Development and Evaluation
Of
‘Varnya’ formulation from the medicinal plants of Ayurvedic importance

THESIS SUBMITTED

By

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List of Publications:

1. **Sharma, A.,** Katiyar, C.K., Banerjee, S., Chanda, J., Kar, A., Biswas, S., Mukherjee P.K., 2020. RP-HPLC and HPTLC Methods for Analysis of Selected Herbs Used as Complexion Promoters in Ayurveda and Unani Systems of Medicine. ***Journal of AOAC international***. 103 (3), 2020.

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2. Mukherjee, P.K., Biswas, R., **Sharma, A.,** Banerjee, S., Biswas, S., Katiyar, C.K., 2018. Validation of medicinal herbs for anti-tyrosinase potential. ***Journal of Herbal Medicine***. 14, 1-16.

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Certificate

This is to certify that the thesis entitled "**Development and Evaluation of '*Varnya*' formulation from the medicinal plants of Ayurvedic importance**" submitted by Mrs. Akanksha Sharma (Index Number: D-7/ISLM/46/17), who got her name registered on 22/12/2017 for the award of Ph. D (Pharmacy) degree of Jadavpur University is absolutely based upon his own work under the supervision of Prof. Pulok K. Mukherjee and that 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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Statement of Originality

I Akanksha Sharma registered on 22. 12. 2017, do hereby declare that this thesis entitled contains literature survey and original research work done by the undersigned candidate as part of Doctoral studies.

All information in this thesis have been obtained and presented in accordance with existing academic rules and ethical conduct. I declare that as required by these rules and conducts, I have fully cited and refereed all materials and results that are not original to this work.

I also declare that I have checked this thesis as per the policy on Anti Plagiarism, Jadavpur University, 2019, and the level of similarity as checked by iThenticate software is 9%

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Declaration

I hereby declare that my research work embodied in this Ph.D. thesis entitled “Development and Evaluation of ‘Varnya’ formulation from the medicinal plants of Ayurvedic importance” have been carried out by me in the School of Natural Product Studies, Dept. Pharm. Tech., Jadavpur University, West Bengal, Kolkata, India under the direct supervision of Prof. Pulok K. Mukherjee, Director, School of Natural Product Studies, Dept. Pharm. Tech., 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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Content

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(Mrs. Akanksha Sharma)

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LIST OF ABBREVIATIONS USED

Abb. form	Explanation	Abb. form	Explanation
AMPK	5'-adenosine monophosphate activated protein kinase	ECG	(-)-epicatechin 3-0-gallate
UV	Ultraviolet	GCG	(-)-gallocatechin 3-0-gallate
PIH	Post-inflammatory Hyperpigmentation	EGCG	(-)-epigallocatechin 3-0-gallate
ROS	Reactive Oxygen Species	mRNA	Messenger Ribonucleic Acid
L-dopa	L-3,4-dihydroxyphenylalanine	DNA	Deoxyribonucleic acid
IQ	Indole-5, 6-quinone	GR	Glycyrrhizin
Tyrp-2	Tyrosinase-Related Protein-2	GC analysis	Gas Chromatography
DHICA	5, 6-dihydroxyindole-2-carboxylic acid	GSK-3β	Glycogen Synthase Kinase-3β
Tyrp-1	Tyrosinase-Related Protein-1	HQ	Hydroquinone
IQCA	Indole-2-carboxylic acid-5, 6-quinone	HHQ	Hydroxyhydroquinone
MITF	Microphthalmia-associated transcription factor	BQ	Benzoquinone
DAG	Diacylglycerol	RH	Rhododendrol
PKC	Protein Kinase-C	TCM	Traditional Chinese Medicines
DGK-ζ	Diacylglycerol Kinase-ζ	Cd	Cadmium
ACTH	Adrenocorticotropic Hormone	As	Arsenic
MC1R	Melanocortin Receptor-1	Hg	Mercury
cAMP	Adenosine Monophosphate	Pb	Lead
MEK	Mitogen-activated Protein Kinase	GLP-1	Glucagon-like peptide-1
ERK	Extracellular Signal-regulated Kinase	DPP-IV	Dipeptidyl peptidase-IV
PI3K	Phosphatidylinositol 3-kinase	MMP-9	Matrix metallopeptidase-9
Akt	Serine-threonine Protein Kinase	MEBA	Methanol extracts of *B. aristata*
EGF	Epidermal Growth Factor	AFBA	Aqueous fraction of *B. aristata*
PPO	Polyphenol Oxidase	EFBA	Ethyl acetate fraction of *B. aristata*
IC_{50}	50% Inhibitory concentration	HFBA	Hexane fraction of *B. aristata*
α-MSH	α-Melanocyte-stimulating Hormone	IL-6	Interleukin-6
EC	(-) epicatechin	RP-HPLC	Reverse phase High Performance Liquid Chromatography
C	(+) catechin	LOD	Limit of detection
LOQ	Limit of quantification	FPP	Finished pharmaceutical product
GAE	Gallic acid equivalents	HR	Hausner ratio
QrE	Quercetin equivalents	PE	Petroleum ether
%RSD	Relative standard deviation	TOF-MS-ES+	Time-of-flight mass spectrometry
ICH	International Conference on Harmonisation	BA	Betulinic acid
LB plot	Lineweaver–Burk plot	RCSB	Research collaboratory for structural bioinformatics
SD	Standard deviation	TPC	Total phenolic content
CFRC	Chloroform fraction of *R. cordifolia*	TF	Total flavonoids

HFRC	Hexane fraction of *R. cordifolia*	DR	Dragendorff's reagent
DMSO	Dimethyl sulfoxide	EGC	Epigallocatechin
OD	Optical density	API	Active pharmaceutical ingredient
K_i	Inhibition constant	WHO	World health Organization
CD	Circular dichroism	API	Ayurvedic Pharmacopoeia of India
MVD	Molegro Virtual Docker	AUL	Absorbency under load
PDB	Protein data bank	ppm	Parts per million
MMV	Molegro Molecule Viewer	BIS	Bureau of Indian Standards
ANS	1-Anilino-8-naphthalene sulfonate	SPF	Sun protection factor
RMSD	Root Mean Square Deviation	USM	Unani System of Medicine
NCEs	New chemical entities	% LOD	Percentage loss on drying
V_{ss}	Steady-state rate	CFU	Colony forming units
MEDI	Methanol extracts of *D. indica*	dwf	Dry weight formulation
AFDI	Aqueous fraction of *D. indica*	DPPH	Diphenyl 2-picrylhydrazyl
CFDI	Chloroform fraction of *D. indica*	RP	Reducing power
HFDI	Hexane fraction of *D. indica*	HPTLC	High Performance Thin Layer Chromatography
PAF	Platelets activating factor	AAS	Atomic absorption spectroscopy
CCRIHM	Central Council of Research in Indian and Homeopathic Medicine	TAC	Total alkaloid Content
CCRUM	Central Council for Research in Unani Medicine	LB plot	Lineweaver–Burk plot
AbTyr	*Agaricus bisporus* tyrosinase	HLB	Hydrophilic-lipophilic balance

Dedicated

to

My Mother

Chapter - 1

1. Skin whiting & Tyrosinase inhibition – the role of Ayurvedic (Varnya) Herbs

1.1. Concept of Varnya
1.2. Tyrosinase Enzyme
1.3. Tyrosinase: A key player in melanogenesis
1.4. Traditionally used tyrosinase inhibitors
1.5. Varnya herbs with tyrosinase inhibition properties
1.6. Summary and conclusion

1.1 Concept of Varnya

Varnya is a classical term used for the ancient concept dealing with skin whitening and brightening. Varnya herbs are considered to have good skin whitening properties. As there are many cosmetological aspects explained in antient literature and other classical texts of Ayurveda, concept of varnya is one of the topics which deals with both cosmetology and traditional medicinal system (Patwardhan, et. al., 2009). There are a total of 45 drugs having varnya properties. The total number of kesya dravyas screened are 21 and tvachya drugs are 9 in number. Now-a-days cosmetology is a huge demanding subject in the era of modernization and beautification. Therefore, an effort is made to screen Brihatrayee along with all other available classical texts and recent related scientific articles to reveal the ancient wisdom regarding cosmetology in easy and lucid manner. The result of this tedious work is intended for both literary purpose and practical usage which will be beneficial for research scholars (Narasanagi et. al., 2016)

Acquired hyper-pigmentation disorders of the skin are among the most common complaints in a general dermatology clinic. Among those, melasma is known for causing significant impact on quality of life, including a negative effect on the patient's emotional well-being and social life. Despite the advent of powerful pigment-targeting lasers, the treatment for melasma remains challenging. In the United States alone, approximately 5–6 million individuals are afflicted with melasma of which majority are females (90–95%). In Asia, it is a common diagnosis and can reach an incidence of 0.25–4% of cases seen in any dermatology institution (Biswas et al., 2016). Melasma should not be dismissed as simply a cosmetic entity because it often evokes emotional distress. In addition, stigma may be associated with melasma, particularly in Asian cultures.

