobile phase optimization. Methanol extract of *L. innermis* was re-dissolved (1 mg/mL) using mobile phase and was filtered through 0.45 µm syringe filter prior to injection into the HPLC column. Amount of gallic acid present in the methanol extract was calculated after triplicate injection (n=3) by comparing and interpolating the extract peak area (response) with respect to the standard marker.

4.5.5.4: Calibration curve (linearity)

The linear calibration plot of gallic acid was constructed by means of linear regression analysis between peak areas and concentrations in the range of 10-500 µg/mL of standard solution. The amount of standard compound in the solution was obtained by constructing the calibration curve of standard.

4.5.5.5: Specificity

Chromatograms of gallic acid and methanol extract were verified in terms of specificity; according to the ICH recommendation to minimize the errors possibly happens during the experiment or the contamination of the sample with impurity. The purity of the peaks was checked by multivariate analysis by comparison of retention times and peak area of standard compounds and extract.

4.5.5.6: Limits of detection and quantification (LOD and LOQ)

The LOD and LOQ were calculated by the method based on the standard deviation (σ) and the slope (S) of the calibration plot, using the formula LOD = 3:1σ/S and LOQ = 10:1σ/S (ICH, 2005 and FDA, 1994); where, σ = standard deviation of the response from a number of blank run and S = slope of the calibration plot.

4.5.5.7: Robustness

The robustness of the proposed method was investigated by analyzing the samples under different experimental conditions such as variation of flow rate, mobile phase composition, detection wavelength, column temperature and using different column of same configuration to determine their influence on the retention time.

4.5.5.8: Results

The retention time (R_t) of gallic acid was found to be 5.081 min. The calibration curves were obtained by plotting peak areas against concentrations. Standard compound exhibited good linearity in the concentration range of 10 - 100 µg/ml. The correlation coefficient (r^2) was estimated to be 0.993. Instrumental precision was noted account by repetitive injection of the same sample of three concentrations.

The relative standard deviation (R.S.D.) was estimated to assess instrumental precision. Intra-assay precision was performed by making five standard solutions of three different concentrations and measuring the R.S.D. values. The R.S.D. values of both instrumental precision and intra-assay precision of gallic acid were estimated to be <2.5%, indicating the RP-HPLC analytical method for these two standard compounds were precise. Intermediate precision of the method was optimized by calculating intra-day and inter-day repeatability. The R.S.D. values in all tested groups were found to be <3%, which is acceptable. The sensitivity of RP-HPLC method was determined by limit of

detection (LOD) and limit of quantification (LOQ). LOD is the lowest concentration of the sample, the method can detect and LOQ is the lowest concentration that can be quantified accurately by the method. The LOD and LOQ of standard compound was estimated to be 0.3 and 0.8 µg/mL for gallic acid resulted that the method was sensitive to detect the compounds in sample.

The recovery measures the closeness between the theoretically added amount and the practically obtained amount and was performed by spiking with a known amount of standard compounds in sample. The recovery of gallic acid at different concentrations was found to be 90 to 105% with R.S.D. values <2% (data not shown). It indicates that standard compound can be fully recovered in the crude extract. The amount of gallic acid present in extract was estimated to be about 1.71 % (w/w), respectively. The percentage amounts of standard constituents present in the crude extract were expressed by its weight (µg/mg).

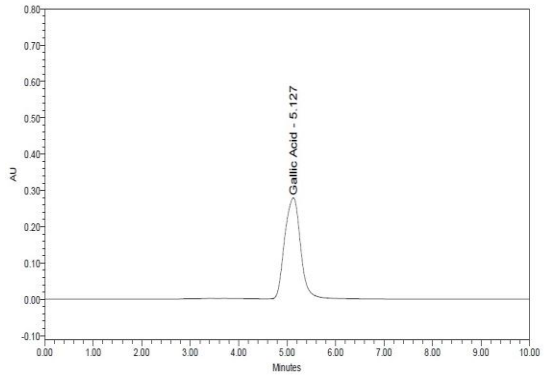


Fig.4 .5.a: HPLC Chromatogram of Gallic acid

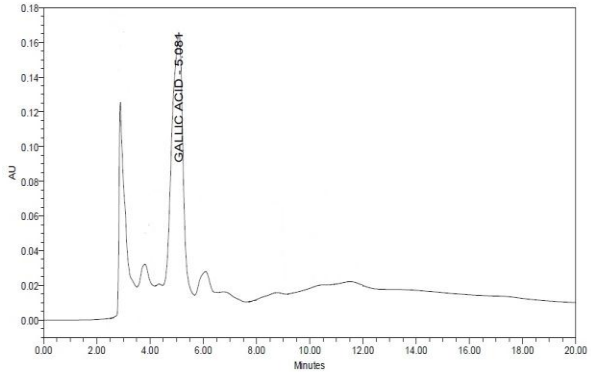


Fig. 4.5.b: HPLC Chromatogram of LIHE

4.6.: Literature review of *Tagetes erecta*

Tagetes is a genus (family Asteraceae) containing about 50 species of annual or perennial herbaceous plant. The plant *Tagetes erecta* Linn. locally known as Genda Phul (Marigold). It is stout, branching herb, native to Mexico and other warmer parts of America and neutralized elsewhere in the tropics and subtropics including India and Bangladesh. These are rapid-growing annual flowering plants in height ranging from dwarfs of 6-8 inch, to medium and taller and erect-growing plants with heights from 10 in to 3ft, bearing large pompon-like double flower up to 5 in across and has a shorter flowering period from midsummer to frost. It is very popular as a garden plant and yields a strongly aromatic essential oil (tagetes oil), which is mainly used for the compounding of high-grade perfumes.

Botanical classifiction:

- Kingdom : Plantae
- Order : Asterales
- Family : Asteraceae
- Genus : Tagetes
- Species : Tagetes erecta

Vernacular names:

- Hindi: Genda
- Telegu: Banti
- Sanskrit: Jhandu
- Tamil: Banti
- English: African marigold

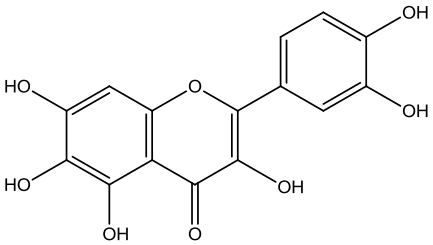
Plant description:

The plant *Tagetes erecta* Linn locally known as Genda Phul.Marrigold belongs to the family Compositae (Asteraceae). It is a stout, branching herb, native of Mexico and other warmer parts of America and naturalized elsewhere in the tropics and subtropics including Bangladesh and India (Kirtikar and Basu,1987).*Tagetes erecta* is an annual herb which prefers a warmlow humidity climate and grows well upto 5 meters in the tropical Andes. Flowering commences 90 to 100 days from field establishment and harvesting of fully developed flowers is carried out regularly throughout the season.

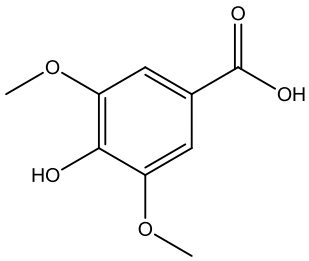
Phytochemistry

Studies of its different parts have resulted in the isolation of various chemical constituents such as thiophenes, flavonoids, carotenoids and triterpenoids. The plant *Tagetes erecta* has been shown to contain quercetagetin, a glucoside of quercetagetin (27), phenolics, syringic acid (28), methyl-3,5-dihydroxy-4-methoxy benzoate, quercetin, thienyl and gallic acid (30).Lutein (29) is an oxycarotenoid, or xanthophyll, containing 2 cyclic end groups (one beta and one alpha-ionone ring) and the basic C-

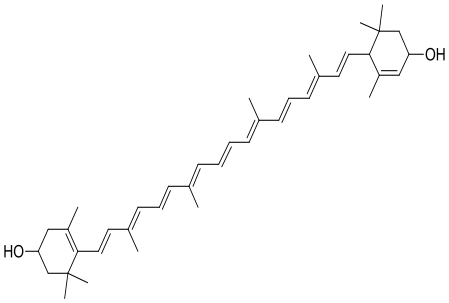
40 isoprenoid structure common to all carotenoids. It is one of the major constituents and the main pigment of Tagetes erecta.



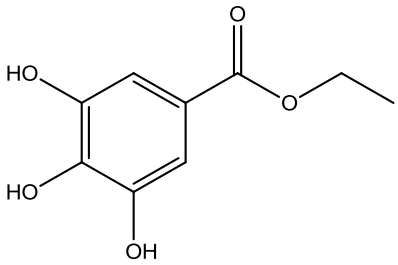
Quercetagetin (27)



Syringic acid (28)



Lutein (29)



Ethyl gallate (30)

Pharmacology

Antibacterial activity: Kiranmai et al., (2012) carried out investigation of antibacterial effect of different extracts of leaves and flowers of Tagetes erecta Linn. After performing preliminary Phytochemical screening and thin layer chromatography, antibacterial study was evaluated according to the agar diffusion method by using gram positive B. cereus, S. aureus and gram negative E.coli, P. aeruginosa. Study was showed that petroleum ether extract of leaves and ethyl acetate extract of flower of Tagetes erecta significantly inhibited the growth of bacteria dose dependently .Rhama and Madhavan reported the anti-bacterial activity of different solvents of *Tagetes erecta* flowers against Alcaligens faecalis, Bacillus cereus, Campylobacter coli, Escherchia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Proteus vulgaris, Streptococcus mutans and Streptococcus pyogenes. The flavonoid possesses anti-bacterial activity against all the tested strains and shows maximum zone of inhibition for Klebsiella pneumoniae (29.50 mm). The flavonoid Patulitrin is one of the potential elements for its anti-bacterial activity.Wound healing activity: Chatterjee et al., (2011) compared the efficacy of hydroalcoholic extracts of leaves of Tagetes erecta (T. erecta) and aerial

parts of Centella asiatica (C. asiatica) on excision, incision and dead space wound models in albino rats. Extract of T. erecta and C. asiatica (P <0.001) showed significant increase in rate of wound contraction, epithelization and formation of scar faster in excision wound model compare to control group. T. erecta extract (250 and 500 mg/kg) showed significantly increased the wound breaking strength in incision wound model and wet and dry granulation tissue weights, breaking strength in a dead space wound model compare to control and C. asiatica treated group (P < 0.001).Kiranmai et al., (2011) carried out the screening of wound healing activity of carbopol gels prepared from hydro alcoholic extracts of Gymnema sylvestere (GE) and Tagetes erecta Linn. (TE) in excision wound model and burn wound models,showed significant reduction in period of epithelization and wound contraction and combined gel showed accelerated wound healing activity may be because of synergism. The enhanced wound healing activity of hydro alcoholic extracts may be due to free radical scavenging action and the phytoconstituents (flavonoids) present in it which either due to their individual or additive effect fastened the process of wound healing.Chivde et al., (2011) performed in Vitro antioxidant study on the ethanolic extract of Tagetes erecta flowers. During the study preliminary phytochemical analysis were carried out on ethanolic extract of flowers of Tagetes erecta and found the presence of alkaloids,flavonoids, proteins, steroids and tannins. For in Vitro antioxidant activity three different assays like DPPH, reducing power and super oxide radical scavenging activity at different concentrations were used. In all the three assay, Tagetes erecta showed better reducing power than the standard (i.e. ascorbic acid), and super oxide anion scavenging activity and DPPH antioxidant activity showed less than standard. However, ethanolic extract of Tagetes erecta demonstrated antioxidant property in all the in Vitro models.Raghuveer et al., (2011) carried out studies on hydro alcoholic extract of Tagetes erecta its anti-diabetic activity by inducing diabetes using single intra-peritoneal injection of streptozotocin (60 mg/kg b.w).Treatment with standard drug Glibenclamide, blood glucose rose at 30 min followed by subsequent fall up to 120 min. From present study, it was observed that administration of Tagetes erecta extracts showed increase in glucose levels after 30 min and hypoglycaemia effect was observed only after 120 minutes.

Anti Hyperlipedemic activity: Raghuveer et al., (2011) investigated the anti hyperlipedemic activity of hydro alcoholic extract of Tagetes erecta in hyperlipedemic rats at a dose of 200 and 400 mg/kg. Hyperlipidemia was induced by cholesterol 25mg/kg/day. Lovastatin (10mg/kg/day) was used as standard. Blood samples were collected from rats in all the groups on 30th day and estimated for their serum cholesterol, serum triglyceride, serum HDL and serum LDL levels using standard procedures. From the study it was observed that administration of Tagetes erecta extracts significantly decreased all the hyperlipedemic parameters in rats.Kumaret al., (2011) giving emphasis on use of many folk

remedies from plant origin for their potential antioxidant and hepatoprotctive liver damage in experimental animal models, carried out hepatoprotective activity in Carbontetrachloride (CCl4)-induced hepatotoxicity model using 80% ethanolic soxhlet extract. In the experiment Wistar albino rats (150-250g) of either sex were used for the activity. The ethanolic extract of Tagetes erecta was found to show significant increase in serum ALT, AST, ALP and bilirubin levels in carbon tetrachloride intoxicated groups compared to the normal control group. Ethyl acetate fraction of T. ereta at the dose of 400mg/kg oraly significantly decreased the elevated serum marker enzymes and level of bilirubin almost to the normal levels compared to carbon tetrachloride intoxicated group .

Ovicidal and Repellent activity: Elango et al., (2011) assessed the ethyl acetate, acetone and methanol extracts of Andrographis paniculata, Eclipta prostrata and Tagetes erecta leaves for oviposition-deterrent, ovicidal and repellent activities against malaria vector, Anopheles subpictus Grassi (Diptera: Culicidae) and emphasized on Mosquito control facing a threat due to the emergence of resistance to synthetic insecticides and potential Insecticides of plant origin which may serve as suitable alternative biocontrol techniques in the future .

Nematocidal activity: Wang et al., (2007) carried out the studies on nematocidal activity of marigold. This plant produces number of potentially bioactive compounds, among which αtherthienyl is recognized as one of the most toxic.This sulfur-containing compound is abundant in marigold tissues, including roots. It has nematicidal, insecticidal, fungicidal, antiviral, and cytotoxic activities, and it is believed to be the main compound responsible for the nematicidal activity of marigold. Nematicidal compounds apparently permeate from marigold root tissues into nematodes attached to the root, but they are also believed to kill nematodes found in the rhizosphere, the soil near marigold roots. Thus, marigold is believed to be most effective suppressing plant-parasitic nematodes.

Husain et al., 2011 reported the nematicidal efficacy of four medicinal plants viz. Azadirachta indica, Calotropis procera, Datura stramonium and Tagetes erecta was ascertained for the control of M. incognita. All leaf amendments at different dosages significantly improved the plant growth characteristics of okra and reduced root-knot infections compared with the untreated control.

Insecticidal activity: Nikkon et al reported the insecticidal activity in Tagetes erecta flowers against a stored product insect pest, Tribolium castaneum (Herbst). The chloroform fraction showed highest toxicity against both the larvae and adults of Tribolium castaneum followed by petroleum ether fraction and ethanol extract. The LC values of chloroform fraction against first, second, third, fourth, fifth and sixth instar larvae were 11.64, 14.23, 19.26, 29.02, 36.66, 59.51 µg/cm2 (72 h.), respectively

and for adults the value was 65.93 µg/cm2 (72 h.). No mortality was observed in control. Finally they concluded that the flower of Tagetes erecta might be a pesticide against Tribolium castaneum.