Melasma not only is a cosmetic entity but also it evokes emotional distress. In addition, stigma may be associated with melasma, particularly in Asian cultures. Melasma is a acquired cutaneous and persistent relapsing hyper melanosis showing hyperpigmented patches on sun-exposed areas of the face, neck, and forearms. Exposure to ultraviolet (UV) radiation is believed to be the leading factor in its development. Ayurveda refers to this condition as Vyanga where in Vata and Pitta Dosha as well as Manasika Nidanas

(psychological & etiological factors) such as Krodha (anger), Shoka (sorrow), and Ayasa (mental exertion) are the main culprits (Silagi, et. al.,).

1.2 Tyrosinase Enzyme

Plants and animals contain a multifunctional copper-containing enzyme called tyrosinase (EC 1.14.18.1). The enzyme greatly influences melanogenesis in melanocytes. In addition to determining the color of skin and hair, melanin also protects against harmful UV rays. Melanin pigment is synthesized from the precursor of quinones catalyzed by tyrosinase. Due to overproduction and accumulation of melanin pigments in the skin, dermatological conditions such as solar lentigo, melasma, post-inflammatory hyperpigmentation (PIH), and Linea nigra may occur. It can also occur due to hormonal imbalances, such as lupus, Nelson's syndrome (abnormal secretion of hormones and enlargement of pituitary gland) and Addison's disease which is a primary adrenal insufficiency is a rare long-term endocrine disorder. Kojic acid has been generally used as tyrosinase inhibitor. As tyrosinase inhibitors, arbutin and kojic acid are commonly used in cosmetics to whiten the skin. In clinical practice, these depigmenting agents are used as hyperpigmentation treatments. At therapeutic concentrations, kojic acid causes dermal sensitization while arbutin is potentially cytotoxic (Sarkar et al., 2013; Burnett et al., 2010; Zhu and Gao, 2008).

Hyperpigmentation can probably be controlled with the use of plant extracts and Phyto-constituents. Research supports the herbs can be used as the replacement for synthetic drugs managing the health issues and problems (Zhu and Gao, 2008; Sarkar et al., 2013; Mukherjee and Wahile, 2006). Insects' defensive mechanisms rely on tyrosinase. In addition, tyrosinase also contributes to the browning of vegetables and fruits. Plant-derived food products usually lose their color when they are browned, indicating that their nutritional value has been compromised. Food can be protected from excessive tyrosinase activity by using tyrosinase inhibitors. As well, these inhibitors are beneficial in reducing reactive oxygen species (ROS), e.g. melanoma caused by UV radiation (Mukherjee et al., 2011; Rao et al., 2013). The currently available inhibitors of tyrosinase have been summarized in various review articles (Loizzo et al., 2012 & Chang, 2009).

1.3 Tyrosinase: A key player in melanogenesis

The name “melanin” originates from the word “melanos,” meaning dark. These are chemical variables derived from the oxidation of phenolic precursors. Melanin is synthesized via a pathway called the Raper-Mason pathway. Tyrosinase catalyzes the first step of melanogenesis, which is the oxidation of tyrosine and dopa. Melanin synthesis begins with this first step (Chang, 2009).

The reduction in melanin synthesis is aided by tyrosinase inhibitors, which help to counteract melanogenesis. The oxidation of L-tyrosine as well as L-dopa, this enzyme forms dopaquinone and cyclizes quinine which is the precursor to leucodopachrome. with the use of redox exchange, leucodopachrome (cycloDOPA) is converted to dopachrome. Also, with the help of tyrosinase, dopachrome is decarboxylated and converted to indole-5, 6-quinone (IQ). Tyrosinase or Tyrp-1 oxidizes DHICA into indole-2-carboxylic acid-5, 6-quinone (IQCA) the same way dopachrome is oxidized into DHICA. Polymerization of DHICA, IQCA, and IQ again results in eumelanin (black/brown in color). A thio-group containing amino acid cysteine is added to dopaquinone in another sub-pathway, which results in the formation of pheomelanin (reddish/yellow in color). Eumelanin and pheomelanin mix to form neuromelanin. Catecholaminergic neurons contain neuromelanin. The biosynthetic pathway for melanin formation represents in Figure 1.1 (Solano, 2014).

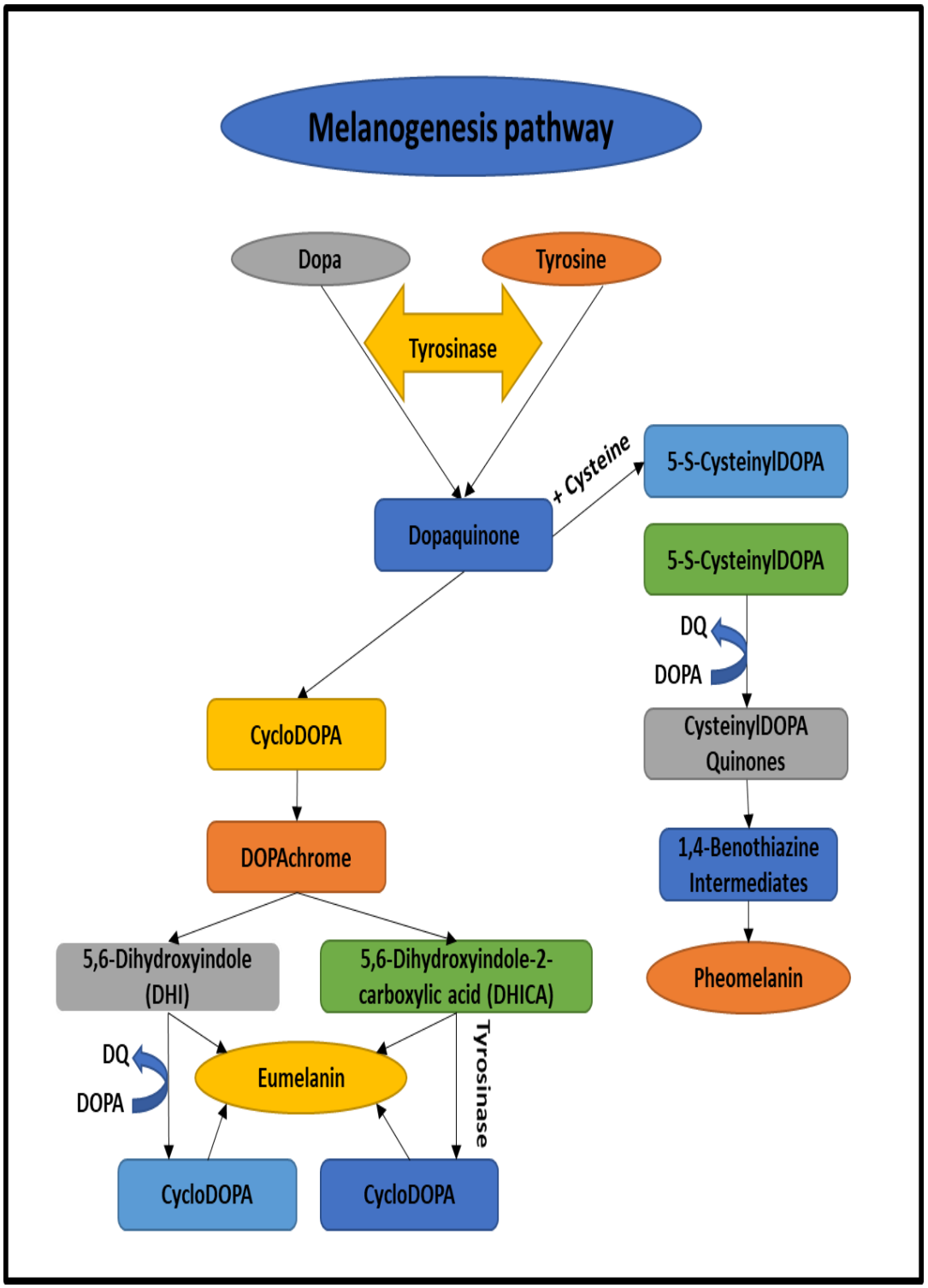
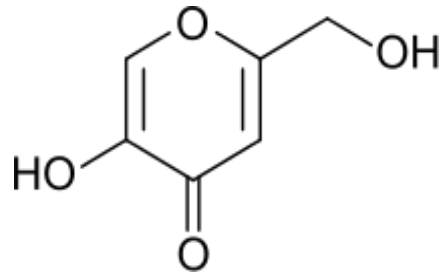


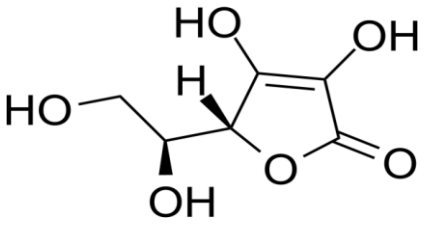
Figure 1.1. Melanin formation- The biosynthetic pathway

1.4 Traditionally used tyrosinase inhibitors

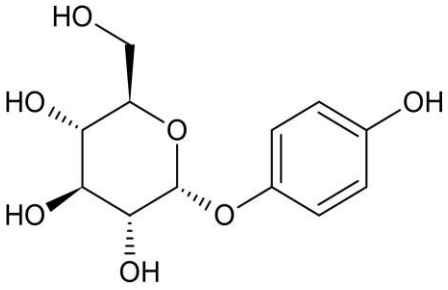
The use of scientifically validated tyrosinase inhibitors as a positive standard is common in most studies conducted to find new tyrosinase inhibitors. Figure 1.2 illustrates these conventional mushrooms tyrosinase inhibitors.