Anti-oxidant and Analgesic activity: Bashir and Gilani reported the in vitro anti-oxidant and in vivo analgesic activities (acetic-acid-induced abdominal writhing) on flower extracts of Tagetes erecta. The results revealed the presence of pronounced antioxidant potential on dosedependent (100 and 300 mg/kg) and analgesic effect also. The antioxidant and analgesic activities obtained seem to be in good accordance with the medicinal uses of Aztec marigold as an antiinflammatory and analgesic.

Cytotoxic activity: Curcumin and lutein were isolated from rhizomes of Curcuma longa and petals of Tagetes erecta. The isolated pigments were quantified spectroscopically and separated by thin layer chromatography. The active components of the pigments were further purified and identified by high performance liquid chromatography. In vitro cytotoxic activity of both extracts against Hep2 cancer cell lines were evaluated. Furthermore, the activities of both pigments in different concentrations against Hep2 cancer cell line were compared. The test sample showing cell viability of more than 97% at 0.078 mg/ml were considered to be less active at minimum concentration. The maximum viability of Hep2 cell line were 3.27% (curcumin) and 8.88% (lutein), respectively, which are most suitable to perform cytotoxic studies.

Antiepileptic Activity: Shetty et al. reported central nervous system (CNS) stimulatory activity of ethanolic extract of flowers of Tagetes erecta in rats. The findings suggested that ethanolic extract may reduce the seizure threshold in epileptic patients.

Fungitoxic activity: Kishore and Dwivedi reported fungitoxic activity of the essential oil of leaves of Tagetes erecta exhibited complete inhibition of the growth Pythium aphanidermatum Fitz., the damping-off pathogen, at a concentration of 2000 ppm.

Antimutagenic activity: Majia et al. reported antimutagenic activity of xanthophylls extracted from Aztec Marigold (Tagetes erecta) on 1-nitropyrene (1-NP) mutagenicity using the Salmonella typhimurium tester strain YG1024 in the plate-incorporation test.

4.6.2: Collection, authentication and extraction of *Tagetes erecta* whole plant

4.6.2.1: Collection and authentication

The fresh whole plant flowers of *Tagetes erecta* were purchased from local vendor present in Jadavpur supermarket near Jadavpur University Kolkata. The voucher specimens (specimen no. SNPS/JU/2016/1103) was deposited at School of Natural Product Studies, Jadavpur University, and Kolkata, India for future references. The plant material was authenticated.

Fig:4.6.a: Picture and Herbarium specimen of *Tagetes erecta*

4.6.2.2: Extraction of Tagetes erecta flower

4.6.2.2.1: Preparation of plant extract of Tagetes erecta:

100gm fresh plant materials were thoroughly washed under running water and chopped into fine pieces. Then it was dried. 100gm Then it was cold macerated using ethanol : water=60:40 with occasional stirring. The material was filtered using a Whatman No. – 45 filter paper. The liquid extract obtained was concentrated under pressure in rotary evaporator, IKA – HB 10 (IKA® India Private Limited, India), for 40 mins at 44ºC, 700mm Hg (pressure) and 30 revolutions per minute. The concentrated extract was further freeze dried under pressure in a lyophilliser (INST IND, Kolkata, India) at -36 ºC for 1 hr. Then it was transferred to a glass screw cap container, labeled and stored in a desiccator for future use.

Yield: 17.1% of freeze dried *Tagetes erecta* was obtained.

4.6.3: HPTLC standardization of *Tagetes erecta*

4.6.3.1:Equipments and reagents

The CAMAG HPTLC system consisting of WINCATS software, LINOMAT V automatic sample applicator, and automatic development chamber, scanning densitometer CAMAG scanner 3 and photo documentation apparatus CAMAG reprostar 3 were used. All the solvents were used of analytical grade.100 µl syringe (HAMILTON, Switzerland) was used for sample application on HPTLC plates. Stationary phase used was Aluminium based silica gel plate 60 F254 (Merck, Mumbai) with 20 cm x 10 cm in a particle size of 5-10 µm. Tolune and ethyl acetate, of analytical grade were purchased from Merck (Mumbai, India). Whatman's syringe filter (NYL 0.45 µ) was used for the filtration of samples and standard.

4.6.3.2: Chromatographic conditions oleanolic acid

HPTLC analysis of *T.erecta* was performed with biomarker oleanolic acid using isocratic technique by external methods. Mobile phase was optimized with petroleum ether and ethyl acetate in a ratio of 6:4 v/v. The temperature was kept at 25°c and mobile phase was developed in a twin trough glass chamber. Standard stock solution was applied consequently in the range of 2-10 µl with 2 µl gradual increment. All total 25 tracks in HPTLC plates were used for standardization including standard and sample solution respectively in a band wise fashion. After development, plates were dried by hand dryer. The coloured bands were observed at 254 to 366 nm.

4.6.3.3: Preparation of Standard/Sample Solutions
4.6.3.3.1: Standard Solution
Oleanolic acid, 1.0 mg/ml

About 1 mg of *Oleanolic acid* standard was weighed and put in to 2 mL eppendorf tube. The standard was dissolved in methanol and volume was made upto 1 ml with methanol. It was then mixed in vortex mixture and put to ultrasonication bath till the material completely dissolved. It was then filtered through 0.45µ syringe filter and kept for further study.

4.6.3.3.2: Preparation of sample solution

About 10 mg of *Tagetes erecta* extract of each variety was dissolved in 1 ml methanol in two different eppendorf tubes. Then the extract was dissolved in methanol and subjected to ultrasonication till the extracts were completely dissolved. It was then filtered through 0.45µ syringe filter and kept for further study.

4.6.3.4: Optimization of Mobile Phase

The mobile phase was optimized for the HPTLC analysis. Out of the many mobile phases had been tried and following mobile phase gave the better separation thus optimized mobile phase was toluene and methanol in a ratio of 90:10 (v/v).

4.6.3.5: Application of standard and extracts

The external standard calibration curve for oleanolic acid was prepared with calibration solutions in a concentration range of 200 to 1000 ng/ml. Then 50 µl of standard solution was drawn into CAMAG LINOMAT syringe and put to linomat applicator to give the concentrations of standard required. The same method applied for the extract.

4.6.3.6: Development

The plate was then dried and developed in a CAMAG twin trough glass chamber with the mobile phase. After development, the plate was dried and scanned in camag TLC scanner 3 at a wavelength of 254 and 366 nm.

Software used : WINCATS

Analysis mode : Peak area versus concentration of standard and extracts

Calculation : By using following equation by WINCATS.

$$Y = m X + C$$

Where,

X = Concentration of Analyte/ metabolite in ng/ml

Y = Peak area

m = Slope of calibration Curve

C = Intercept.

4.6.4: Method validation for HPTLC study

Validation of the HPTLC method was done as recommended by the International Conference on Harmonisation (ICH) guidelines (ICH, 1996, 2005) and FDA (1994) Guidelines defining the Linearity, Specificity, Limits of Quantification and detection, precision, accuracy and robustness.

4.6.4.1: Specificity

The results for HPTLC standardization profiles were checked in terms of specificity according to the ICH guidelines to minimize errors due to the contamination of the sample. The specificity of the method was determined by analysing the standard and test samples. The purity of peaks was checked using multivariate analysis by comparison of retention times and peak area of standard compound with extract and fractions.

4.6.4..2: Limits of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ were calculated by the method based on standard deviation (σ) and the slope (S) of the calibration plot, using the formula LOD=3:1 σ/S and LOQ= 10:1 σ/s (ICH, 1996, 2005 and FDA, 1994); where, σ =standard deviation of the response from a number of blank run and S = slope of calibration plot.

4.6.4.3: Accuracy

Accuracy of the method was determined by percentage recovery of marker in the plant extract and fractions. The method was studied by performing standard addition technique and it is expressed in terms of percentage relative standard deviations (%RSD) from mean recovery of the theoretical concentrations. Prior to injection, the tests were spiked with two different known amounts of standard compounds in doublets. Analyses were done under the sent ambient condition to calculate the overall average recovery. The mean amounts of the markers achieved were taken as real values to calculate the spike recoveries.

4.6.4.4: Precision

The precision of the method was assessed by injecting four replicates at two different concentrations for the reference compound, the extract and fractions. Values were represented as % RSD of intraday and inter-day runs. The mean amount and RSD values were calculated. The intra-day precision of the assay was determined by analysing two concentrations in one day. Also, the intra-day

precision was resolute over two successive days by analyzing the same concentrations. Injections were done in four replicates to determine the repeatability of the process.

4.6.4.5: Robustness

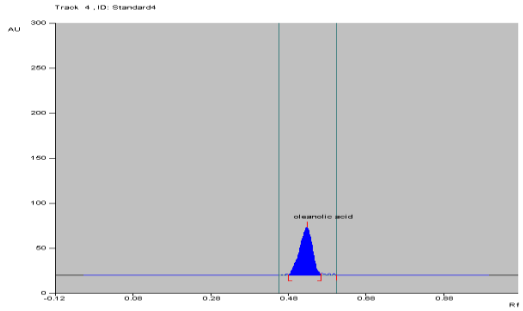
The robustness of the proposed method was investigated by an analysis of samples under different experimental conditions. The test solutions were analysed with variation of flow rate, mobile phase composition, detection wave length and column temperature and using different columns of same configuration to determine their influence effect on the retention time.

4.6.4.8 Statistical Analysis

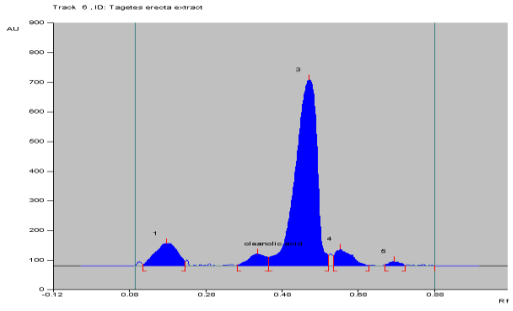
Statistical analysis was performed using the graph pad prism Version 5.0. The results were represented as the mean ± SD.

4.6.4.9. Results and Discussion

The $R_f$value of *Oleanolic acid* was found to be 0.59 . The percentage content of *Oleanolic acid* in *Tagetes erecta* extract was found to be as mentioned 5.72 %. This was determined by a calibration curve with the equation of Y = 3447.759*X - 1121.100 (correlation coefficient = 0.9686 and standard deviation = ± 3.9%) as shown in fig. where X represents amount of *Oleanolicacid* and Y represents area under the curve.



4.6.b: HPTLC chromatogram of oleanolic acid



4.6.c: HPTLC chromatogram of *Tagetes erecta*

4.6.5: RP-HPLC standardization of *Tagetes erecta* extract

4.6.5.1: Chemicals and reagents

Solvents used for chromatography were HPLC grade, viz., Methanol: Water: Acetic acid = 20:79:1 (v/v). Membrane filters 0.45 µm pore size from Millipore and Whatman NYL 0.45 µm syringe filter was used for the filtration of samples. *Oleanolic acid* standard marker compound (Sigma Aldrich, USA),

4.6.5.2: HPLC instrument and chromatographic conditions

HPLC system consisted of a Waters (Milford, MA, USA) 600 quaternary HPLC pump, a Rheodyne-7725i injection valve (USA) with a sample loop of 20 µL, a Waters 2489 UV-Vis dual wavelength detector and the max-plot containing the peaks were obtained using Empower™2 software. A Waters Spherisorb (Ireland) C18 column (250 × 4.6 mm, 5 µm particle size) was used as stationary phase. Mobile phase composition was optimized as (channel A) Acetonitrile: (channel B) Methanol by means of isocratic elution (1 mL/min) and detection at 210 nm. Analysis of the *Oleanolic acid* and pet ether. extract was performed using the above protocol and the chromatograms were recorded. Peaks were identified by comparison of retention times and peak area (response) of standard compounds and extract.

4.6.5.3: Standard and sample solution

A primary stock solution (1 mg/mL) containing β-sitosterol was prepared by dissolved in methanol and subsequently diluted to 10-500 µg/mL and was used for mobile phase optimization. Methanol extract of *E. alba* was re-dissolved (1 mg/mL) using mobile phase and was filtered through 0.45 µm syringe filter prior to injection into the HPLC column. Amount of *Oleanolic acid* present in the hydro alcoholic extract was calculated after triplicate injection (n=3) by comparing and interpolating the extract peak area (response) with respect to the standard marker.

4.6.5. 4: Calibration curve (linearity)

The linear calibration plot of *Oleanolic acid* was constructed by means of linear regression analysis between peak areas and concentrations in the range of 10-500 µg/mL of standard solution. The amount of standard compound in the solution was obtained by constructing the calibration curve of standard.

4.6.5. 5: Specificity

Chromatograms of *Oleanolic acid* and pet. ether extract were verified in terms of specificity; according to the ICH recommendation to minimize the errors possibly happens during the experiment or the contamination of the sample with impurity. The purity of the peaks was checked by multivariate analysis by comparison of retention times and peak area of standard compounds and extract.

4.6.5.6: Limits of detection and quantification (LOD and LOQ)

The LOD and LOQ were calculated by the method based on the standard deviation (σ) and the slope (S) of the calibration plot, using the formula LOD = 3:1σ/S and LOQ = 10:1σ/S (ICH, 2005 and FDA, 1994); where, σ = standard deviation of the response from a number of blank run and S = slope of the calibration plot.

4.6.5.7: Robustness

The robustness of the proposed method was investigated by analyzing the samples under different experimental conditions such as variation of flow rate, mobile phase composition, detection wavelength, column temperature and using different column of same configuration to determine their influence on the retention time.

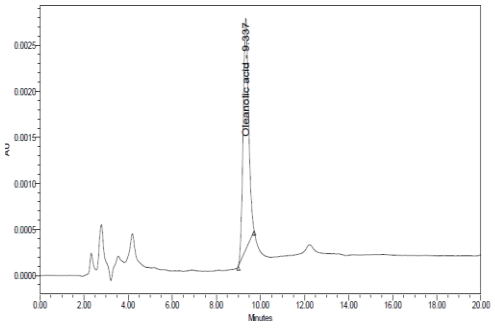
4.6.5.8: Results

The retention time (R_t) of *Oleanolic acid* was found to be 9.19 min. The calibration curves were obtained by plotting peak areas against concentrations. Standard compound exhibited good linearity in the concentration range of 10 - 100 µg/mL. The correlation coefficient (r^2) was estimated to be 0.9923. Instrumental precision was noted account by repetitive injection of the same sample of three concentrations.

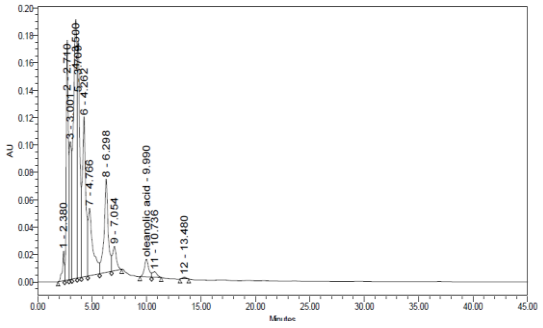
The relative standard deviation (R.S.D.) was estimated to assess instrumental precision. Intra-assay precision was performed by making five standard solutions of three different concentrations and measuring the R.S.D. values. The R.S.D. values of both instrumental precision and intra-assay precision of *Oleanolic acid* were estimated to be <2.5%, indicating the RP-HPLC analytical method for these two standard compounds were precise. Intermediate precision of the method was optimized by calculating intra-day and inter-day repeatability. The R.S.D. values in all tested groups were found to be <3%, which is acceptable. The sensitivity of RP-HPLC method was determined by limit of

detection (LOD) and limit of quantification (LOQ). LOD is the lowest concentration of the sample, the method can detect and LOQ is the lowest concentration that can be quantified accurately by the method. The LOD and LOQ of standard compound was estimated to be 0.3 and 0.8 µg/mL for *Oleanolic acid* resulted that the method was sensitive to detect the compounds in sample.