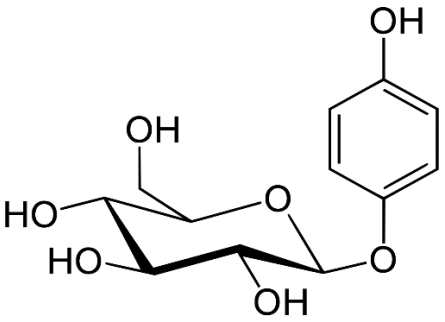
Kojic acid (1)



L-Ascorbic acid (2)



α-Arbutin (3)



β-Arbutin (4)

Figure 1.2: Traditionally used tyrosinase inhibitors

1.4.1 Kojic acid

Kojic acid **(1)** is an extensively studied tyrosinase inhibitor, used as a positive control in tyrosinase inhibitory assays. This fungus metabolite is used both as a cosmetic skin-whitening agent and as a food additive for preventing enzymatic browning. The observed competitive inhibitory effect may well be explained by its ability to chelate copper within the enzyme active site. The protein monophenolase activity of mushroom tyrosinase was inhibited by kojic acid in a competitive manner, whereas the diphenolase activity was inhibited by a mixed inhibitory mechanism (Chang, 2009). Due to its severe thyroid toxicity and potential skin sensitizing effect, its use is, however, restricted (Burnett et al., 2010).

1.4.2 L-ascorbic acid

Because of its anti-browning effects, it was extensively used as tyrosinase inhibitors over the years. In addition, it alters the flavor of most beverages due to transient stability (Komthong et al., 2007). By reducing o-dopaquinone to L-DOPA, ascorbic acid

(2) prevents the formation of dopachrome and melanin (Chang, 2009).

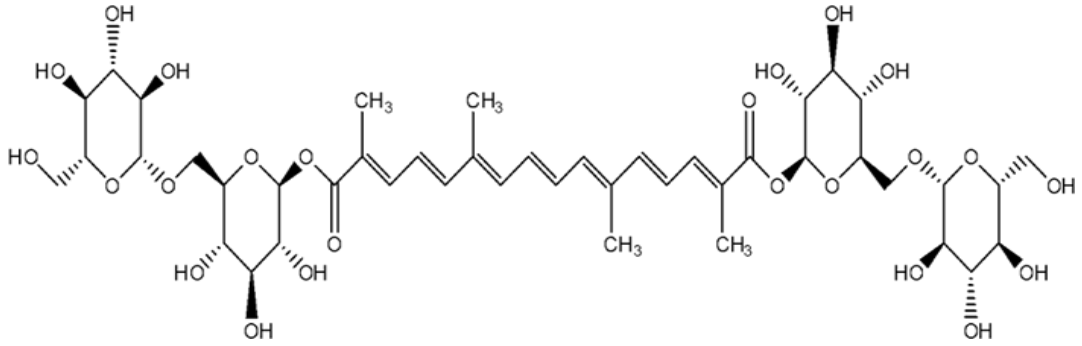
1.4.3 Arbutin

A hydroquinone-α-D-glucopyranoside of hydroquinone from *Arctostaphylos uva-ursi* (bearberry) plant. The monophenolase activity of tyrosinase was inhibited by β-arbutin **(4)** (Tomita et al., 1990). Hori and colleagues reported in 2004 that β-arbutin itself exhibits slow oxidizing potential upon L-tyrosinase with L-dopa as a cofactor. In 2014, Qin and colleagues demonstrated that α-arbutin **(3)** inhibits monophenolase activity, but activates diphenolase activity. The activity was mixed Varnya herbs with tyrosinase inhibition properties.

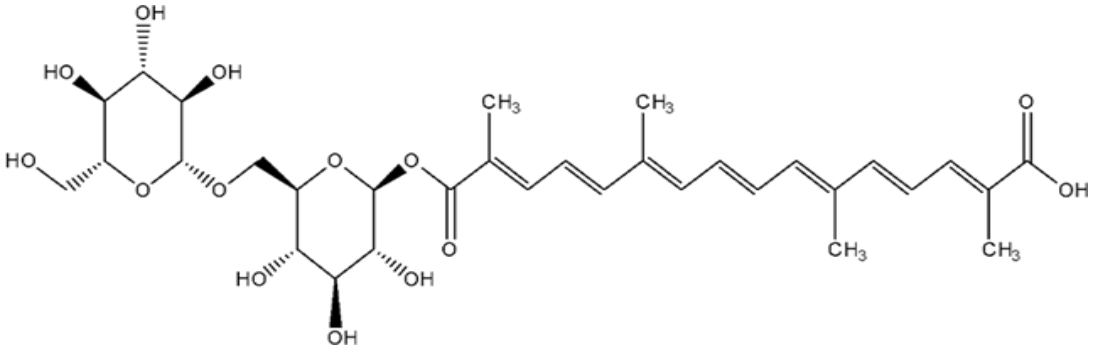
Up to now, many tyrosinase inhibitors have been identified from medicinal plants. Since last decade there have been a major development in the field of cosmetology using the concepts from antient traditional medicinal systems. One of the most used models is the Varnya. Many have highlighted varnya herbs in their work. For the purpose of this study, some of the majorly used Varnya herbs are mentioned below:

1.4.4 *Crocus sativus* L.

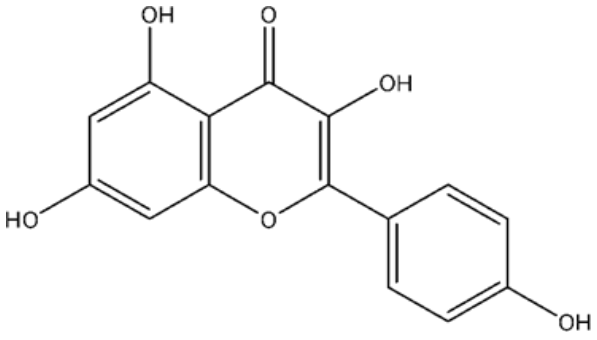
Also Known as saffron (Iridaceae) is used extensively for skin whitening formulations. The components of *Crocus sativus* like crocin-1 **(5)**, and crocin-3 **(6)** have shown good tyrosinase inhibition. crocin-1 (approximately 0.15 mM) has shown to have IC50 valve as low as kojic acid. Isolated kaempferol **(7)**. A concentration of 67 g/mL was determined to be the 50% enzyme inhibition concentration (IC50) of kaempferol. Kaempferol-3-O- glycoside has no activity. When tested on mouse melanoma cell-line, it did not significantly reduce melanin production when compared to kojic acid (Anantharaman et al., 2015). Li and associates isolated 35 compounds from saffron petals in 2004. Crocusatin-K showed a strong tyrosinase inhibitory effect.



Crocin-1 (5)



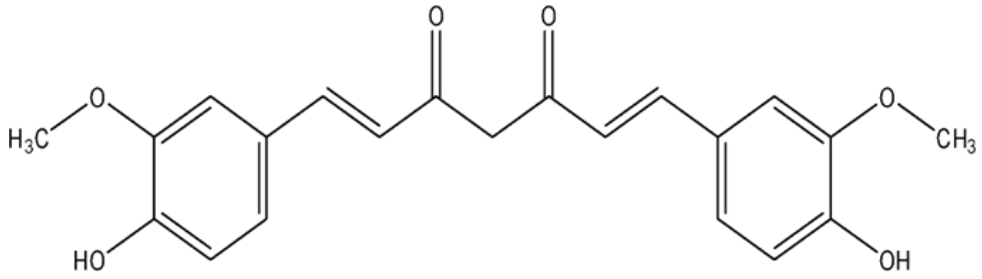
Crocin-3 (6)



Kaempferol (7)

1.4.5 *Curcuma longa* L.