The recovery measures the closeness between the theoretically added amount and the practically obtained amount and was performed by spiking with a known amount of standard compounds in sample. The recovery of *Oleanolic acid* at different concentrations was found to be 90 to 105% with R.S.D. values <2% (data not shown). It indicates that standard compound can be fully recovered in the crude extract. The amount of *Oleanolic acid* present in extract was estimated to be about 2.78% (w/w), respectively. The percentage amounts of standard constituents present in the crude extract were expressed by its weight (µg/mg).



4.6.d: RP-HPLC Chromatogram of Oleanolic acid



4.6.e: RP-HPLC Chromatogram of *Tagetes erecta*

4.7.1: Review of *Acacia concinna*

Taxonomy

Family: Fabaceae

Kingdom: Plantae

Order: Fabales

Vernacular name

Common name: Shikakai

Hindi: Kochi

Telugu: cheekaya

Tamil: Shika

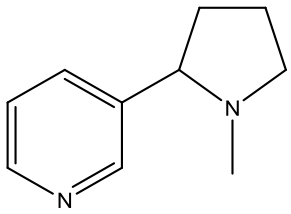
Acacia concinna Linn. (Leguminosae) is a medicinal plant that grows in tropical rainforests of southern Asia and the fruits of this plant are used for washing hair An attempt has been carried out in respect to the authenticity and assay of Shikakai (Acacia Concinna Linn.) fruit.

Shikakai is a climbing, most well-known for the natural shampoo derived from its pod. Thorny branches have brown smooth stripes - thorns are short, broad-based, flattened. Leaves with caducous stipules but are not thorn-like. Leaf stalks are 1-1.5 cm long with a prominent gland about the middle. Leaves are double-pinnate, with 5-7 pairs of pinnae, the primary rachis being thorny, velvety. Each pinnae has 12-18 pairs of leaflets, which are oblong-lance shaped, 3-10 mm long, pointed, obliquely rounded at base. Inflorescences is a cluster of 2 or 3 stalked rounded flower-heads in axils of upper reduced leaves, appearing paniculate. Stalk carrying the cluster is 1-2.5 cm long, velvety. Flower-heads about 1 cm in diameter when mature. Flowers are pink, without or with reduced subtending bracts. Pods are thick, somewhat flattened, stalked, 8 cm long, 1.5-1.8 cm wide.

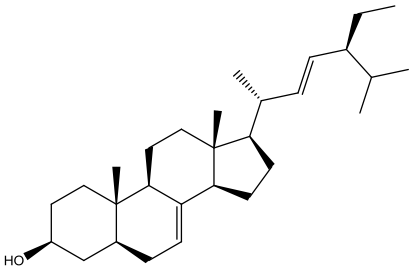
Fig. 4.7.a. Herbarium specimen of *Acacia concinna* pods

Phytochemistry

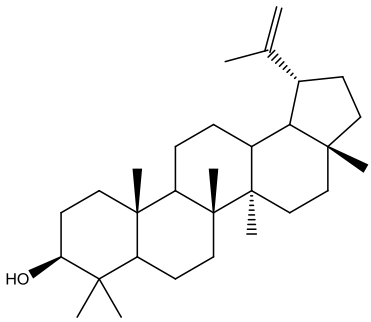
The major saponis present are Concinnosides A, B, C, D, and E. The pods post hydrolysis yield lupeol (33), spinasterol (32), acacic acid, glucose, arabinose and rhamnose. presence of oxalic, tartaric, citric, succinic betulinic acid (34) and ascorbic acids, as well as two alkaloids, calyctomine and nicotine (31), have been detected in the leaves (Siegler, 2001 and Wilbur,2005).



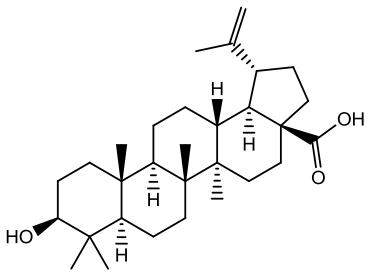
Nicotine (31)



Spinasterol (32)



Lupeol (33)



Betulinic acid (34)

Pharmacology

Its leaves are used in malarial fever, decoction of the pods are used to relieve biliousness and acts as a purgative. An ointment, prepared from the ground pods, is good for skin diseases. The powder or the extract from the bark, leaves or pods is used as a hair cleansing agent. It is very effective in removing oil and dirt from hair. The normal practice is to apply oil to hair and scalp and allow it soak for some time. This will keep the body cool, reducing body heat and also prevent the scalp from getting dry. The powdered *Shikakai* is used as a shampoo to wash off the oil. Extracts of the ground pods have been used for various skin diseases. An extract of Shikakai leaves is used to cure malarial fever and decoction of the pods relieves biliousness and acts as a purgative. The ethanolic extract

from the pod of *A. concinna* has been used in anti-dandruff shampoo due to its anti-dermatophyte and antibacterial activities. Saponins in the pods acts as detergent . Moreover, an ointment of the extract has been used for skin disease. *Shikakai* is a commonly used herb that has many remedial qualities. It is popularly referred as "pod for the hair" as it has a naturally mild pH, that gently cleans the hair without stripping it of natural oils. *Shikakai* is used to control dandruff, promoting hair growth and strengthening hair roots. Since *Shikaka*i is naturally low in pH, therefore it is extremely mild, and does not strip hair of its natural oils. Usually no rinse or conditioner is used since it also helps in the disentangling of the hair. The barks and pods of Shikakai is high on saponins these act as foaming agents. Therefore Shikakai is such a good cleaning agent and hence has been traditionally used as a detergent. Owing to the presence of this group of chemical compounds *Shikakai* is used in Bengal for poisoning fish and are documented to be potent marine toxins. Apart from this the leaves are also used as an infusion in anti-dandruff preparations (Ediriweera *et al.*,2014).

4.7.2: Collection, authentication and extraction of *Acacia concinna* pods

4.7.2.1: Collection and authentication

The pods of *Acacia concinna* were purchased from local vendor present in Jadavpur supermarket near Jadavpur University Kolkata. Voucher specimens (specimen no. SNPS/JU/2016/1103) was deposited at School of Natural Product Studies, Jadavpur University, and Kolkata, India for future references.

4.7.2.2: Extraction of A. concinna pods

Preparation of hydro-alcoholic extract of A. concinna

100gm fresh plant materials were thoroughly washed under running water and chopped into fine pieces. Then it was freeze dried in a lyophilliser (INST IND, Kolkata, India) at -36 ºC for 2hrs. 100gm of the freeze dried product was cold macerated using a hydro alcoholic mixture of methanol and water (70:30) for 4 days with occasional stirring. The material was filtered using a Whatman No. – 45 filter paper. The liquid extract obtained was concentrated under pressure in rotary evaporator, IKA – HB 10 (IKA® India Private Limited, India), for 40 mins at 44ºC, 700mm Hg (pressure) and 15 revolutions per minute. The concentrated extract was further freeze dried in a lyophilliser (INST IND, Kolkata, India) at -36 ºC for 1 hr. Then it was transferred to a glass screw cap container, labeled and stored in a desiccator for future use.

Yield: 19.5% of freeze dried ACHE was obtained.

4.7.3: Phytochemical Screening of *A. concinna* Hydro-alcoholic extract

Table 4.7.A. Results of Phytochemical Screening of EOHE By different above mentioned tests; (+ : present; - : absent)
The methodology is described in section 4.1

Carbohydrate	Phytosterol	Alkaloids	Terpenoids	Flavonoids	Tannins & Phenolics	Saponins	Volatile Oils
+	+	+	+	+	+	+	-

4.7.4: HPTLC standardization of *Acacia concinna*

4.7.4.1: Equipments and reagents

The CAMAG HPTLC system consisting of WINCATS software, LINOMAT V automatic sample applicator, and automatic development chamber, scanning densitometer CAMAG scanner 3 and photo documentation apparatus CAMAG reprostar 3 were used. All the solvents were used of analytical grade.100 µl syringe (HAMILTON, Switzerland) was used for sample application on HPTLC plates. Stationary phase used was aluminium based silica gel plate 60 F254 (Merck, Mumbai) with 20 cm x 10 cm in a particle size of 5-10 µm. Tolune and ethyl acetate, of analytical grade were purchased from Merck (Mumbai, India). Whatman's syringe filter (NYL 0.45 µ) was used for the filtration of samples and standard.

4.7.4.2: Chromatographic conditions

HPTLC analysis of *Acacia concinna* cultivars was performed with biomarker betulinic acid using isocratic technique by external methods. Mobile phase was optimized with chloroform, methanol and formic acid = 49: 1: 1 (v/v/v). The temperature was kept at 25°c and mobile phase was developed in a twin trough glass chamber. Standard stock solution was applied consequently in the range of 2-10 µl with 2 µl gradual increment. All total 25 tracks in HPTLC plates were used for standardization including standard and sample solution respectively in a band wise fashion. After development, plates were dried by hand dryer. The coloured bands were observed at visible light.

4.7.4.3: Preparation of Standard/Sample Solutions

4.7.4.3.1:Standard Solution

Betulinic acid, 1.0 mg/ml

About 1 mg of betulinic acid standard was weighed and put in to 2 mL eppendorf tube. The standard was dissolved in methanol and volume was made up to 1 ml with methanol. It was then mixed in vortex mixture and put to ultrasonication bath till the material completely dissolved. It was then filtered through 0.45µ syringe filter and kept for further study.

4.7.4.3.2: Preparation of sample solution

About 10 mg of *Acacia concinna* extract of each variety was dissolved in 1 ml methanol in two different eppendorf tubes. Then the extract was dissolved in methanol and subjected to ultrasonication till the extracts were completely dissolved. It was then filtered through 0.45µ syringe filter and kept for further study.

4.7.4.4: Optimization of Mobile Phase

The mobile phase was optimized for the HPTLC analysis. Out of the many mobile phases had been tried and following mobile phase gave the better separation thus optimized mobile phase was chloroform, methanol and formic acid = 49: 1: 1 (v/v/v)

4.7.4.5: Application of standard and extracts

The external standard calibration curve for betulinic acid was prepared with calibration solutions in a concentration range of 200 to 1000 ng/ml. Then 50 µl of standard solution was drawn into CAMAG LINOMAT syringe and put to linomat applicator to give the concentrations of standard required. The same method applied for the extract.

4.7.4.6: Development

The plate was then dried and developed in a CAMAG twin trough glass chamber with the mobile phase. After development, the plate was dried, sprayed with anisaldehyde reagent and heated in a hot air oven. After that it was scanned in Camag TLC scanner 3 at a wavelength of visible light.

Software used	:	WINCATS
Analysis mode	:	Peak area versus concentration of standard and extracts
Calculation	:	By using following equation by WINCATS.

$$Y = m X + C$$

Where,

X = Concentration of Analyte/ metabolite in ng/ml

Y = Peak area

m = Slope of calibration Curve

C = Intercept.

4.7.4.7: Method validation for HPTLC study

Validation of the HPTLC method was done as recommended by the International Conference on Harmonisation (ICH) guidelines (ICH, 1996, 2005) and FDA (1994) Guidelines defining the Linearity, Specificity, Limits of Quantification and detection, precision, accuracy and robustness.

4.7.4.7.1: Specificity

The results for HPTLC standardization profiles were checked in terms of specificity according to the ICH guidelines to minimise errors due to the contamination of the sample. The specificity of the method was determined by analysing the standard and test samples. The purity of peaks was checked using multivariate analysis by comparison of retention times and peak area of standard compound with extract and fractions.

4.7.4.7.2: Limits of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ were calculated by the method based on standard deviation (σ) and the slope (S) of the calibration plot, using the formula LOD=3:1 σ/S and LOQ= 10:1 σ/s (ICH, 1996, 2005 and FDA, ;1994); where, σ =standard deviation of the response from a number of blank run and S = slope of calibration plot.

4.7.4.7.3: Accuracy

Accuracy of the method was determined by percentage recovery of marker in the plant extract and fractions. The method was studied by performing standard addition technique and it is expressed in terms of percentage relative standard deviations (%RSD) from mean recovery of the theoretical concentrations. Prior to injection, the tests were spiked with two different known amounts of standard

compounds in doublets. Analyses were done under the sent ambient condition to calculate the overall average recovery. The mean amounts of the markers achieved were taken as real values to calculate the spike recoveries.

4.7.4.7.4: Precision

The precision of the method was assessed by injecting four replicates at two different concentrations for the reference compound, the extract and fractions. Values were represented as % RSD of intraday and inter-day runs. The mean amount and RSD values were calculated. The intra-day precision of the assay was determined by analysing two concentrations in one day. Also, the intra-day precision was resolute over two successive days by analysing the same concentrations. Injections were done in four replicates to determine the repeatability of the process.

4.7.4.7.5: Robustness

The robustness of the proposed method was investigated by an analysis of samples under different experimental conditions. The test solutions were analysed with variation of flow rate, mobile phase composition, detection wave length and column temperature and using different columns of same configuration to determine their influence effect on the retention time.

4.7.4.8 Statistical Analysis

Statistical analysis was performed using the graph pad prism Version 5.0. The results were represented as the mean ± SD.

4.7.4.9. Results and Discussion

The R_f value of betulinic acid was found to be 0.42. The percentage content of betulinic acid in *Acacia concinna* hydro-alcoholic extract was found to be as mentioned 1.29 %. This was determined by a calibration curve with the equation of Y = 140.780 + 0.365 * X (correlation coefficient = 0.98061 and standard deviation = ± 2.96%) as shown in fig. where X represents amount of betulinic acid and Y represents area under the curve.

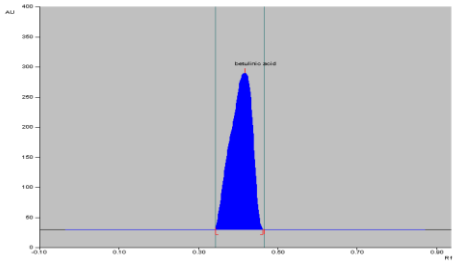


Fig. 4.7.b: HPTLC Chromatogram of Betulinic acid

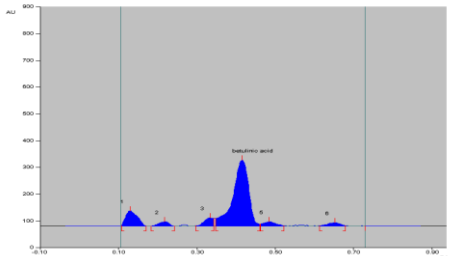


Fig. 4.7.c: HPTLC Chromatogram of *Acacia concinna*

4.7.5: RP-HPLC standardization of *A. concinna* extract

4.7.5.1: Chemicals and reagents

Solvents used for chromatography were HPLC grade, viz., acetonitrile:water (1% glacial acetic acid) =80:20 (v/v). Membrane filters 0.45 µm pore size from Millipore and Whatman NYL 0.45 µm syringe filter was used for the filtration of samples. betulinic acidstandard marker compound (Sigma Aldrich, USA),

4.7.5.2: HPLC instrument and chromatographic conditions

HPLC system consisted of a Waters (Milford, MA, USA) 600 quaternary HPLC pump, a Rheodyne-7725i injection valve (USA) with a sample loop of 20 µL, a Waters 2489 UV-Vis dual wavelength detector and the max-plot containing the peaks were obtained using Empower™2 software. A Waters Spherisorb (Ireland) C18 column (250 × 4.6 mm, 5 µm particle size) was used as stationary phase. Mobile phase composition was optimized as (channel A) water: (channel B) acetonitrile by means of isocratic elution (1mL/min) and detection at 272nm. Analysis of the betulinic acid, methanol extracts and fractions were performed using the above protocol and the chromatograms were recorded. Peaks were identified by comparison of retention times and peak area (response) of standard compounds and extract.

4.7.5.3: Standard and sample solution

A primary stock solution (1 mg/mL) containing betulinic acid was prepared by dissolved in methanol and subsequently diluted to 10-500 µg/mL and was used for mobile phase optimization. Methanol extract of *A. concinna* was re-dissolved (1 mg/mL) using mobile phase and was filtered through 0.45 µm syringe filter prior to injection into the HPLC column. Amount of betulinic acid present in the methanol extract was calculated after triplicate injection (n=3) by comparing and interpolating the extract peak area (response) with respect to the standard marker.

4.7.5.4: Calibration curve (linearity)

The linear calibration plot of betulinic acid was constructed by means of linear regression analysis between peak areas and concentrations in the range of 10-500 µg/mL of standard solution. The amount of standard compound in the solution was obtained by constructing the calibration curve of standard.

4.7.5.5: Specificity

Chromatograms of betulinic acid and hydro-alcoholic extract were verified in terms of specificity; according to the ICH recommendation to minimize the errors possibly happens during the experiment or the contamination of the sample with impurity. The purity of the peaks was checked by multivariate analysis by comparison of retention times and peak area of standard compounds and extract.

4.7.5.6: Limits of detection and quantification (LOD and LOQ)

The LOD and LOQ were calculated by the method based on the standard deviation (σ) and the slope (S) of the calibration plot, using the formula LOD = 3:1σ/S and LOQ = 10:1σ/S (ICH, 2005 and FDA, 1994); where, σ = standard deviation of the response from a number of blank run and S = slope of the calibration plot.

4.7.5.7: Robustness

The robustness of the proposed method was investigated by analyzing the samples under different experimental conditions such as variation of flow rate, mobile phase composition, detection wavelength, column temperature and using different column of same configuration to determine their influence on the retention time.

4.7.5.8: Results

The retention time (R_t) of betulinic acid was found to be 9.489 min. The calibration curves were obtained by plotting peak areas against concentrations. Standard compound exhibited good linearity in the concentration range of 10 - 100 µg/ml. The correlation coefficient (r^2) was estimated to be 0.99864. Instrumental precision was noted account by repetitive injection of the same sample of three concentrations.

The relative standard deviation (R.S.D.) was estimated to assess instrumental precision. Intra-assay precision was performed by making five standard solutions of three different concentrations and measuring the R.S.D. values. The R.S.D. values of both instrumental precision and intra-assay precision of betulinic acid were estimated to be <2.5%, indicating the RP-HPLC analytical method for these two standard compounds were precise. Intermediate precision of the method was optimized by calculating intra-day and inter-day repeatability. The R.S.D. values in all tested groups were found to be <3%, which is acceptable. The sensitivity of RP-HPLC method was determined by limit of

detection (LOD) and limit of quantification (LOQ). LOD is the lowest concentration of the sample, the method can detect and LOQ is the lowest concentration that can be quantified accurately by the method. The LOD and LOQ of standard compound was estimated to be 0.3 and 0.8 µg/mL for betulinic acid resulted that the method was sensitive to detect the compounds in sample.

The recovery measures the closeness between the theoretically added amount and the practically obtained amount and was performed by spiking with a known amount of standard compounds in sample. The recovery of betulinic acid at different concentrations was found to be 90 to 105% with R.S.D. values <2% (data not shown). It indicates that standard compound can be fully recovered in the crude extract. The amount of betulinic acid present in extract was estimated to be about 0.96% (w/w), respectively. The percentage amounts of standard constituents present in the crude extract were expressed by its weight (µg/mg).

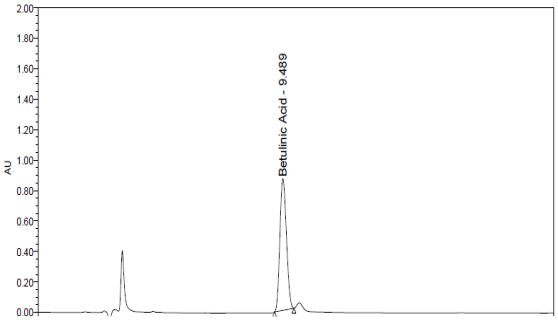


Fig 4.7.d: RP-HPLC Chromatogram of betulinic acid

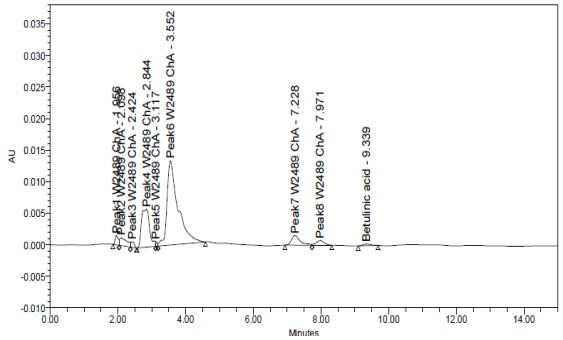


Fig 4.7.e: RP-HPLC Chromatogram of *Acacia concinna*

Chapter 5

Development and evaluation of polyherbal cream

5.1. Need for herbal cream

5.2: Selection of Botanicals

5.3: Development of polyherbal cream

5.4. Evaluation parameter of polyherbal cream

5.5: Evaluation of Marketed Formulations

5.1: Need for herbal cream

A glowing skin, voluptuous and shiny hair reflects general well-being. For such purposes, consumers spend enormous sums on skin-care and hair care products. Consumer needs may be divided into two sections: protection and repair/treatment. At present a variety of personal care products are available according to individual's preference. Conventional drugs like ZPTO may be excreted through breast milk in lactating mothers and in turn is transferred to the new born causing severe allergic manifestations. Such manifestations have also been noticed in adults who have had severe allergic reactions accompanied by skin irritations. Ketoconazole is a favourite among dermatologists and has been found to cause abnormal hair texture, discolouration, irritation, small eruptions on the scalp, etc. So there is a need to look for alternatives which are present around us in nature, explore their anti dandruff property and thereby search for leads which would be safer to the above mentioned synthetic derivatives. Herbs/herbal extracts are an ancient methodology as its references have been discovered in holy Vedas and in Unani scriptures. Herbals extracts are processed for curing several remedies and serve other health prospective. Treatment of many herbs can be used for topical pathological manifestations because of their wide safety range. Several botanical treatments for cutaneous diseases have stood the test of time for their effectiveness as documented by modern scientific evidence. Herbals extracts are processed for curing several remedies and serve other health prospective. Treatment of many herbs can be used for topical pathological manifestations because of their wide safety range. Several botanical treatments for cutaneous diseases have stood the test of time for their effectiveness as documented by modern scientific evidence.

Formulations are applied topically mainly for their local action. Topical cleansing cosmetic formulations are presented in liquid and semi-solid dosage forms. These are often most prescribed for management of hair and skin disorders because of their lower side effects, cost and availability. These are easier to use and comfortable to apply. Out of them shampoos, body washes, face washes and hand washes have gained popularity in the personal care segment.

5.2: Selection of Botanicals:

Tagetes erecta (Marigold) contains many potent antioxidants and anti-inflammatory compounds that fight infections, decrease swelling, improve blood flow, reduce muscle spasm, slow down effects of free radical damage/aging and more.

The miraculous healing properties of holy basil come mainly from its essential oils and the phytonutrients in it. Holy basil is an excellent antibiotic, germicidal, fungicidal, and disinfectant agent and very effectively protects our body from all sorts of bacterial, viral, and fungal infections.

Cucumis sativus (cucumber) fruit extract is used in cosmetics and personal care products due to its antioxidant, anti-inflammatory, and skin-conditioning properties.

Papaya has skin lightening properties that help clear blemishes and pigmentation. The enzyme papain, along with the alpha-hydroxy acids, acts as a powerful exfoliator and dissolves inactive proteins and dead skin cells. This, in turn, can make your skin lighter and softer.

5.3: Development of polyherbal cream

A cream is a preparation usually for application to the skin. Creams for application to mucous membranes such as those of the rectum or vagina are also used. Creams may be considered pharmaceutical products as even cosmetic creams are based on techniques developed by pharmacy and unmedicated creams are highly used in a variety of skin conditions (dermatoses). The use of the finger tip unit concept may be helpful in guiding how much topical cream is required to cover different areas.

Creams are semi-solid emulsions of oil and water. They are divided into two types: oil-in-water (O/W) creams which are composed of small droplets of oil dispersed in a continuous water phase, and water-in-oil (W/O) creams which are composed of small droplets of water dispersed in a continuous oily phase. Oil-in-water creams are more comfortable and cosmetically acceptable as they are less greasy and more easily washed off using water. Water-in-oil creams are more difficult to handle but many drugs which are incorporated into creams are hydrophobic and will be released more readily from a water-in-oil cream than an oil-in-water cream. Water-in-oil creams are also more moisturising as they provide an oily barrier which reduces water loss from the stratum corneum, the outermost layer of the skin.

5.3.1: Requirements of a cream

- The provision of a barrier to protect the skin
- This may be a physical barrier or a chemical barrier as with sunscreens
- To aid in the retention of moisture (especially water-in-oil creams)
- Cleansing
- Emollient effects
- As a vehicle for drug substances such as local anaesthetics, anti-inflammatories (NSAIDs or corticosteroids), hormones, antibiotics, antifungals or counter-irritants.

Creams are semisolid dosage forms containing more than 20% water or volatile components and typically less than 50% hydrocarbons, waxes, or polyols as vehicles. [Thera, 1944, 82, 377-90.] They may also contain one or more drug substances dissolved or dispersed in a suitable cream base. This term has traditionally been applied to semisolids that possess a relatively fluid consistency formulated as either water-in-oil (e.g., cold cream) or oil-in-water (e.g., fluocinolone acetonide cream) emulsions. However, more recently the term has been restricted to products consisting of oil-in-water emulsions or aqueous microcrystalline dispersions of long-chain fatty acids or alcohols that are water washable and more cosmetically and aesthetically acceptable.

Creams can be used for administering drugs via the vaginal route (e.g., Triple Sulfa vaginal cream). Creams are also used to treat sun burns (J Dermatol 2009; 36:583–6.)

5.3.2: Selection of ingredients for the cream base

For selection of ingredients for the cream base extensive literature review and marketed formulations were surveyed. The cream essentially contains

- Humectants, emollient and moisture
- Cleansing ingredient
- Stabilizing agent and thickening agent
- emulsifier
- Active ingredient
- Preservatives

The following ingredients were selected for the development of the cream base.

Table 5.A: Ingredients for cream base

Sl.No.	Name	CAS No.	Role
1.	Ethyl alcohol	9004-82-4	Cleansing ingradient
2.	Glycerin	8043-29-6	Humectant
3.	Stearic acid Cetyl alcohol	50-70-4	stabilizer, thickner
4.	EDTA	6381-92-6	Sequestering Agent, co-preservative, pH stabilizer
5.	Methyl paraben	9003-01-4	Prreservative
6.	Glycol stearate		Opacifier

5.3.3: Formulae for polyherbal cream used in the study

No. 1 – Cream-A

Table 5.B: Formula for Cream-A

Sl.No.	**Name**	**Quantity**
1.	Steric acid	**10%**
2.	Cetosteryl alcohol	**2%**
3.	Olive oil	**2%**
4.	EDTA	**0.1%**
5.	Triethanolamine	**1.3%**
6.	Propylene glycol	**5%**
7.	Glycol	**5%**
8.	Rose water	**.250ml**
9.	Carica papaya hyro-alcoholic extract	**50 mg**

10.	Propyl paraben	**2%**
11.	Distilled water	**Upto 50ml**

No. 2 – Cream-B

Table 5.C: Formula for polyherbal Cream-B

Sl.No.	**Name**	**Quantity**
1.	Steric acid	**10%**
2.	Cetosteryl alcohol	**2%**
3.	Olive oil	**2%**
4.	EDTA	**0.1%**
5.	Triethanolamine	**1.3%**
6.	Propylene glycol	**5%**
7.	Glycol	**5%**
8.	Rose water	**.250ml**
9.	Carica papaya hyro-alcoholic extract	**500 mg**
10.	Tagetes erecta hydro alcoholic extract	**500mg**
11.	Propyl paraben	**2%**
12.	Distilled water	**Upto 50ml**

No. 3 – Cream-C

Table 5.C: Formula for polyherbal Cream-C

Sl.No.	**Name**	**Quantity**
1.	Steric acid	**10%**
2.	Cetosteryl alcohol	**2%**
3.	Olive oil	**2%**
4.	EDTA	**0.1%**
5.	Triethanolamine	**1.3%**
6.	Propylene glycol	**5%**
7.	Glycol	**5%**
8.	Rose water	**.250ml**
9.	Cucumis sativas aquous extract	**2ml**
10.	Oscimum sanctum hydro alcoholic extract	**500mg**
11.	Propyl paraben	**2%**
12.	Distilled water	**Upto 50ml**

5.3.4: Procedure involved in the making of polyherbal cream

Prepare three separate phases A , B and C :

Phase A: Oil phase

- In a clean beaker stearic acid, cetosteryl alcohol and olive oil melted together

Phase B: water phase

- Triethanolamine, propylene glycol, EDTA is mixed together with water.

Phase C:

- Add the weighed amount plant extracts to the oil or water phase depending on their solubility.
- Phase A was transferred to a clean beaker and was stirred using a mechanical controlled stirrer (REMI). To it slowly Phase B was added with continuous stirring. The mixture was stirred to obtain a uniform blend. Once a uniform mixture was obtained it was transferred to a container and adequately labeled.

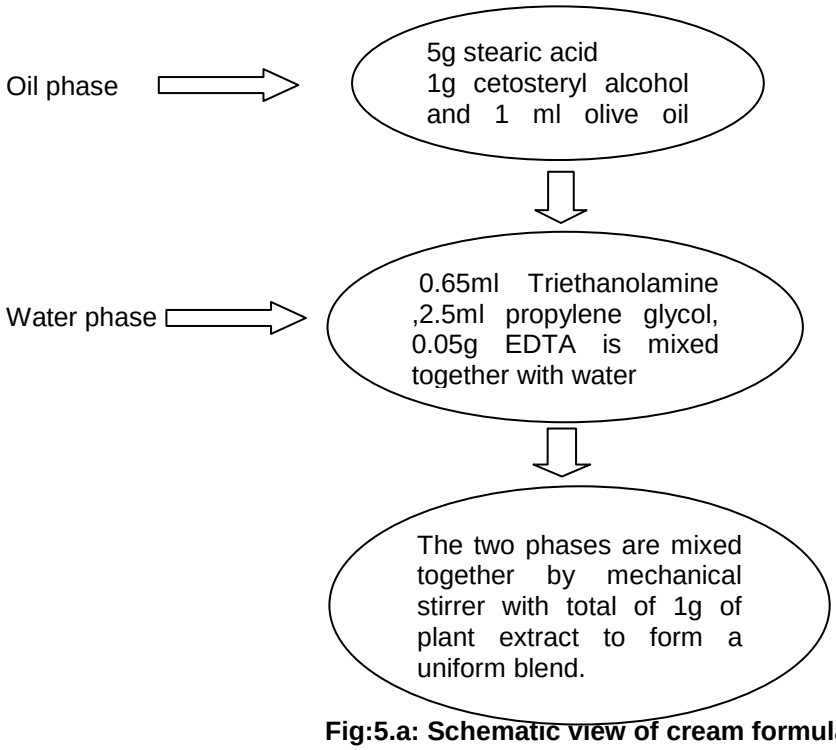


Fig:5.a: Schematic view of cream formulation

5.4. Evaluation parameter of polyherbal cream:

- **pH of the Cream**

The pH meter was calibrated using standard buffer solution. About 0.5g of the cream was weighed and dissolved in 50.0 ml of distilled water and its pH was measured.