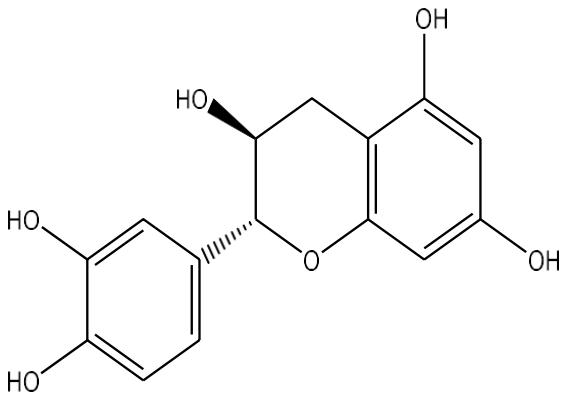
Longa, a member of the Gingeraceae family, is found in a semisolid herbal preparation called 'Ubtan' that enhances brightness of the skin (Dixit and Goyal, 2011 & Mukherjee et al., 2015;). Curcumin **(8)** found in the rhizome of *Curcuma longa.* Turmeric curcuminoids have been shown to inhibit mushroom tyrosinase by Du and colleagues. The tyrosinase inhibition percentages of curcuminoids (IC50 in *M) are listed as follows: curcumin < dimethyl curcumin < bis-dimethyl curcumin. The tyrosinase inhibitory activity of curcumin-bis-α-D-glycoside was better (Prasad et al., 2014 & Du et al., 2011).



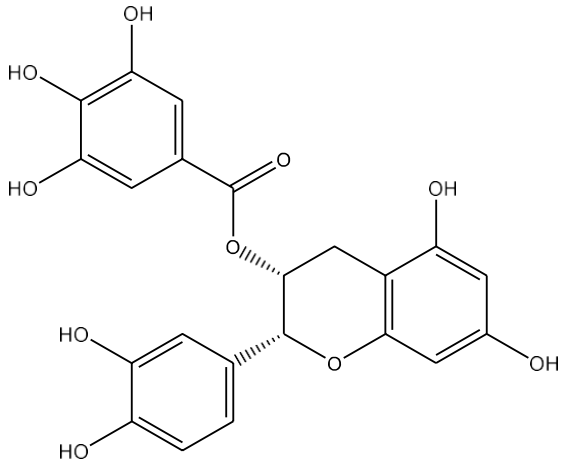
Curcumin (8)

1.4.6 *Camellia sinensis* (L.)

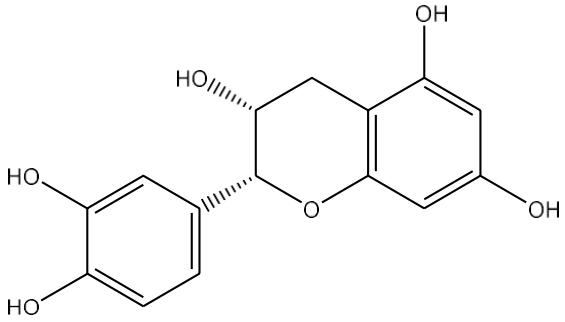
It is also known as green tea belonging to the family Theaceae, it is a popular ingredient in skin care products. Polyphenolic compounds in green tea extract provide significant antioxidant and anti-inflammatory benefits, as well as UV protection. Upon investigating some of the Tea polyphenol, like (+) catechin (C) (**9**), (-)-epicatechin 3-0-gallate (ECG) (**10**), (-) epicatechin (EC) (**11**), epigallocatechin (EGC) (**12**), it was observed that EGCG, ECG and GCG showed highly competitive monophenolase inhibition. Altogether, EGCG, EGC and C Along with another polyphenol (gallic acid), it was tested in B16 melanoma cells. They all inhibited melanogenesis by decreasing the expression of tyrosinase, with the exception of C. EGCG, the polyphenol most abundant in green tea, inhibits melanogenesis by decreasing the expression of MITF.



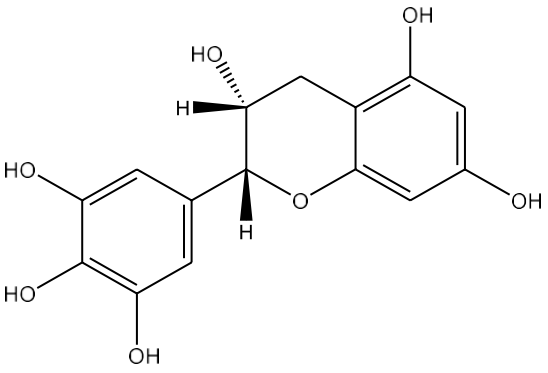
(+) catechin (C) (9)



(-)-epicatechin 3-0-gallate (ECG) (10)



(-) epicatechin (EC) (11)



Epigallocatechin (EGC) (12)

1.4.7 Other Herbs

Citrus fruits are cultivated widely and are the most conventional organic products on earth. They belong to the Rutaceae family. Traditional Chinese medicine uses a few essential Chinese lemons like *Citrus limon* Burmann forma Lisbon (Lisbon lemon), *C. limon* Burmann forma Eureka (Eureka lemon), and *Citrus keraji* Hort. ex. Tanaka (Keraji). They are rich in volatile oils like citral and myrcene. Based on a Lineweaver-Burk plot, citral inhibits tyrosinase non-competitively, while myrcene inhibits it competitively.

Distillation co-product of citrus hydrosol was found to inhibit mixed type tyrosinase in a recent study. In the GC analysis of hydrosols, some of the essential oils were detected, such as myrcene, sabinene, geraniol, and citral (Lante and Tinello, 2015). As a by-product of the citrus juice industry, nobiletin and hesperidin are citrus flavonoids obtained from citrus peel. In a study of mushroom tyrosinase inhibitory mechanisms, nobiletin and hesperidin were characterized as competitive and noncompetitive, respectively (Zhang et al., 2007).

Ginsenosides or panaxosides are steroid glycosides and triterpene saponins derived from natural sources. Both white ginseng and red ginseng are rich in ginsenosides. Based on its ability to regulate protein kinase A (PKA) pathway and cAMP level, ginsenoside Rh4 from Korean red ginseng exhibited depigmentation activity in B16 melanoma cells. Inhibits melanin synthesis as a result. One of the useful compounds obtained from P. ginseng leaves was p-coumaric acid, which inhibited the activity of mushroom tyrosinase. Interestingly, it inhibited human and murine tyrosinase and not mushroom tyrosinase. In comparison with kojic acid and arbutin, its inhibition potential for human tyrosinase was significantly higher. According to enzyme kinetics analysis, p-coumaric acid is a mixed type or competitive inhibitor of human tyrosinase (for L-tyrosine). In addition, it inhibits human epidermal melanogenesis. The roots and seeds of P. ginseng contain cinnamic acid. Cinnamic acid reduces melanin content and tyrosinase activity in the Melan-A cell, and it also depigmented the UVB-tanned skin of brown guinea pigs (Kim, 2015). As a result of the above facts, several medicinal plants with tyrosinase inhibitory activity have been compiled in Table 1.1.

Table 1.1. Important tyrosinase inhibitors from Ayurvedic Varnya herbs

Plant name (Family)	Phyto-constituent	Plant part used	Mechanism of Inhibition	References
Xanthoceras Sorbifolia Bunge (Sapindaceae)	Saponins	Nutshell	Mixed	(Zhang and Zhou, 2013)
Lawsonia inermis L. (Lythraceae)	Lawsone (2-hydroxy-1,4 napthaquinone)	Leaves	Mixed	(Gholamhoseinian and Razmi, 2012)
Berberis aristata DC. (Berberidaceae)	Berberine	Steam and Bark	Mixed	(Biswas et al., 2015a)
Rhizophora stylosa Griff. (Rhizophoraceae)	Polyphenols like tannin	Barks	N.D	(Suh et al., 2014)
Madhuca latifolia (J.Konig) J.F.Macbr. (Sapotaceae)	Ursolic acid, p-hydroxy-acetophenone, hydroquinone, taxifolin, madhushazone, madhusalmone, madhucic acid	Fruit pulp and seeds	Dose dependent inhibition	(Khan, et al., 2015)
Piper nigrum L. (Piperaceae)	Piperonylic acid	Fruits	Mixed	(Si et al., 2013)

1.5 Summary

In addition to dermatology, biomedical research, food and agricultural science, and insect physiology, tyrosinase inhibition studies are another active area of research. In our search for articles related to tyrosinase inhibition plant, Varnya herbs, we have found more than 3000 citations in PubMed, SciFinder, Scopus, Science Direct, and Google Scholar up to April 2021. This chapter discusses commercially important medicinal herbs and their Phyto molecules used in whitening cosmetic formulations. Hyperpigmentation can also be treated with these plants. Although they have been successful in inhibiting tyrosinase, there remain some limitations.

Varnya is an age-old concept, mentioned and practiced by the traditional medicinal system, Ayurveda. In Ayurveda, the herbs are enlisted in various categories, for the ease of identifying and understanding their functions and uses. Varnya concept is one of the most recognized classes of the system. Varnya means complexion and the herbs falling under this category are considered as Varnya Dravyas (complexion enhancing herbs).