The pH of the cream was found to be in range of 5.6 to 6.8 which is good for skin pH. The herbal formulation was shown pH nearer to skin required i.e pH 6.3.

- **Viscosity**

Viscosity of the formulation was determined by Brookfield Viscometer at 100 rpm, using spindle no.4.

The viscosity of cream was in the range of 28001 – 27025 cps which indicates that the cream is easily spreadable by small amounts of shear. The herbal formulation was shows viscosity within the range.

- **Dye test**

The scarlet red dye is mixed with the cream. Place a drop of the cream on a microscopic slide covers it with a cover slip, and examines it under a microscope. If the disperse globules appear red the ground colourless. The cream is o/w type. The reverse condition occurs in w/o type cream i.e. the disperse globules appear colourless in the red ground.

- **Homogeneity**

The formulations were tested for the homogeneity by visual appearance and touch.

- **Appearance**

The appearance of the cream was judged by its colour, pearlscence and roughnes s and graded. When formulation were kept for long time, it found that no change in color of cream.

- **After feel**

Emolliency, slipperiness and amount of residue left after the application of fixed amount of cream was checked.

- **Type of smear**

After application of cream, the type of film or smear formed on the skin were checked.

- **Removal**

The ease of removal of the cream applied was examined by washing the applied part with tap water.

- **Saponification value**

Introduce about 2 gm of substance refluxed with 25 ml of 0.5 N alcoholic KOH for 30 minutes, to this 1 ml of phenolphthalein added and titrated immediately, with 0.5 N HCl. Saponification value = (b-a)*28.05/w a - volume in ml of titrant, b - Volume in ml of titrant, w - Weigh of substance in gm.

- **Acid value**

Take 10 gm of substance dissolved in accurately weighed, in 50 ml mixture of equal volume of alcohol and solvent ether, the flask was connected to reflux condenser and slowly heated, until sample was dissolved completely, to this 1 ml of phenolphthalein added and titrated with 0.1N

NaOH, until faintly pink color appears after shaking for 30 seconds.

Acid value = n*5.61/w

n = the number of ml of NaOH required.

w = the weight of substance

- **Irritancy test**

Mark an area (1sq.cm) on the left hand dorsal surface. The cream was applied to the specified area and time was noted. Irritancy, erythema, edema, was checked if any for regular intervals up to 24 hrs and reported.

- **Accelerated stability testing**

Accelerated stability testing of prepared formulations was conducted for 2 most stable formulations at room temperature, studied for 7 days. They were formulation number 1 and 2 at 40 ± 100 C for 20 days. The formulations were kept both at room and elevated temperature and observed on 0th, 5th, 10th, 15th and 20th days.

5.D. Accelarated stability studies of cream -A

Time interval	Homogeneity	Type of smear	Viscosity(cp)	Physical changes	pH
0th day	+++	+++	12.005	No change in color and odour	6.1
5th day	++	+++	12.004	No change in color and odour	6.1
10th day	++	+++	12.005	No change in color and odour	6.3
15th day	++	+++	12.005	No change in color and odour	6.1
20th day	++	+++	12.005	No change in color and odour	6.2

5.E. Accelarated stability studies of cream -B

Time interval	Homogeneity	Type of smear	Viscosity(cp)	Physical changes	pH
0th day	+++	+++	12.521	No change in color and odour	5.7
5th day	+++	+++	12.498	No change in color and odour	5.7
10th day	++	+++	12.514	No change in color and odour	5.9

15th day	++	+++	12.520	No change in color and odour	5.9
20th day	++	+++	12.518	No change in color and odour	5.8

5.F. Accelarated stability studies of cream -C

Time interval	Homogeneity	Type of smear	Viscosity(cp)	Physical changes	pH
0th day	+++	+++	12.014	No change in color and odour	5.7
5th day	+++	+++	12.021	No change in color and odour	6.1
10th day	+++	+++	12.019	No change in color and odour	5.8
15th day	+++	+++	12.020	No change in color and odour	5.9
20th day	++	+++	12.018	No change in color and odour	6.1

+ = average

++ = good

+++ = excellent

- **Spreadibility test**

Sample was applied between two glass slides and was compressed to uniform thickness by placing 100gm weight for 5minutes. Weight was added to the pan. The time required to separate the two slides, i.e. the time in which the upper glass slide moved over the lower slide was taken as measure of spreadability.

Spreadability =m*l/t

m = Weight tide to upper slide

l = length moved on the glass slide

t = time taken.

Prepared polyherbal cream formulation:

Fig:5.b: Prepared polyherbal cream formulation

Chapter 6

Development and evaluation of polyherbal shampoo

6.1. Significance of shampoo

6.2: Selection of Botanicals

6.3: Development of polyherbal shampoo

6.4. Evaluation parameter of polyherbal shampoo

6.5: Evaluation of Marketed Formulations

6.1: Significance of shampoo

Shampooing is the most common form of hair treatment. Shampoos are primarily products aimed at cleansing the hair and scalp.

Types of shampoo:

1. Clear liquid shampoo
2. Liquid cream shampoo
3. Solid cream shampoo
4. Antidandruff and medicated shampoo
5. Oil shampoo
6. Powder shampoo
7. Aerosol shampoo
8. Dry shampoo (powder type)
9. Dry shampoo (liquid type)

Formulating a Shampoo

Developing a detergent mixture that is not selective, not too detersive, and well adapted to scalp and hair is a delicate compromise.The variability of the substrate and the duality of the desired aim need to be taken into consideration. Thus, multiple problems of compatibility, dosing, and balance have to be solved. To cleanse without causing dryness, to lubricate dry hair while cleansing, to impart a clean beauty or a specific treatment, to ensure a delicate sequential replacement of soil by conditioning are a multiparameter equation. This multiparameter equaequation necessarily leads to multiple solutions, according to the prevalence given to one or another criterion. It also suggests that the simplest shampoo formulation will be composed of a variety of ingredients.

A shampoo comprises the following:

1. A mixture of several surfactants, as rarely can a single surfactant ensure the required properties of wetting, emulsifying and rinsing. The

most widely used are fatty alcohol sulfates.

2. Foam stabilizers or softeners or both. Foam is an attribute highly prized by consumers. Psychologically, it is associated with the detergent

effect, but it is also an indicator that the cleansing job has been achieved (dosing element). Foaming qualities are composed of the upstart

of foam, its volume, softness, texture, stability, and its removal by rinsing. These properties are mostly brought out by adding fatty acid alkanolamides, which impartacreamyfeel and a softer and more stable foam.

3. Thickeners. Consistency and richnessare provided by natural gums (karaya, Itragacanth), cellulose hydrocolloids (hydroxymethyl or hydroxyethyl or carboxymethyl celluloses), acrylic polymers (such as carbomer), or salts such as sodium or ammonium chloride.

4. Pearlescents and opacifiers. They are added to change the appearance of shampoos, but they may also play a softening part. The most frequently used are long-chain fatty alcohol sulfates or fatty acid esters.
5. Conditioning agents. They are intended to bring softness and gloss, to reduce flyaway, and to enhance disentangling. They are particularly

useful in shampoos for dry and damaged hair. Their role in a shampoo is not as effective as that of a conditioner or a rinse because of the multiple functions a shampoo has to comply with, the likelihood of incompatibilities or even conflicts resulting in a compromise. They, however, have an essential role on the feel, appearance, manageability, and esthetic and cosmetic qualities of hair after shampooing.

A great number of compounds may be added according to the type of formulation and cleansing base, the purpose and care and beautifying aims. They are mostly fatty ingredients (fatty alcohols, lanolin derivatives, vegetable or mineral oils, lecithins), proteins, and cationic polymers. A special mention should be made here of cationic polymers, which have brought a true breakthrough in the domain of shampoos. As discussed later, cationic surfactants are the preferred agents to level out the surface of the hair, to soften it, and to improve compatibility. Such properties could not be made profitable in shampoos because of incompatibility between cationic and anionic surfactants, which are the primary cleansers. The advent of cationic polymers compatible with anionic surfactants has opened a new era for conditioning from the formulator's point of view.

6. Special care ingredients. They are aimed at modifying unesthetic events such as dandruff or greasy hair and are most often based on pyridinethione salts, piroctone olamine, sulfur-containing compounds, tars, and essential oils.
7. Chelating agents. They are intended to trap traces of metal salts in order to prevent the formation of insoluble complexes or salts, as well as catalytic decomposition of fragrance and colorants.
8. Preservatives. Preservatives are used to inhibit any bacteriologic contamination.
9. Fragrances and colorants. Fragrances and colorants are intended to individualize shampoo perception.

Surfactants used in Shampoos

Surfactants are compounds that are both lipophilic and hydrophilic. They are lipophilic by their hydrocarbon so-called fatty chain, which links to fatty materials. They are hydrophilic by the polar group that is located at the end of the fatty chain and that renders it water soluble and helps rinse away the fatty material retained. Surfactants constitute four main chemical categories according to the type of the polar end: anionic, cation &, amphoterics and nonionics.

Anionic

Their hydrophilic polar group is negatively charged. The first anionic detergent used was soap, which is a salt of a fatty acid obtained by alkali treatment of vegetable or animal greases and oils.

Soaps have two main drawbacks:

First, they tend to hydrolyze in water solution, whereby alkali is released and the pH is increased, which is detrimental both to the skin and hair surface. Second, in hard water, they precipitate into calcium salts, which get stuck onto the hair, leading to a dull appearance, brittle feel, and poor disentangling. This is the reason why soaps have almost been given up and replaced by synthetic surfactants. These compounds have a much stronger polarity and are thus freed from the shortcomings inherent in soaps formulation.

Cationics

Their polar group has a positive charge. They show high affinity for the hair fiber to which they impart softness, and compatibility while reducing fly-away. The cosmetic characteristics of cationics and their beneficial effect to hair surface condition make them attractive for dyed or bleached hair. They are, however, poor detergent and lathering agents, and they may lead to some re-deposition of the soil onto the hair. A lack of compatibility with anionic surfactants further limits the extent of potential use in shampoo formulation (Liberman and Lachman,Industrial pharmacy, 2001).

Amphoterics

They bear on the same chain both an anionic and a cationic group; therefore, they exhibit intermediary properties. They behave as cationics at lower pH values and as anionics at higher pH values. They complex with anionics and thus reduce their tendency to adsorb onto proteins. Theyare generally combined with other surfactants in the formulation of mild shampoos to control the properties and to adjust the effects according to the performances desired. The ones most often used are betaines, sultaines, and imidazolinium derivatives (marketed as Miranols). Amine oxides, which provide useful cationic properties at an acidic pH, also should be mentioned(Liberman and Lachman,Industrial pharmacy, 2001).

Nonionics

In contrast to the previous surfactants, nonionics have no polar end. The hydrophilic element in these surfactants is a repeated oxyethylenic pattern. They are generally considered as the mildest of all surfactants. They have good dispersing, emulsifying, and detergent properties, but their use has been restricted because of poor foaming potential. They serve more often as auxiliary detergents. Their high skin tolerance has found application in their combination with alkylether sulfatesor amphoterics in the formulation of very mild shampoos.The compounds best known are polyoxyethylene fatty alcohols and polyoxyethylene sorbitol esters (marketed as Tweens [ICI Americas, Wilmington, DE]). More recently, another class of nonionics has been developed that show good foaming properties together with mildness for the scalp and cleansing efficiency. They contain a polyglycerol moiety instead of polyoxyethylene as hydrophilic part.In addition to these cleansing compounds,other nonionics called alkanolamides are worth mentioning. They are used as foam boosters or stabilizers or as thickeners (Liberman and Lachman,Industrial pharmacy, 2001).

Natural Surtactants

They mainly consist of saponins, which are found in various vegetal species, namely, soap bark, soapwort, sarsaparilla, and ivy. The hydrophilic component is a sugar, which likens them to nonionics, but their lipophilic moiety, either a steroid or a triterpene, often bears an acidic or basic group. Such is the case with soapbark, whose lipophilic moiety is the quillaic acid. These saponins make a very good lather but have less than average cleansing properties. High concentrations are therefore needed to yield sufficient detergency. At these concentrations, saponins are not devoid of aggressiveness and may even show unfavorable cosmetic characteristics. As a consequence, natural surfactants are often associated with synthetic surfactants to ensure good cleansing and satisfactory cosmetic quality (Liberman and Lachman,Industrial pharmacy, 2001).

Preservatives:

The material recommended includes-

p-hydroxy benzoic acid and its ester

Formaldehyde

Phenyl mercuric ester

2-bromo nitropropane 1:2 di ol

6.2: Selection of Botanicals:

Acacia concinia (Shikakai) has antifungal properties that help get rid of dandruff for good. This prevents issues like follicle-clogging and aggravation, leading to unhindered hair growth. It also effectively deals with dryness and itching.

Lawsonia inermis (Henna) The natural ingredients in henna do not cause hair fall, and it actually promotes better hair growth. It is an excellent hair conditioner that works like other synthetic conditioners.

Tagetes erecta (Marigold) contains many potent antioxidants and anti-inflammatory compounds that fight infections, decrease swelling, improve blood flow, reduce muscle spasming, slow down effects of free radical damage/aging and more.

Ayurveda recognises the hibiscus flower as an important source for hair growth. The leaves, roots and flowers have various medicinal properties.

The miraculous healing properties of holy basil come mainly from its essential oils and the phytonutrients in it. Holy basil is an excellent antibiotic, germicidal, fungicidal, and disinfectant agent and very effectively protects our body from all sorts of bacterial, viral, and fungal infections.

6.3: Development of polyherbal Shampoo

Washing the hair and scalp has become a near-universal practice. The method of doing so varies depending on both geographic and economic factors. Shampoos assumed importance as a product category with the advent of synthetic detergents. These were developed in the 1930s, became widely used in laundry markets by the mid-1940s and appeared in a shampoo format during the

1950s. Shampoos are probably the most widely used hair products today; based on synthetic detergents they are relatively insensitive to water hardness, thus allowing for efficient rinsing since there are no scum residues. In the early days a shampoo could be defined as an effective cleansing agent for hair and scalp, but today the shampoo must do much more. It must leave the hair easy to comb, lustrous and controllable whilst being convenient and easy to use.

6.3.1: Requirements of a shampoo

1. To remove sebum (the secretion of the sebaceous glands) and atmospheric pollutants from the hair and scalp.
2. To remove the residues of previously applied hair treatments, e.g. polymeric constituents from styling lotions and hair sprays.
3. To deliver an optimum level of foam to satisfy the expectation of the user.
4. To leave the hair in a satisfactory condition after rinsing so that it can be combed easily both in the wet and dry state.
5. To perform as a vehicle for the deposition of beneficial materials onto the hair and scalp.
6. To be non-toxic and non-irritating to the hair and the scalp.
7. To be non-damaging to the tissues of the eye if inadvertently splashed.

6.3.2: Selection of ingredients for the shampoo base

For selection of ingredients for the shampoo base extensive literature review and marketed formulations were surveyed. The shampoo essentially contains

- Main detergents
- Foam boosters and stabilizers
- Opacifiers
- Viscosity modifiers, including hydrocolloids and electrolytes
- Special additives for hair condition
- Special additives for scalp health, including antidandruff additives
- Sequestering agents.

The following ingredients were carefully selected for the development of the shampoo base.

Table 6.A: Ingredients for Shampoo base

S.l. No.	Name	CAS No.	Role
1.	Sodium Lauryl Ether Sulphate	9004-82-4	Main Detergent
2.	Glycerin	8043-29-6	Humectant
3.	Sorbitol	50-70-4	Humectant, stabilizer, conditioner, thickner
4.	EDTA	6381-92-6	Sequestering Agent,

			co-preservative, pH stabilizer
5.	Coco Betaine	61789-40-0	Co-surfactant, foam booster, viscosity enhancer, conditioning agent, antistatic agent, moderate emulsifier.
6.	Sodium Benzoate	9003-01-4	Prreservative
7.	Sodium chloride	7647-14-5	Thickner
8.	Glycol stearate		Opacifier

6.3.3: Formulae for Shampoos

No. 1 – SH-A

Table 6.B: Formula for polyherbal shampoo SH-A

S.l. No.	Name	Quantity
1.	Sodium Lauryl Sulphate	**25%**
2.	Glycerin	**2%**
3.	Sorbitol	**5%**
4.	EDTA	**0.1%**
5.	Coco Betaine 30% v/v	**10%**
6.	Sodium Benzoate	**1%**
7.	Sodium chloride	**2%**
8.	*Oscimum sanctum* hyro-alcoholic Extract	**500 mg**
9.	*Lawsonia innermis* hyro-alcoholic Extract	**50 mg**
10.	*Acacia Concinna* hyro-alcoholic Extract	**50 mg**
11.	Methyl paraben	**0.01 g**
12.	Distilled water	**Upto 50ml**
13.	Fragrance	**q.s**

No. 2 – SH-B

Table 6.C: Formula for polyherbal shampoo SH-B

S.l. No.	Name	Quantity
1.	Sodium Lauryl Sulphate	**25%**
2.	Glycerin	**2%**
3.	Sorbitol	**5%**
4.	EDTA	**.1%**

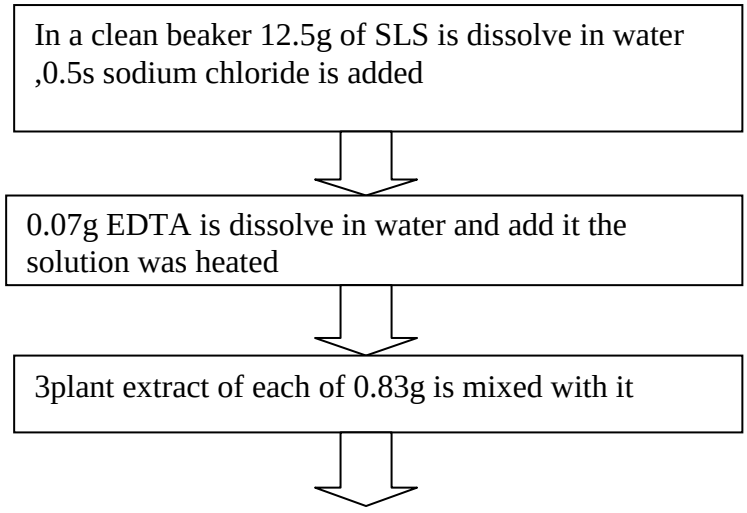
5.	Sodium Benzoate	**1%**
6.	Sodium chloride solution 0.5%	**2ml**
7.	Glycol stearate	**2%**
8.	*Hibiscus rosa sinensis* hyro-alcoholic Extract	**500mg**
9.	Olive oil	**0.6%**
10.	Coconut oil	**0.1%**
11.	Distilled water	**Upto 50ml**
12.	Fragrance	**q.s**

No. 3 – SH-C

Table 6.D: Formula for polyherbal shampoo SH-C

S.l. No.	**Name**	**Quantity**
1.	Sodium Lauryl Sulphate	**25%**
2.	Glycerin	**2%**
3.	Sorbitol	**5%**
4.	EDTA	**.1%**
5.	Coco Betaine	**10%**
6.	Sodium Benzoate	**1%**
7.	Ethyl alcohol	**2ml**
8.	Sodium chloride	**2%**
9.	Glycol stearate	**2%**
10.	Olive oil	**0.50ml**
11.	Coconut oil	**0.50 ml**
12.	Castor oil	**0.50ml**
13.	Distilled water	**Upto 50ml**

6.3.4: Procedure involved in the making of shampoo



0.0125g methyl paraben, 2.5ml glycerine,2.5ml sorbitol, 1g sodium benzoate is added on it and volume make upto 50 ml

Fig 6.a: Schematic diagram of shampoo formulation

Prepare three separate phases A,B and C:

Phase A: SLES, Coco Betaine, Glycol stearate.

- In a clean beaker SLES was dissolved in water and to it slowly coco betaine solution was added with mild stirring. To it 2ml of 0.5% NaCl solution was added.
- EDTA was dissolved in sufficient amount of water and added to the mixture. The formed mixture was heated to 60ºC with mild stirring.
- In a clean beaker glycol stearate was heated to 60ºC and added to the above mixture.
- Avoid formation of foam.

Phase B: Sodium benzoate, fragrance and colour.

- Sodium benzoate was dissolved in sufficient amount of water.
- Add fragrance and non-bleeding colour.

Phase C: Sorbitol, glycerine and plant extracts.

- Dissolve sorbitol in sufficient amount of water and add glycerine.
- Add the weighed amount plant extracts to the above mixture and heat-sonicate for 15 min.

Maintain Phase A, B and C at 40ºC.

Phase A was transferred to a clean beaker and was stirred using a mechanical controlled stirrer (REMI) and the RPM was optimized to avoid formation of foam and appearance changes. To it slowly Phase C was added with continuous stirring. The mixture was stirred to obtain a uniform blend. To it Phase B was added slowly with continuous stirring. Volatile oils were added with continuous stirring. Check pH and add citric acid solution if necessary. Once a uniform mixture was obtained it was transferred to a bottle and adequately labeled.

6.5.Evaluation of polyherbal shampoo:

- **Physical appearance/visual inspection:**

The formulations prepared were evaluated in terms of theirclearity, thick, semi white transparent in colour, great foam producing ability and fluidity.

- **Determination of pH:**

The pH = 6.5 of 10% herbal HS solution in distilled water was determined at room temperature 25°C.

- **Determine percent of solids contents:**

A clean dry evaporating dish was weighed and added 1grams of HS to the evaporating dish. The dish and HS was weighed. The exact weight of the HS was calculated only and put the evaporating dish with HS was placed on the hot plate until the liquid portion was evaporated. The 0.01g weight of the HS only (solids) after drying was calculated.

- **Wetting time:**

The canvas was cut into 1-inch diameter discs having an average weight of 0.44g. The disc was floated on theSurface of HS solution 1%w/v and the stopwatch started. The time required for the disc to begin to sink was measured accurately and noted as 3 sec wetting time.

- **Foam Formation (Shake Test):**

Took 50 ml of the 1% shampoo solution in a 250 ml graduated cylinder and recorded the volume. Then covered the cylinder with hand and shaken 10 times. The total volume of the contents was recorded after shaking. Calculated the volume of the foam and recorded the size of the bubbles.

- **Foam Quality and Retention:**

Immediately after the Shake Test (Test C), time was recorded. Recorded the volume of foam at 1-minute intervals for 5 minutes.

- **Rheological evaluations:**

The viscosity of the HS was determined by using Brookfield Viscometer set at different spindle speeds from 0.3 to 10 rpm3. The viscosity of the HS was measured by using spindle T95. The temperature and sample container's size was kept constants during the study.

- **Dirt dispersion**:

Two drops of HS were added in a large test tube contain 10 ml of distilled water. 1 drop of India ink was added; the test tube was stoppered and shakes it ten times. The amount of ink in the foam was estimated as Light.

- **Cleaning action:**

5 grams of wool yarn were placed in grease, after that it was placed in 200 ml. of water containing 1 gram of HS in a flask. Temperature of water was maintained at 35 . The flask was shaked for 4 minutes at the rate of 50 times a minute. The solution was removed and sample was taken out,dried and weighed. The amount of grease removed was calculated by using the following equation.

DP=100(1-T/C)

In which, DP is the percentage of detergency power, 1g is the weight of sebum in the control sample and the cleansing action HS is found to be 15 %.

- **Surface tension measurement:**

Measurements were carried out with a 10% HS dilution in distilled water at room temperature. Thoroughly clean the stalagmometer using chronic acid and purified water. Because surface tension is highly affected with grease or other lubricants.

$R_2=[(W_3-W_1)n_1/(W_2-W_1)n_2] \times R_1$

Where,

W_1 is weight of empty beaker.

W_2 is weight of beaker with distilled water.

W_3 is Weight of beaker with HS solution.

N_1 is no. of drops of distilled water.

N_1 is no. of drops of HS solution.

R_1 is surface tension of distilled water at room temperature.

R2 is surface tension of HS solution.

- **Detergency ability:**

The Thompson method was used to evaluate the detergency ability of the samples. Briefly, a crumple of hair were washed with a 5% sodium lauryl sulfate (SLS) solution, then dried and divided into 3g weight groups. The samples were suspended in a nhexane solution containing 10% artificial sebum and the mixture was shaken for 15 minutesat room temperature. Then samples were removed, thesolvent was evaporated at room temperature and theirsebum content determined. In the next step, each samplewas divided into two equal parts, one washed with 0.1 ml ofthe 10% test HS and the other considered as thenegative control. After drying, the resided sebum onsamples was extracted with 20 ml n-hexane and reweighed. Finally, the percentage of detergency power was calculated using the following equation:

DP=100(1-T/C)

- **Skin irritation test:**

Applied the solution of prepared shampoo on skin and kept for 5 min and observed for redness of skin and irritation there, were no any red coloration and the irritation to the skin.

- **Visual stability:**

The prepared shampoo was tested for the visual stability for 21 days at room temperature with relative humidity 65±5, and observed for color change and pH. There were no changes in color and pH of shampoo within 21 days and no any phase separation between oil and water.

- **Viscosity:**

Viscosity was determined by using the Ostwald viscometer.

Table 6.5.A: Evaluation of Shampoo

Formulation No.	Extractive values (%w/w)	Moisture content	pH	Cleaning action (%)	Foaming capacity					Dirt dispersion	Wetting time	Nature of hair after wash	Pour-ability
					0 (min)	5 (min)	30 (min)	60 (min)	Avg. Value				
SH-A	23.1	65.2	5.8	33.6	276.2	208.5	186.5	173.8	211.25	Good	2m 22 s	Soft, manageble	Good
SH-B	25.6	68.6	5.7	31.6	261.8	204.3	177.4	164.5	202	Moderate	2m 46s	Soft, manageble	Good
SH-C	24.8	70.3	6.2	30.6	254.6	201.3	174.9	161.8	198.1	Moderate	2min 56s	Soft, manageble	Good
SH-M	27.2	73.6	6.0	28.5	252.3	200.2	165.5	148.5	191.2	Good	3m 01s	Soft, manageble	Good

Table 6.5.B: Stability testing of Shampoo

Formulation No.	Ph	Foaming capacity	Pour-ability	Viscosity (η Pa/s)	Total Viable count (*10^2cfu/ml)
SH-A	5.8,5.8,5.83	211.25, 210.8, 209, 9	Good, Good, Good	2.27, 2.21, 2.18	0, 0, 0
SH-B	5.7, 5.7, 5.74	202, 200.6, 197	Good, Good, Good	2.06, 1.98, 1.65	0, 0, 1
SH-C	6.2, 6.23, 6.31	198.1, 197.3, 195.2	Good, Good, Good	2.13, 2.06, 1.93	0, 0, 1

6.5.2: Evaluation of Marketed Formulations.

The above prepared Formulations were compared with similar formulations available in the commercial market. These were evaluated on the above mentioned parameters.

Evaluation of commercial Shampoo (SH-1)

A commercial shampoo (SH-M) was selected out of the big list of consumer products available. SH-M was procured from the local departmental store, Its constituents are are follows:

Aqua, SLS, COCO betaine, sodium chloride, polysorbate-80, piroctone olamine, panthenol, sodium benzoate, citric acid, propylene glycol, dimethicone propyl pg betaine, PEG-120, Methyl glucose dioleate, guar, Hydroxypropyl trimonium chloride, sodium salicylate, Disodium EDTA, Linalool, Citronellol, citral, benzyl alcohol, Salix alba bark extract, betula alba bark extract and Zingiber officinale root extract. The results have been summarized in table.

6.5.3: Results and Discussion:

Herbal extracts and volatile oils were used to prepare topical cleansing preparations for maintaining, hygiene and beauty. Details about prepared formulations and marketed formulations are as follows:

- Moisture content was determined for all twelve prepared formulations and three marketed formulations. In the Shampoo section SH-A had the lowest moisture content of 23.1% whereas the marketed formulation SH-M had a content of 27.2% w/w. It is believed that moisture is a cause of microbial growth and hence will interfere with the stability of the formulation on storage. Therefore SH-A and Cream-A are less prone to degradation.
- The pH of the formulations should be in between 5.5 to 6.5 for shampoos and from 5.5 to 6.0 for rest of the formulations else irritation and rashes may develop. All the formulations tested, i.e., marketed and prepared, were in the range, and hence are good candidates for consumer complianc

Prepared Shampoo formulation:

Fig 6.c: Diagram of prepared polyherbal shampoo formulation

Chapter 7 Development and evaluation of polyherbal lotion

7.1. Need for using lotion

7.2: Selection of Botanicals

7.3: Development of polyherbal lotion

7.4. Evaluation parameter of polyherbal lotion

7.5: Evaluation of Marketed Formulations

7.1: Need for using polyherbal lotion

Many confuse moisture and hydration. Moisture holds hydration in and hydration is water. Moisturizers act as a shield to keep water from evaporating away from the skin- it's sort of like how you would keep bread covered so it doesn't get all tough and stale from sitting out. Our skin can become tough and cracked from drying out without a moisturizer. Dry skin will cause fine wrinkles and eventually deep ones and breakouts. Dry and sensitive skinned people certainly know the feeling of being dried out. Oily skin types tend to think they don't need moisturizer because most moisturizers are way to heavy for them and either break them out or make them feel very oily. It can become a slippery slope because they'll want to keep their faces very clean to keep the oil away but then won't use a moisturizer and leave their skin exposed to loose it's water. Dermalogica carries two great oily skin moisturizers: Active Moist and Oil Control Lotion. I'm sure if you google oil skin moisturizer there are plenty of other great products to suit our needs.

7.2: Selection of Botanicals:

Tagetes erecta (Marigold) contains many potent antioxidants and anti-inflammatory compounds that fight infections, decrease swelling, improve blood flow, reduce muscle spasming, slow down effects of free radical damage/aging and more.

Cucumis sativus (cucumber) fruit extract is used in cosmetics and personal care products due to its antioxidant, anti-inflammatory, and skin-conditioning properties.

Papaya has skin lightening properties that help clear blemishes and pigmentation . ... The enzyme papain, along with with the alpha-hydroxy acids, acts as a powerful exfoliator and dissolves inactive proteins and dead skin cells. This, in turn, can make your skin lighter and softer.

7.3: Development of polyherbal Lotion:

A lotion is a low-viscosity topical preparation intended for application to the skin. By contrast, creams and gels have higher viscosity, typically due to lower water content. Lotions are applied to external skin with bare hands, a brush, a clean cloth, or cotton wool.While a lotion may be used as a medicine delivery system, many lotions, especially hand lotions and body lotions are meant instead to simply smooth, moisturize, soften and perhaps perfume the skin.