Varnya Dravya or skin whitening and brightening herbs are those herbs which are considered to have properties to improve skin complexion and glow. These herbs are used very much by people in day-to-day life.

To present a link between the modern and ayurvedic concept, this study explores the relation between the claims of age-old medicinal system and modern medicinal system. In modern pharmaceutical point of view, the skin complexion completely depends upon melanin. The increase and decrease in the melanin secretion depends upon tyrosinase enzyme. To relate both the concepts, tyrosinase inhibition potential of the Varnya herbs are studied.

Food and beverages, such as tea, coffee, and berries, contain natural hydroquinone (HQ) or benzene-1,4-diol. The tyrosinase inhibition effect of these ingredients makes them popular in cosmetic formulations. By acting as an alternative substrate, it inhibits tyrosinase. A catalyzed product can cause irreparable damage to melanocytes and melanosomes. Due to this, long-term use of HQ may cause permanent depigmentation, exogenous ochronotic, and skin allergies. The mutagenic and carcinogenic potential of these chemicals may, however, result in more severe side effects (Lee et al., 2015).

A large number of phenols have been reported as tyrosinase inhibitors. In a similar way to catechol's, many phenols are oxidized to form ortho-quinones by tyrosinase. They serve as nucleophiles. Michal accepts thiol group containing enzymes can be attacked, leading to reactive oxygen species. By binding with melanosome proteins, it can also trigger allergic reactions by generating neo-antigens. In *Acer maximowiczianum* Miq, a phenolic compound called rhododendrol (RH) or 4-(4- hydroxyphenyl)-2-butanol was isolated. (Family- Sapindaceae). It was used in Japan as a skin-whitening agent. Similar to ortho-quinone, it was shown to be toxic. As a result of RH quinones toxicity, many consumers developed leukoderma on their face, neck, and hands.

Studies of tyrosinase inhibition have been conducted mostly with the mushroom tyrosinase *Agaricus campestris*. Crystal structure of tyrosinase inhibited by tropolone from Aspergillus has been developed (Ismaya et al., 2011) and deposited in Protein Data Bank (PDB) with access code 2Y9X. In-silico modeling of mushroom tyrosinase has extensive use in screening synthetic and natural lead molecules based on its X-ray crystallographic structure. Yet, it is not known what the 3D structure of human tyrosinase looks like. Homology modeling has been used to devise computational drugs. Due to their structural similarities, mushroom tyrosinase is more popular than human tyrosinase. Human tyrosinase contains a domain like epidermal growth factor (EGF), which is structurally similar to mushroom tyrosinase. Moreover, human tyrosinase's two copper ions were coordinated at its catalytic site by six residues of histidine, like a mushroom. While the tetrameric mushroom tyrosinase shares a few amino acids with human monomeric tyrosinase, the amino acid sequences share very little (Chang, 2009; Ismaya et al., 2011). 50 endemic Korea plants were extracted with ethanol and screened with human tyrosinase from HEK293-TYR cells using ethanol extraction and tyrosinase from HEK293-TYR. As a result of this study, *Vaccinium bracteatum* Thunb extracts exhibited greater inhibition of human tyrosinase compared to mushroom tyrosinase. Kim et al. (2012) found that *Morus bombycis* Koidz exhibited higher tyrosinase inhibition potential than human tyrosine (Kim et al., 2012). Human tyrosinase is inhibited more by thujaplicins than mushroom.

1.6. Conclusion

It is this research's specific objective to translate preclinical findings into clinical applications. There is an urgent need for alternative depigmentation drugs that are more effective than hydroquinone due to its toxicity. As an important component of this process, medicinal plants and phytochemicals played an important role. For the topical treatment of hyperpigmentation, Phyto-molecules like n-butyl resorcinol, ellagic acid, arbutin, and azelaic acid have been clinically investigated (Leyden et al., 2011). Research on tyrosinase inhibitors is useful for improving nutritional value and food quality, limiting pests, inhibiting pigmentation disorders and other melanin-related health issues in humans. Different types of compounds from medicinal sources have been tested in order to obtain better inhibitors. Overall, however, much more research is needed to confirm tyrosinase inhibiting herbs and Varnya herbs are essential for skin care and health. There must be more research around this genre of work.

Publication

Mukherjee, P.K., Biswas, R., **Sharma, A.,** Banerjee, S., Biswas, S., Katiyar, C.K., 2018. Validation of medicinal herbs for anti-tyrosinase potential. ***Journal of Herbal Medicine***. 14, 1-16. https://doi.org/10.1016/j.hermed.2018.09.002

Chapter - 2

2. Scope, objective, and plan of work

2.1. Scope and rationale of the present study

2.2. Objective of the work

2.3. Study framework

2.1. Scope and rational of present study

In both plants and animals, polyphenol oxidase, or tyrosinase, is a multifunctional copper-containing enzyme. This enzyme regulates melanogenesis (melanin synthesis) within melanocytes. Among its main functions is the oxidation of L-tyrosine and L-dopa to dopaquinone, followed by the oxidation of dopaquinone to dopachrome. The color of the skin is regulated by melanin, a heterogeneous mixture of biopolymers. Excessive amounts of melanin cause hyperpigmentation. The tyrosinase enzyme performs other functions in addition to producing melanin, such as sclerotizing insect cuticles and detoxifying symbiotic bacteria's defensive substances. Furthermore, enzymatic browning reactions caused by tyrosinase damage plants-derived foods, such as fruits and vegetables. In the past, sulphites and ascorbic acid were widely used as anti-browning agents since they inhibit tyrosinase. In addition, prolong consumption of sulphites can harm the nutritional quality of food and cause unwanted side-effects in the human body, such as allergies and gastrointestinal distress. In a similar way, ascorbic acid can alter the smell of most beverages, and its effect is temporary. The food industry has therefore made it a priority for researchers to develop new potent and safe tyrosinase inhibitors for use in anti-browning procedures. Additionally, these inhibitors are effective in treating hyper-pigmentation syndromes as well.

The advantages of natural products and their synthetically modified derivatives are their broad spectrum of biological activities, higher safety margins, and lower costs than their synthetic equivalents. The possible uses of herbs as tyrosinase inhibitors should be investigated further with suitable models and approaches. A plant's therapeutic potential depends on the types of bioactive compounds present in the species. Collection of species, ranges of extraction techniques, processing steps and handling generally lead to wide variation in quality of commercial products; therefore, this presents the greatest challenge to the assurance of consistent quality products. To achieve the optimal concentrations of known active constituents in herbs or herbal preparations, standardization with analytical techniques such as high-performance liquid chromatography (HPLC) is essential. The use of traditional and other plants from natural resources should be evaluated in terms of potential for treating hyperpigmentation disorders. Cosmetic products can also contain them as an ingredient

for skin-lightening effects. It is not desirable to have hyperpigmented skin or fruits that have enzymatically browned.

2.2. Objective of the work

The present study was an attempt to investigate selected three medicinal plant species *Berberis aristata*, *Rubia cordifolia and Dillenia indica* for their phytochemical and biological potential related to tyrosinase inhibition. The work was divided into several major sections including collections and authentication of the plant materials; enzyme inhibition kinetics analysis; assessment of mechanism of inhibition; extraction and bio-assay guided fractionation followed by standardization using marker components through HPLC method.

Several medicinal plant species have been investigated in this study, including Berberis aristata, Rubia cordifolia and Dillenia indica, in order to determine their phytochemical and biological effects on the inhibition of tyrosinase. In this study, the work was divided into a number of major sections that included the collection and authenticity of the plant materials; assessment of the mechanism of inhibition; extraction, and standardization using marker components using HPLC method.

The works primarily focused on the following aspects:

- The selection of Varnya herbs based on their ethno-meditative uses.
- Acquiring and authenticating the selected plants.
- Based on a literature review and the condition of the raw material, extract plant materials using methanol as the solvent.
- Varnya herbs were evaluated for their tyrosinase inhibitory potential.
- Analysis of extracts using reverse phase high performance liquid chromatography (RP-HPLC) and a specific solvent system.
- To develop topical tyrosinase-inhibiting formulations using selected medicinal plants.

- Formulation evaluation through physical, chemical, microbial, and functional evaluation.
- Evaluation of formulation stability.

The design of the research was based on the ethno-medicinal perspective, and four selected medicinal plants were evaluated for their anti-tyrosinase activity, and a poly-herbal formulation was developed to stimulate skin lightening or whitening.

2.3. Study framework

The works mainly design for evaluating each of tyrosinase inhibitory medicinal plants and a formulation preparation. The plan of work is represented in the schematic diagram (Figure 2.1).

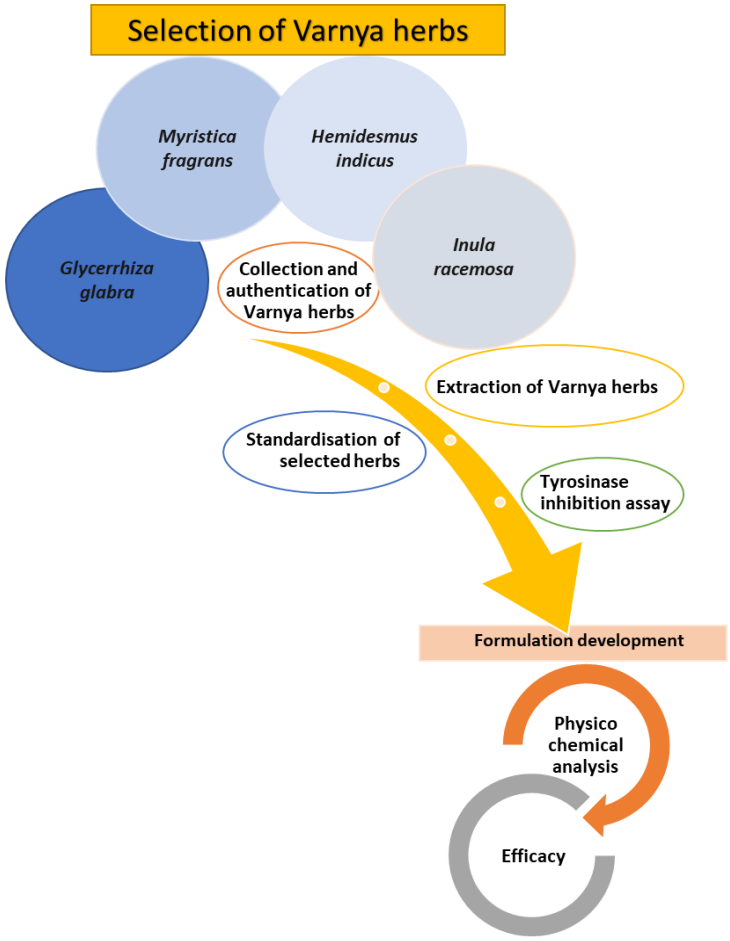


Figure 2.1 Work plan for the study

Chapter - 3

3. Standardization of Varnya herbs using RP-HPLC and HPTLC

3.1. Plant profile of selected Varnya herbs

3.2. Extraction of herbs

3.3. Marker analysis and quantification of Phyto-molecules by validated HPLC & HPTLC analysis

3.4. Results

3.5. Discussion

3.6. Conclusion

3.1. Plant profile of selected Varnya herbs

In Ayurveda there are many plants enlisted under the Varnya group of herbs, out of those, the following herbs were selected, and their plant profiles were shown in this chapter.

3.1.1. *Glycyrrhiza glabra* L.

Liquorice or the root of *G. glabra* is traditionally and commercially used for skin whitening formulations. Glycyrrhiza glabra grows in Eurasia, Southwest Asia, and the Mediterranean. As reported by Hayashi (2009) and Hayashi and Sudo (2009), *G. Glabra* was found in Southern Europe (Spain, Italy), Turkey, Iran, Iraq, Central Asia, and northwestern China, and *G. Urarensis* was found in the center. Only in the Xinjiang Uygur Autonomous Region, northeastern China, *G. inflata* found throughout Asia, Mongolia, and northwestern and northeastern China. There are two distinct types of G. glabra: Spanish licorice (G. glabravar Typica) and Russian licorice (G. glabravar Glandulifera).

Scientific classification			**Vernacular names**		
Kingdom	:	Plantae	English	:	Liquorice
Division	:	Tracheophyta	Sanskrit	:	Yeshtmadhu
Class	:	Dicotyledons	Hindi	:	Yeshtmadhu
Order	:	Fabales	Bangali	:	Jesthimadhu
Family	:	Fabaceae	Tamil	:	Atimaduram
Genus	:	*Glycyrrhiza*	Telugu	:	Yashtimadhukkam
Species	:	*G. glabra*	Gujrati	:	Jethimadhu

Fig 3.1a: Roots of *G. glabra L.*

Fig 3.1b: Plant and flower of G*. glabra L.*

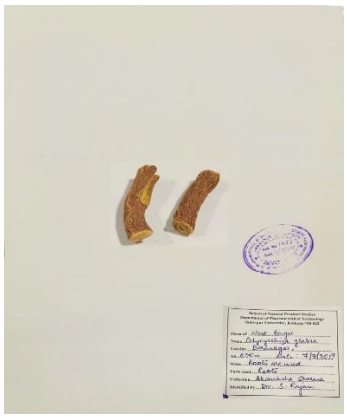


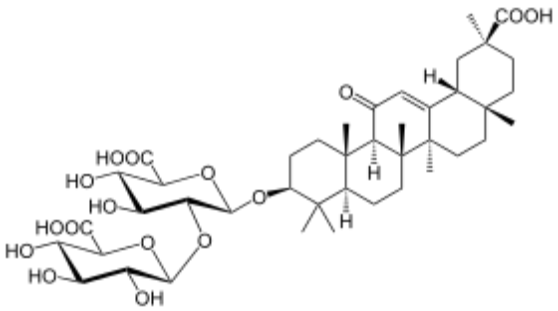
Fig 3.2: Herbarium of *G. glabra*

i. Plant description

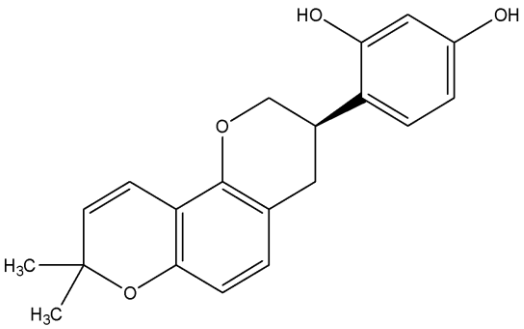
A herbaceous perennial with a 0.5–1.5 m tall stem that is woody at the base and thickly scaly glandular punctuated by two stoloniferous roots. Imparipinnate leaves with 9–17 ovate-oblong, oblong-lanceolate, or elliptic leaflets, 7–15 cm long. Abaxially thickly scaly glandular punctate and pubescent on veins, adaxially glabrescent or pilose, 1.7–4.0 by 0.8–2.0 cm (Plate1). Stipules are caducous and linear, about 1–2 mm in length. The inflorescence is open, racemose, and many flowers have bloomed. Flowers are 0.8–1.2 cm in length. Corolla purple or pale whitish blue, 9–12 mm, standard ovate or oblong, 1–1.1 cm, base clawed, wings 8–9 mm, keel straight, 7–8 mm; ovary glabrous. Calyx campanulate, 5–7 mm, 5-toothed, upper 2 teeth mostly connected; corolla purple or pale whitish blue, 9–12 mm, standard ovat 2–3 cm long, oblong, flat, glabrous or slightly hairy legume with 2–8 dark green, smooth seeds, 2 mm across (Lee et al., 2005).

ii. Chemical constituensts

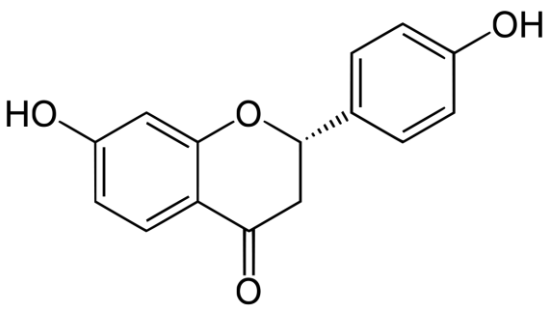
Total phenols, total flavonoids, and total tannins in licorice extracts of G. glabra roots at two distinct harvest dates ranged from 72.10 to 107.93 mg/g, 18.42 to 44.2 mg/g, and 4.8 to 12.78 mg/g, respectively, in licorice extracts of G. glabra roots (Cheel et al. 2013). The primary components of licorice extract, liqueritin and glycyrrhizin, ranged from 28.65 to 62.80 mg/g and 41.84 to 114.33 mg/g, respectively. Glycyrrhizin (1), glabridin (2), glabrene, and liquiritigenin (3) derivative proportions ranged from 0.88 to 11.38 percent, 1.86 to 10.03 percent, 1.80 to 18.40 percent, and 5.53 to 16.31 percent, respectively. Treatment of 65-day-old G. glabra plantlets with 0.1–2 mM methyl jasmonate and 0.1mM and 1mM salicylic acid increased glycyrrhizin synthesis by 3.8 and 1.8 times, respectively. When compared to the controls, the results were.4.1 times higher (Shabani et al. 2009). Increased glycyrrhizin levels in roots treated with methyl jasmonate hindered root growth, but salicylic acid increased glycyrrhizin levels without affecting root growth.



Glycerrhizin (1)



Glabridin (2)



Liquiritigenin (3)

Fig 3.3: Chemical constituents of *Glycyrrhiza glabra* L.