7.3.1: Requirements of a lotion

This medication is used as a moisturizer to treat or prevent dry, rough, scaly, itchy skin and minor skin irritations (e.g., diaper rash, skin burns from radiation therapy). Emollients are substances that soften and moisturize the skin and decrease itching and flaking. Some products (e.g., zinc oxide, white petrolatum) are used mostly to protect the skin against irritation (e.g., from wetness).

Dry skin is caused by a loss of water in the upper layer of the skin. Emollients/moisturizers work by forming an oily layer on the top of the skin that traps water in the skin. Petrolatum, lanolin, mineral oil and dimethicone are common emollients. Humectants, including glycerin, lecithin, and propylene glycol, draw water into the outer layer of skin. Many products also have ingredients that soften the horny substance (keratin) that holds the top layer of skin cells together (e.g., urea, alpha hydroxy acids such as lactic/citric/glycolic acid, and allantoin). This helps the dead skin cells fall off, helps the skin keep in more water, and leaves the skin feeling smoother and softer**.**

7.3.2: Selection of ingredients for the lotion base

For selection of ingredients for the lotion base extensive literature review and marketed formulations were surveyed. The lotion essentially contains

- Humectant, emolient and moisture
- Cleansing ingredient
- Stabilizing agent and thickening agent
- emulsifier
- Active ingredient
- Preservatives

The following ingredients were carefully selected for the development of the lotion base.

Table 7.3A: Ingredients for Lotion base

S.l. No.	**Name**	**CAS No.**	**Role**
1.	Ethyl alcohol	9004-82-4	Cleansing ingradient
2.	Glycerin	8043-29-6	Humectant
3.	Stearic acid Cetyl alcohol	50-70-4	stabilizer, thickner
4.	EDTA	6381-92-6	Sequestering Agent, co-preservative, pH stabilizer
5.	Methyl paraben	9003-01-4	Prreservative
6.	Glycol stearate		Opacifier

7.3.3: Formulae for polyherbal Lotion

No. 1 – Lotion-A

Table 7.3.B: Formulation of lotion-A

S.l. No.	Name	Quantity
1.	Stearic acid	**10%**
2.	Cetosteryl alcohol	**2%**
3.	Olive oil	**2%**
4.	EDTA	**0.1%**
5.	Triethanolamine	**1.3%**
6.	Propylene glycol	**5%**
7.	Glycol	**5%**
8.	*Carica papaya* hydro alcoholic extract	**800mg**
9.	*Tagetes erecta* hydro alcoholic extract	**700mg**
10.	Propyl paraben	**2%**
11.	Distilled water	**Upto 100ml**

No. 2 – Lotion-B

Table 7.3.C: Formula for polyherbal Lotion-B

S.l. No.	Name	Quantity
1.	Stearic acid	**10%**
2.	Cetosteryl alcohol	**2%**
3.	Olive oil	**2%**
4.	EDTA	**0.1%**
5.	Triethanolamine	**1.3%**
6.	Propylene glycol	**5%**
7.	Glycol	**5%**
8.	Coconut oil	**1ml**
9.	Olive oil	**1ml**
10.	Propyl paraben	**2%**
11.	Distilled water	**Upto 100ml**

No. 3 – Lotion-C

Table 7.3.D: Formula for polyherbal Lotion-C

S.l. No.	Name	Quantity
1.	Stearic acid	**10%**
2.	Cetosteryl alcohol	**2%**
3.	Olive oil	**2%**
4.	EDTA	**0.1%**
5.	Triethanolamine	**1.3%**

6.	Propylene glycol	**5%**
7.	Glycol	**5%**
8.	Tagetes erecta hydro alcoholic extract	**700mg**
9.	Rose water	**.250ml**
10.	Propyl paraben	**2%**
11.	Distilled water	**Upto 100ml**

7.3.4: Procedure involved in the making of Lotion:

Prepare three separate phases A ,B and C :

Phase A: Oil phase

- In a clean beaker stearic acid ,cetosteryl alcohol and olive oil melted together

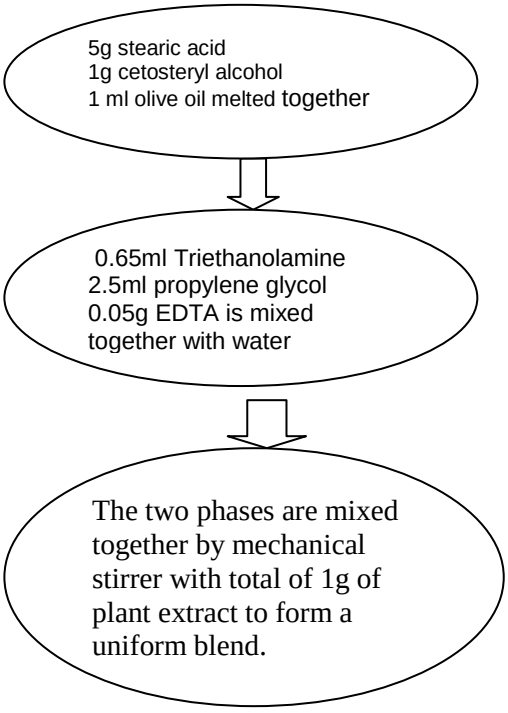
Phase B: water phase

- Triethanolamine ,propylene glycol, EDTA is mixed together with water.

Phase C:

- Add the weighed amount plant extracts to the oil or water phase depending on their solubility.

Phase A was transferred to a clean beaker and was stirred using a mechanical controlled stirrer (REMI). To it slowly Phase B was added with continuous stirring. The mixture was stirred to obtain a uniform blend. Once a uniform mixture was obtained it was transferred to a container and adequately labeled.



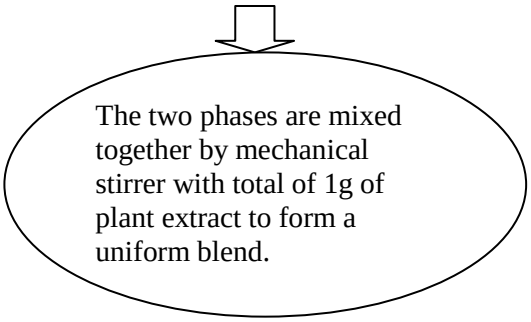


Fig 7.a: Flow chart of preparation of polyherbal lotion

Evaluation of polyherbal lotion:

- **pH of the lotion**

The pH meter was calibrated using standard buffer solution. About 0.5g of the cream was weighed and dissolved in 50.0 ml of distilled water and its pH was measured.

The pH of the cream was found to be in range of 5.6 to 6.8 which is good for skin pH. The herbal formulation was shown pH nearer to skin required i.e pH 6.3.

- **Viscosity**

Viscosity of the formulation was determined by Brookfield Viscometer at 100 rpm, using spindle no.4.

The viscosity of cream was in the range of 28001 – 27025 cps which indicates that the cream is easily spreadable by small amounts of shear. The herbal formulation was shows viscosity within the range.

- **Dye test**

The scarlet red dye is mixed with the cream. Place a drop of the cream on a microscopic slide covers it with a cover slip, and examines it under a microscope. If the disperse globules appear red the ground colourless. The cream is o/w type. The reverse condition occurs in w/o type cream i.e. the disperse globules appear colourless in the red ground.

- **Homogeneity**

The formulations were tested for the homogeneity by visual appearance and touch.

- **Appearance**

The appearance of the cream was judged by its colour, pearlscence and roughness and graded.

When formulation were kept for long time, it found that no change in color of cream.

- **After feel**

Emolliency, slipperiness and amount of residue left after the application of fixed amount of cream was checked.

- **Type of smear**

After application of cream, the type of film or smear formed on the skin were checked.

- **Removal**

The ease of removal of the cream applied was examined by washing the applied part with tap water.

- **Saponification value**

Introduce about 2 gm of substance refluxed with 25 ml of 0.5 N alcoholic KOH for 30 minutes, to this 1 ml of phenolphthalein added and titrated immediately, with 0.5 N HCL. Saponification value = (b-a)*28.05/w a - volume in ml of titrant, b - Volume in ml of titrant, w - Weigh of substance in gm.

- **Acid value**

Take 10 gm of substance dissolved in accurately weighed, in 50 ml mixture of equal volume of alcohol and solvent ether, the flask was connected to reflux condenser and slowly heated, until sample was dissolved completely, to this 1 ml of phenolphthalein added and titrated with 0.1N

NaOH, until faintly pink color appears after shaking for 30 seconds.

Acid value = n*5.61/w

n = the number of ml of NaOH required.

w = the weight of substance

- **Irritancy test**

Mark an area (1sq.cm) on the left hand dorsal surface. The cream was applied to the specified area and time was noted. Irritancy, erythema, edema, was checked if any for regular intervals up to 24 hrs and reported.

- **Accelerated stability testing**

Accelerated stability testing of prepared formulations was conducted for 2 most stable formulations at room temperature, studied for 7 days. They were formulation number 1 and 2 at 40 ± 100 C for 20 days. The formulations were kept both at room and elevated temperature and observed on 0th, 5th, 10th, 15th and 20th days.

Table-7.3.D. Accelarated stability studies of lotion -A

Time interval	Homogeneity	Type of smear	Viscosity(cp)	Physical changes	pH
0th day	+++	+++	12.005	No change in color and odour	6.1
5th day	++	+++	12.004	No change in color and odour	6.1
10th day	++	+++	12.005	No change in color and odour	6.3
15th day	++	+++	12.005	No change in	6.1

				color and odour	
20th day	++	+++	12.005	No change in color and odour	6.2

Table 7.3.E. Accelarated stability studies of lotion -B

Time interval	Homogeneity	Type of smear	Viscosity(cp)	Physical changes	pH
0th day	+++	+++	12.521	No change in color and odour	5.7
5th day	+++	+++	12.498	No change in color and odour	5.7
10th day	++	+++	12.514	No change in color and odour	5.9
15th day	++	+++	12.520	No change in color and odour	5.9
20th day	++	+++	12.518	No change in color and odour	5.8

Table-7.3.F. Accelarated stability studies of lotion -C

Time interval	Homogeneity	Type of smear	Viscosity(cp)	Physical changes	pH
0th day	+++	+++	12.014	No change in color and odour	5.8
5th day	++	++	12.021	No change in color and odour	6.1
10th day	++	++	12.019	No change in color and odour	5.9
15th day	+	+	12.020	No change in color and odour	5.7
20th day	+	+	12.018	No change in color and odour	6.1

+ = average

++ = good

+++ = excellent

- **Spreadibility test**

Sample was applied between two glass slides and was compressed to uniform thickness by placing 100gm weight for 5minutes. Weight was added to the pan. The time required to separate the two slides, i.e. the time in which the upper glass slide moved over the lower slide was taken as measure of spreadability.

Spreadability =m*l/t

m = Weight tide to upper slide

l = length moved on the glass slide

t = time taken.

Prepared polyherbal lotion:

Fig:7.b: Prepared polyherbal lotion

Chapter 8

Summary and Conclusion

8.1: Summary

8.2: Conclusion

8.1: Summary

The gaining popularity of herbal remedies demands more scientifically sound evidence for the principles responsible and their effectiveness. Natural products have the properties to rejuvenate and protect the hair and skin from environmental pollutants, chemicals, ultraviolet radiations and microbial manifestations. They have been used since ancient times and still continue in playing a major role in modern cosmetic market. Cosmetic formulations containing herbal components are more appropriate for hyper allergic skin because they are less irritant and more easily adjustable to skin. Conventional drug treatments irritate the skin, causing itching, redness, drying or allergic reactions. Consumers are shifting towards natural alternatives therefore herbalism and traditional medicines are gaining momentum. The majority of the formulations present in the market use ingredients which have been declared unsafe and ever carcinogenic when used for a long periods. Therefore it is of great interest to develop products from natural resources so as to desirable cleansing and therapeutic effects of herbals. The products of plant origin, are prone to contamination, deterioration with variation in their secondary metabolites both in qualitative and quantitative aspects. To ascertain such changes standardization of plant material using various techniques need to be carried out. Cosmetic preparations are for frequent and long term use, therefore the heavy metal content needs to be regulated for such topical formulations. The present thesis highlights on development of herbal topical formulations and their evaluation. Such formulations are free from common allergens, parabens and sls. The chapters have been summarized below.

Chapter 1 describes the skin, hair and considerations for designing formulations. The skin and hair are essential parts of the body. This chapter gives an overview of the anatomy of skin and its underlying structures. It also gives an insight into the hair structure and the phases of hair development. The latter part of the chapter gives an overview of topical herbal formulation and the key aspects that need to be kept in mind while developing such formulations.

Chapter 2 titled “Scope, Objective and Plan of Work”, in its true sense describes the scope and objective of the study along with the objective of the work. In this chapter, frame work of the study has been explained.

Chapter 3 gives an account of overview of topical formulations such as cream, shampoo and lotion with few example of formulation. This chapter also have the plant list which was selected for development of formulation.

Chapter 4 titled Literature review, Extraction, Phytochemical screening and HPTLC and RP-HPLC standardization

"***Carica papaya***" describes the plant and its detail. It is followed by its reported chemical constituents and their pharmacological activities. It discusses in detail regarding its collection, authentication and extraction using a hydroalcoholic mixture and extraction of oil, from the rhizomes using optimised Microwave Assisted Hydro Distillation. The extract post evaporation and lyophillisation yielded 28.3% w/w.

Carica papaya was screened for phytochemicals using various tests. It was tested positive for carbohydrate, phytosterol, alkaloids, terpenoids, flavonoids, tannins, phenols and saponins.

The extract was standardized through HPTLC and RP-HPLC method using ascorbic acid as the phytochemical marker. The developed HPTLC and RP-HPLC methods were validated followed by ICH guidelines. In HPTLC, presence of ascorbic acid in *Carica papaya* was confirmed by the corresponding R_f value of standard ascorbic acid (R_f - 0.38). Under the UV light at 254 nm, the spot of standard ascorbic acid resembled with the spot of the hydroalcoholic extract. Calibration curve was linear in the range of 200-1000 ng/spot with a correlation coefficient (r^2) of 0.99261, which indicated a good linear confidence of the peak area on concentration. Residual standard deviation of the calibration curve was found to be 3.08%, which proves the linearity of the calibration curve having the equation Y = -3788.573+26.061*X. The quantity of ascorbic acid present in the extract was found to be 1.28% (w/w). In RP-HPLC, presence of ascorbic acid in the extract was confirmed by comparing the peaks of marker with the extract for their corresponding retention time 4.3 min. Calibration curve was linear in the range of 50-1000 µg/mL. The content was found to be 1.3% w/w in *Carica papaya*. A retention time of standard was highly repeatable, with %RSD < 2%. The coefficient of determinants (r^2) was 0.997 which represents the data is closest to the line of best fit. A high repeatability in the retention time and area response was obtained for standard and *Carica papaya* even at high concentration. The method was adaptable because of the good precision and excellent repeatability for separation and quality assessment of botanicals.

"***Cucumis sativus***" describes the plant and its fruits. It is followed by its reported chemical constituents and their pharmacological activities. It discusses in detail regarding its collection, authentication and extraction using aquous mixture The extract post evaporation and lyophillisation yielded 15% w/w.

Cucumus sativus aquous extract was screened for phytochemicals using various tests. It was tested positive for carbohydrate, phytosterol, alkaloids, terpenoids, flavonoids, tannins, phenols and saponins.