3.1.2. *Myristica fragrans*

M. fragrans also known as 'Nutmeg', belongs to the family Myristicaceae. The different parts of the plant have been used since long as spices in the food preparations and it has been reported to possess significant antioxidant properties, protein tyrosine phosphatase inhibitory activity, antibacterial activity (Jaiswal, et, al., 2009). It is used as stomachic, digestive, carminative, and cardiac tonic in traditional system of medicine (Sharma, et, al., 1979). M. fragrans is also prescribed for medicinal purposes in Asia to treat many diseases such as rheumatism, muscle spasm, decreased appetite, and diarrhea (Nguyen et al., 2010).

Scientific classification			**Vernacular names**		
Kingdom	:	Plantae	English	:	Nutmeg
Division	:	Angiosperms	Sanskrit	:	Jatiphala
Class	:	Magnoliopsida	Hindi	:	Jatiphala
Order	:	Magnoliales	Bangali	:	Jatiphala
Family	:	Myristicaceae	Tamil	:	Catikkay
Genus	:	*Myristica*	Telugu	:	Jajikaya
Species	:	*M. fragrans*	Gujrati	:	Jayaphala

Fig 3.4a: Seeds of *M. fragrans*

Fig 3.4b: Plant of *M. fragrans*

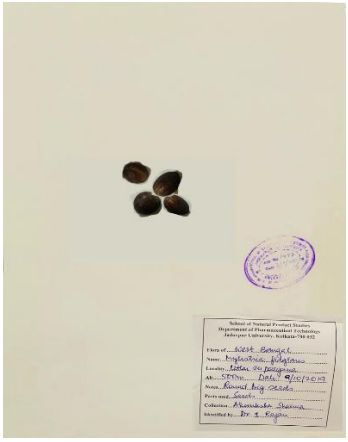


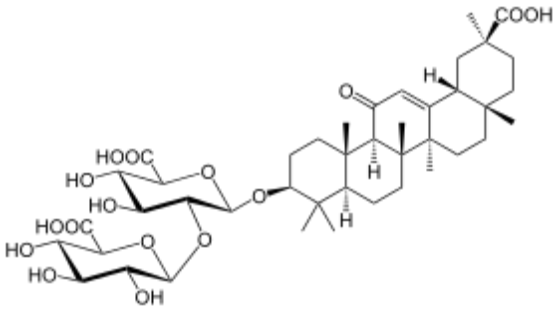
Fig 3.5: Herbarium of *M. fragrans*

i. Plant description

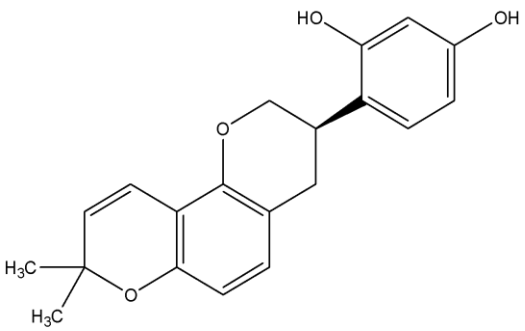
M. fragrans Houtt. (Myristicaceae) is an aromatic evergreen tree cultivated in tropical countries including South Africa, India, and the Philippines. nutmeg is the dried kernels from this plant. Traditionally, this spice has been used in many Western foods since it was introduced to Europe in the 12th century. Myristica fragrans is an evergreen tree, usually standing between 5 and 15 meters (16 to 49 feet) high, but occasionally reaching 20 meters (66 ft) or even 30 meters (98 ft) tall. Alternately arranged leaves measure 5–15 cm long and 2–7 cm wide, with petioles about 1 cm long. The species is dioecious, meaning the staminate flower and the carpellate flower are borne on different plants, although occasionally one plant will produce both kinds of flower. Bell-shaped, pale yellow and somewhat waxy and fleshy, the flowers are small and bell-shaped. Staminate flowers are grouped in groups of one to ten, and each is 5–7 mm (0.2–0.3 in) long; carpellate flowers are in smaller groups, one to three, and larger, being 10 mm (0.4 in) long (Orwa et. al., 2009).

ii. Chemical constituents

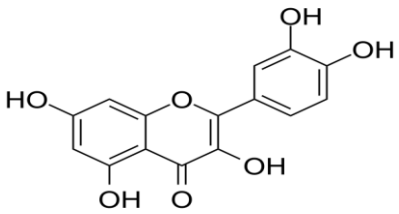
M. fragrans consists of 25–30% fixed oils and 5–15% volatile oils (camphrene, eugenol, etc.), along with other molecules such as myristic acid (4), myristicin (5), quercetin (6) and lignin compounds. One of these molecules, eugenol, is widely used in dentistry, as it is very effective at combating oral bacteria. The effectiveness of nutmeg extract against oral bacteria has been studied only in a few studies (Orwa et. al., 2009).



Myristic acid (4)



Myristicin (5)



Quercetin (6)

Fig 3.6: Chemical constituents of *M. fragrans*

3.1.3. *Hemidesmus indicus*

H. indicus (Anantamool) also known as Sariva in Ayurveda. The major chemical constituents present in it are 2-hydroxy-4-methoxybenzaldehyde vanillin, rutin, β-sitosterol and lupeol. This herb has been reported to be potent tyrosinase inhibitor, antioxidant, antimicrobial and has wound healing properties. In the Ayurvedic system of Medicine, it is widely used in the treatment of oligospermia, gastritis anorexia and menorrhagia (Nagari et al., 2015).

Scientific classification

Kingdom	:	Plantae
Division	:	Angiosperms
Class	:	Tracheophytes
Order	:	Gentianales
Family	:	Apocynaceae
Genus	:	*Hemidesmus*
Species	:	*H. indicus*

Vernacular names

English	:	Indian Sarsaparilla
Sanskrit	:	Anantamul, Sariva
Hindi	:	Anantamul
Bangali	:	Anantamul
Tamil	:	Nannari
Telugu	:	Suganda pala
Gujrati	:	Sariva

Fig 3.7a: Roots of *H. indicus*

Fig 3.7b: Plant of *H. indicus*

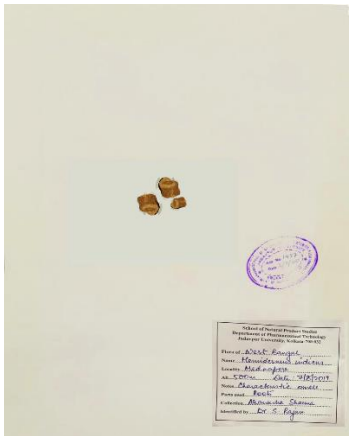


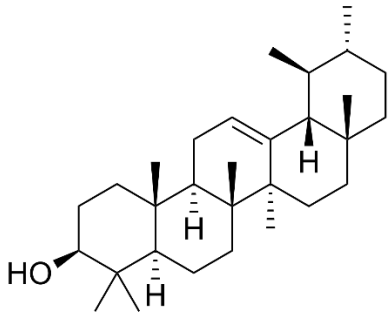
Fig 3.8: Herbarium of *H. indicus*

i. Plant description

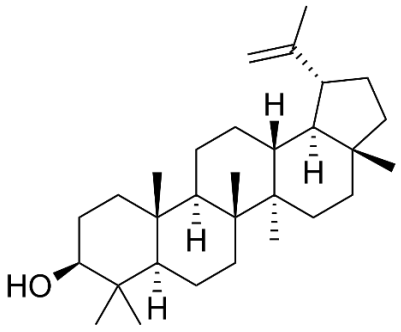
Tropical and subtropical parts of India are home to the plant. Soils rich in humus such as loam and clay loam are suitable for its cultivation. The soil for this plant should have a pH between 7.5 and 8.5. Flower clusters are small, greenish purple, clustered in axillary cymes. The fruits (follicles) are cylindrical, pointed, and paired. They have oblong seeds. It usually blooms sparsely in October and matures in January. It is found in tropical and subtropical parts of India, particularly in the upper Gangetic plains, Bengal, Madhya Pradesh, and the south. Frequently found on slopes of sub-ravines, it twines around shrubs and trees (Das et al., 2013).

ii. Chemical constituents

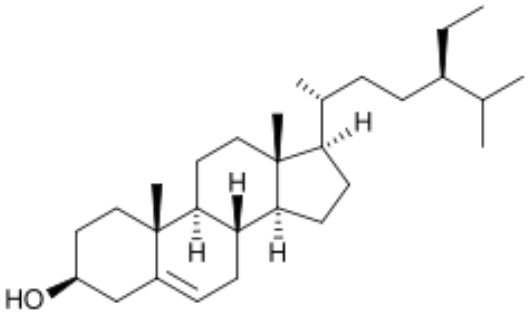
Plant roots of *H.indicus* contain hexatriacontane, ferulic acid, α-amyrin (7), lupeol (8), its octacosanoate, phytin, and sitosterol (9). Additionally, there are two oleanenes, a coumarino-lignoid-hemidesminine, and three ursenes among six pentacyclic triterpenes. It also yielded 3-keto-lup-12-en-21 28-olide as well as lupanone, lupeol-3-α-acetate, hexadecenoic acid, 4-methoxy-3-methoxybenzalaldehyde, 3-methoxy-4-5-methoxybenzalaldehyde glycosides-indicine, and hemidine. The leaves contain tannin, flavonoids, hyperoside, rutin, and coumarin. Leaf lignoids including hemidesminine, hemidesmin I, and hemidesmin II are a rare group of naturally occurring compounds (Anonymous, 2016).