The extract was standardized through HPTLC and RP-HPLC method using chlorogenic acidas the phytochemical marker. The developed HPTLC and RP-HPLC methods were validated followed by ICH guidelines. In HPTLC, presence of chlorogenic acid in *Cucumis sativus* by the corresponding $R_f$value of standard chlorogenic acid (R_f - 0.61). Under the UV light at 254 nm, the spot of standard chlorogenic acid resembled with the spot of the hydroalcoholic extract. Calibration curve was linear in the range of 200-1000 ng/spot with a correlation coefficient (r^2) of 0.95405, which indicated a good linear confidence of the peak area on concentration. Residual standard deviation of the calibration curve was found to be 3.21%, which proves the linearity of the calibration curve having the equation $Y = 8.795 * X + 42.615$. The quantity of chlorogenic acid present in the extract was found to be 3.21% (w/w). In RP-HPLC, presence of chlorogenic acid in the extract was confirmed by comparing the peaks of marker with the extract for their corresponding retention time 9.93 min. Calibration curve was linear in the range of 50-1000 µg/mL. The content was found to be 4.41% w/w in *Cucumis sativus* hyrdo alcoholic extract. A retention time of standard was highly repeatable, with %RSD < 2%. The coefficient of determinants (r^2) was 0.993 which represents the data is closest to the line of best fit. A high repeatability in the retention time and area response was obtained for standard and *Cucumis sativus* hydroalcoholic extracts even at high concentration. The method was adaptable because of the good precision and excellent repeatability for separation and quality assessment of botanicals.

"***Hibiscus rosa sinensis***" describes the herb in detail. It is followed by its reported chemical constituents and their pharmacological activities. It discusses in detail regarding its collection, authentication and extraction using a hydroalcoholic mixture The extract post evaporation and lyophillisation yielded 15.2% w/w. *Hibiscus rosa sinensis* aquous extract was screened for phytochemicals using various tests. It was tested positive for carbohydrate, phytosterol, alkaloids, terpenoids, flavonoids, tannins, phenols and saponins.

The extract was standardized through HPTLC and RP-HPLC method using quercetin as the phytochemical marker. The developed HPTLC and RP-HPLC methods were validated followed by ICH guidelines. In HPTLC, presence of quercetin-3 in *Hibiscus rosa sinensis* was confirmed by the corresponding $R_f$value of standard quercetin (R_f - 0.44). Under the UV light at 254 nm, the spot of standard quercetin resembled with the spot of the hydroalcoholic extract. Calibration curve was linear in the range of 200-1000 ng/spot with a correlation coefficient (r^2) of 0.9929, which indicated a good linear confidence of the peak area on concentration. Residual standard deviation of the calibration curve was found to be 3.08%, which proves the linearity of the calibration curve having the equation $Y = 745.6 + 4.985*X$. The quantity of quercetin present in the extract was found to be 1.02% (w/w). In RP-HPLC, presence of quercetin the extract was confirmed by comparing the peaks of marker with the

extract for their corresponding retention time 9.14 min. Calibration curve was linear in the range of 50-1000 µg/mL. The content was found to be 1.7% w/w in *Hibiscus rosa sinensis* hydroalcoholic extract. A retention time of standard was highly repeatable, with %RSD < 2%. The coefficient of determinants (r^2) was 0.9929 which represents the data is closest to the line of best fit. A high repeatability in the retention time and area response was obtained for standard and *Hibiscus rosa sinensis* hydroalcoholic extract even at high concentration. The method was adaptable because of the good precision and excellent repeatability for separation and quality assessment of botanicals.

"***Ocimum sanctum***" describes the plant and its herbs in detail. It is followed by its reported chemical constituents and their pharmacological activities. It discusses in detail regarding its collection, authentication and extraction from the leaves using maceration. The yield is 16% w/w. Alkaloids, glycosides, tannin, saponnin, flavonoids are present. The extract was standardized through HPTLC and RP-HPLC method using eugenol as the phytochemical marker. The developed HPTLC and RP-HPLC methods were validated followed by ICH guidelines. In HPTLC, The $R_f$value of eugenol was found to be 0.61. The percentage content of eugenol in *Oscimum sanctum* hydro-alcoholic extract was found to be as mentioned 4.41%. This was determined by a calibration curve with the equation of $Y = 8.795 * X + 42.615$ (correlation coefficient = 0.95405 and standard deviation = ± 10.9%) as shown in fig. where X represents amount of eugenol and Y represents area under the curve. The retention time (R_t) of eugenol was found to be 6.029 min. The calibration curves were obtained by plotting peak areas against concentrations. Standard compound exhibited good linearity in the concentration range of 10 - 100 µg/ml. The correlation coefficient (r^2) was estimated to be 0.993. Instrumental precision was noted account by repetitive injection of the same sample of three concentrations.

The relative standard deviation (R.S.D.) was estimated to assess instrumental precision. Intra-assay precision was performed by making five standard solutions of three different concentrations and measuring the R.S.D. values. The R.S.D. values of both instrumental precision and intra-assay precision of eugenol were estimated to be <2.5%, indicating the RP-HPLC analytical method for these two standard compounds were precise. Intermediate precision of the method was optimized by calculating intra-day and inter-day repeatability. The R.S.D. values in all tested groups were found to be <3%, which is acceptable. The sensitivity of RP-HPLC method was determined by limit of detection (LOD) and limit of quantification (LOQ). LOD is the lowest concentration of the sample, the method can detect and LOQ is the lowest concentration that can be quantified accurately by the method. The LOD and LOQ of standard compound was estimated to be 0.3 and 0.8 µg/mL for eugenol resulted that the method was sensitive to detect the compounds in sample.

"*Lawsonia inermis*" describes the plant and its leaves in detail. It is followed by its reported chemical constituents and their pharmacological activities. It discusses in detail regarding its collection, authentication and extraction using a hydroalcoholic mixture. The extract post evaporation and lyophillisation yielded 18.2% w/w.

Lawsonia inermis hydroalcoholic extract was screened for phytochemicals using various tests. It was tested positive for carbohydrate, phytosterol, alkaloids, terpenoids, flavonoids, tannins, phenols and saponins.

The extract was standardized through HPTLC and RP-HPLC method using gallic acid as the phytochemical marker. The developed HPTLC and RP-HPLC methods were validated followed by ICH guidelines. In HPTLC, presence of gallic acid in *L. innermis* was confirmed by the corresponding R_f value of standard gallic acid (R_f - 0.52). Under the UV light at 254 nm, the spot of standard gallic acid resembled with the spot of the hydroalcoholic extract. Calibration curve was linear in the range of 200-1000 ng/spot with a correlation coefficient (r^2) of 0.98308, which indicated a good linear confidence of the peak area on concentration. Residual standard deviation of the calibration curve was found to be 1.64%, which proves the linearity of the calibration curve having the equation Y = -2372.845+ 22.521*X. The quantity of gallic acid present in the extract was found to be 1.64% (w/w). In RP-HPLC, presence of gallic acid in the extract was confirmed by comparing the peaks of marker with the extract for their corresponding retention time 5.081 min. Calibration curve was linear in the range of 50-1000 µg/mL. The content was found to be 1.71% w/w in *Lawsonia inermis* hydroalcoholic extract. A retention time of standard was highly repeatable, with %RSD < 2%. The coefficient of determinants (r^2) was 0.993 which represents the data is closest to the line of best fit. A high repeatability in the retention time and area response was obtained for standard and LIHE even at high concentration. The method was adaptable because of the good precision and excellent repeatability for separation and quality assessment of botanicals.

Tagetes erecta describes the plant and its details, The yield value of the extraction is 17.1%.Olionolic acid is used as biomarker. In HPTLC study R_f value of *Oleonolic acid* was found to be 0.59. The percentage content of *Oleonolic acid* in *Tagetes erecta* extract was found to be as mentioned 5.72%. This was determined by a calibration curve with the equation of Y = 3447.759*X - 1121.100 (correlation coefficient = 0.9686 and standard deviation = ± 3.9%) as shown in fig. where X represents amount of *Oleonolic acid* and Y represents area under the curve.

In HPLC study The retention time (R_t) of *Oleonolic acid* was found to be 9.19 min. The calibration curves were obtained by plotting peak areas against concentrations. Standard compound exhibited good linearity in the concentration range of 10 - 100 µg/mL. The

correlation coefficient (r^2) was estimated to be 0.9923. Instrumental precision was noted account by repetitive injection of the same sample of three concentrations.

The relative standard deviation (R.S.D.) was estimated to assess instrumental precision. Intra-assay precision was performed by making five standard solutions of three different concentrations and measuring the R.S.D. values. The R.S.D. values of both instrumental precision and intra-assay precision of *Oleonolic acid* were estimated to be <2.5%, indicating the RP-HPLC analytical method for these two standard compounds were precise. Intermediate precision of the method was optimized by calculating intra-day and inter-day repeatability. The R.S.D. values in all tested groups were found to be <3%, which is acceptable. The sensitivity of RP-HPLC method was determined by limit of detection (LOD) and limit of quantification (LOQ). LOD is the lowest concentration of the sample, the method can detect and LOQ is the lowest concentration that can be quantified accurately by the method. The LOD and LOQ of standard compound was estimated to be 0.3 and 0.8 µg/mL for *Oleonolic acid* resulted that the method was sensitive to detect the compounds in sample. The amount of *Oleonolic acid* present in extract was estimated to be about 2.78% (w/w), respectively. The percentage amounts of standard constituents present in the crude extract were expressed by its weight (µg/mg).

"***Acacia concinna***" describes the plant and its pods in detail. It is followed by its reported chemical constituents and their pharmacological activities. It discusses in detail regarding its collection, authentication and extraction using a hydroalcoholic mixture. The extract post evaporation yielded 19.5% w/w.

Acacia concinna hydroalcoholic extract was screened for phytochemicals using various tests. It was tested positive for carbohydrate, phytosterol, alkaloids, terpenoids, flavonoids, tannins, phenols and saponins.

The extract was standardized through HPTLC and RP-HPLC method using betulinic acid as the phytochemical marker. The developed HPTLC and RP-HPLC methods were validated followed by ICH guidelines. In HPTLC, presence of betulinic acid in *Acacia concinna* was confirmed by the corresponding $R_f$value of standard gallic acid (R_f - 0.42). Under visible light, the spot of standard betulinic acid resembled with the spot of the hydroalcoholic extract. Calibration curve was linear in the range of 200-1000 ng/spot with a correlation coefficient (r^2) of 0.98061, which indicated a good linear confidence of the peak area on concentration. Residual standard deviation of the calibration curve was found to be 2.96%, which proves the linearity of the calibration curve having the equation Y = 140.780 + 0.365 * X. The quantity of betulinic acid present in the extract was found to be 1.29% (w/w). In RP-HPLC, presence of betuliniic acid in the extractwas confirmed by comparing the peaks of marker with the extract

for their corresponding retention time 9.489 min. Calibration curve was linear in the range of 50-1000 µg/mL. The content was found to be 0.96% w/w in *Acacia concinna* hydroalcoholic extract. A retention time of standard was highly repeatable, with %RSD < 2%. The coefficient of determinants (r^2) was 0.99864 which represents the data is closest to the line of best fit. A high repeatability in the retention time and area response was obtained for standard and *Acacia concinna* hydroalcoholic extract even at high concentration. The method was adaptable because of the good precision and excellent repeatability for separation and quality assessment of botanicals.

Chapter 5,6 and 7 details the "Development of polyherbal cream ,shampoo and lotion formulation and its evaluation" and also deals with strategy, methods and ingredients used for the development of formulations. A total of twelve formulations were developed three in each category viz., shampoo, body wash hand wash and face wash. The common ingredients included a primary surfactant, thickner, humectant, foam booster, preservative, sequestering agent, pH modifier and boatanicals. The ingredients were varied from formulation to formulation keeping in mind its viscosity, foaming ability and therapeutic benefits."Evaluation of formulations", contains evaluation parameters of developed and marketed preparations. The formulations were evaluated on parameters like Moisture content, extractive values, pH, cleansing action, foaming capacity and dirt dispersion, wetting time, nature of hair after wash, nature of skin after wash and pourability. They were also evaluated for heavy metal content, microbial load, rheology and stability studies.Moisture content was determined for all twelve prepared formulations and three marketed formulations. In the Shampoo section SH-A had the lowest moisture content of 23.1% whereas the marketed formulation SH-C had a content of 27.2% w/w. Similar trends were noticed for cream and lotion respectively. It is believed that moisture is a cause of microbial growth and hence will interfere with the stability of the formulation on storage. Therefore SH-A, cream-A, lotion-A are less prone to degradation. The pH of the formulations should be in between 5.5 to 6.5 for shampoos and from 5.5 to 6.0 for rest of the formulations else irritation and rashes may develop. All the formulations tested, i.e., marketed and prepared, were in the range, and hence are good candidates for consumer compliance. Cleaning action was evaluated. The formulations were evaluated for their cleansing property and it was noticed that shampoos had the best cleansing action compared to all the products developed. SH-A had the maximum value among all the samples evaluated.Foaming capacity: A good shampoo should have good foaming property. This attracts the consumers and is also a good remover of unwanted substances.SH-A had the highest foaming property. Most of the formulations developed had satisfactory dirt dispersion property. The products belonging to the A series in each section and the marketed products had good dirt dispersion values. Lesser the wetting time faster would be the action. SH-A

among shampoos, Nature of hair after wash is of high importance to the end user. A good shampoo will always be appreciated if it kept the hair soft and manageable. All of the products developed and marketed maintained the tone of the skin and didn't produce any significant hypersensitive reactions. All formulations showed good pourability.The rheological parameters were studied. Shear stress and shear strain were ramped against the formulations at specific intervals to obtain their changes in dynamic viscosity. It was noticed that the formulations prepared had higher viscosity than than the marketed ones throughout. The formulations belonging to A series in every category exhibited good rheological properties.

It has already been emphasized on the importance of stability studies in formulation develop ment. The samples were evaluated at intervals of 0, 3 and 6 months respectively for pH, foaming capacity, pourability, viscosity and Total Viable Count. The results are displayed below.Slight pH changes were noticed in many. 5.5 – 7 is acceptable for shampoos and for rest of the formulations it should be within 5.5 to 6. About 11 of the 12 prepared were within range exceeded the permissible limit and is not fit for use on storage. Viscosity varied on storage for about all the preparation with the A series having least change. Foaming capacity of most formulations decreased on storage. Pourability remained unaffected. From stability studies we can conclude that the developed formulations SH-A, cream-A, lotion-A were the most stable of the lot.

8.2: Conclusion

The traditional knowledge with its holistic and systematic approach supported through experimental evidence may serve as an innovative and powerful discovery engine for newer, safer and affordable medicines. The work was performed and represented in this thesis deals with the Topical Herbal Formulations Development from Medicinal Plants. The work was based on the approach of exploration of medicinal herbs with multiple potentials ranging from anti-microbial to hair conditioning potential and others from traditional and modern scientific knowledge.

The RP-HPLC and HPTLC standardization methods with respective bio-markers were adaptable owing to their good precision and excellent repeatability. These methods can be used as potential cost-effective, simple and highly selective tools that can ensure both quality and batch-to-batch reproducibility of the products.

Polyherbal formulations (cream, lotion and shampoo) were prepared for the hair, body and face. Stability study supports its safer uses for long time without changes of any product qualities. The developed products were compared to marketed formulations. Comparing against various parameters, formulations SH-A, cream-A and lotion-A were at par or even at times better, in certain parameters, than the marketed formulations. These formulations and the optimized procedures involved in their preparation may be utilized in future to give rise to better personal care products for the huge consumer base.

Chapter 9

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