α-amyrin (5)



Lupeol (6)



Sitosterol (9)

Fig 3.9: Chemical constituents of *H. indicus*

3.1.4. *Inula racemose H.*

I. racemosa belongs to the family Asteraceae, also known as Pushkarmool in Ayurveda; is mainly used in the treatment of several diseases like antifungal agent, antibacterial agent, they are known to be effective in epidermal carcinoma. While in ancient literature it has been reported to be used as immunomodulator, anti-catarrhal, anti-inflammatory, analgesic, anti-diabetic, anti-spasmodic, cardioprotective, diuretic, etc (Singh, et al., 2016). One of the components found in I. racemose, inulavosin 90 has been worked on previously for its anti-melanogenic activity. Inulavosin 90 effectively reduces the melanin content without affecting the tyrosinase enzyme transcription in B16 melanoma cells. It inhibits melanogenesis by missing the target tyrosinase, and instead attacks on lysosomes (Fujita et al., 2009).

Scientific classification			**Vernacular names**		
Kingdom	:	Plantae	English	:	Elicampane
Division	:	Tracheophytes	Sanskrit	:	Padmapatra
Class	:	Angiosperms	Hindi	:	Puskarmool
Order	:	Asterales	Bangali	:	Puskarmool
Family	:	Asteraceae	Tamil	:	Puskkaramulam
Genus	:	*Inula*	Telugu	:	Puskaramul
Species	:	*I. racemosa*	Gujrati	:	Puskarmool

Fig 3.10a: Plant of *I. racemose H.*

Fig 3.10b: Roots of *I. racemose H.*

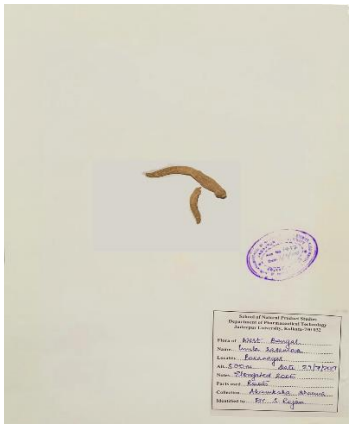


Fig 3.11: Herbarium of *I. racemose H.*

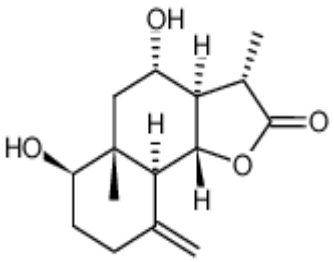
i. Plant description

I. racemosa perennial herb with a prominent root and a fragrant rootstock, racemose grows up to 1.5 m tall. There are many stems that grow from rootstock. In shape, the leaves are elliptic-lanceolate, leathery, and rough above, densely hairy below, and 25–50 cm long and 10–12 cm wide. A terminal raceme has yellow flower heads with bisexual florets. In January–July, flowers bloom, while fruiting occurs in October–November. Achenes (fruits) are about 0.5 cm long and slender (Jabeen et al., 2007).

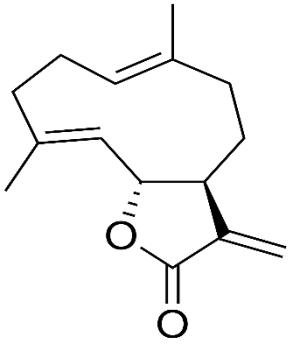
ii. Chemical constituents

A total of 28 compounds are found in Inula. They are mostly sesquiterpenes. Some of the compounds found in I. racemosa are eudesmanolide (10), 2-hydroxy-4-methoxybenzaldehyde (11), elemanolide (12), germacranolide, sesquicaranolide, guainolide, and humulane sesquiterpenes. Oil extracted from the roots of I. racesmosa

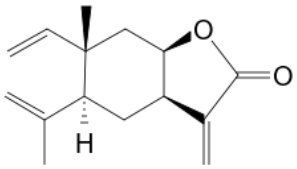
has been investigated and 16 compounds identified. Approximately 60% of the oil's chemical content is sesquiterpenes, the most abundant of which is heptadeca-1,8,11,14-tetraene (22%) (Bokadia et al. 1986).



Eudesmanolide (10)



2-hydroxy-4-methoxybenzaldehyde (11)



Elemanolide (12)

Fig 3.12: Chemical constituents of *I. racemose H.*

3.2. Extraction of plant material

The extraction process started with the collection of *G. glabra*, *M. fragrans*, *H. indicus* and *I. racemosa*, from the local market and vendors of West Bengal. The herbs were authenticated by Dr. S. Rajan, Field botanist, medicinal plant collection unit, Ooty, Tamil Nadu, Govt. of India. For further references the voucher specimen numbers SNPS-1000/2018, SNPS-1001/2018, SNPS-1002/2018 and SNPS-1003/2018 for *M. fragrans*, *H. indicus* and *I. racemosa* respectively were retained in the School of Natural Product Studies, Jadavpur University, Kolkata. The extraction process was carried out in methanol by cold maceration for 72 hours and filtered through Whatman no. 1 filter paper. The filtrate was concentrated on rotary evaporator and extracts were lyophilized and stored at -20°C for further use. The percentage yields of the extracts were calculated.

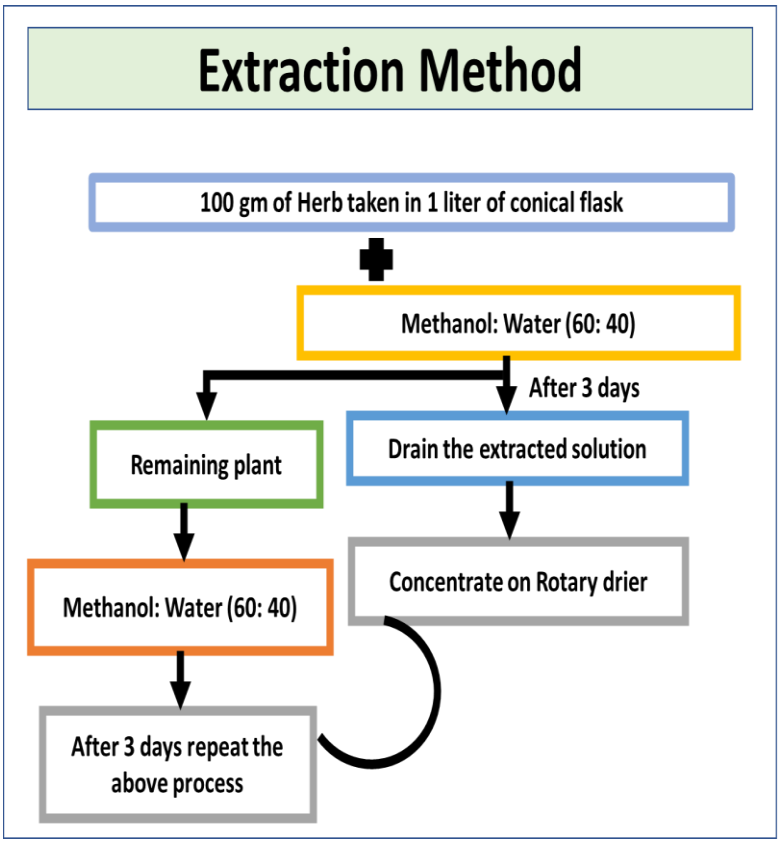


Fig 3.13: Extraction method for Varnya herbs

3.3. Marker analysis and quantification of phytomolecule by validated HPLC & HPTLC analysis

For defining the constituents of the herbs, the most effective method is high performance thin layer chromatography (HPTLC) in combination with high performance liquid chromatography (HPLC), which operates with different kinds of detectors. The RP-HPLC and HPTLC of three potent medicinal plants were evaluated based on their marker profiling. The results obtained from these two methods were compared to observe the metabolic profiling in the extracts of the herbs.

3.3.1. Instrumentation and Reagents

The RP-HPLC system used (Waters, Milford, MA, USA) comprises of a 600-controller pump, a multiple-wavelength ultraviolet–visible (UV-Vis) detector equipped with an in-line degasser AF 2489 and a rheodyne 7725i injector having 20 µl loop. A Milli-Q academic water purification system developed by Bedford, MA, USA equipped with 0.22 mm millipak express filter and Eyela (Tokyo, Japan) rotary vacuum evaporate were used for the analysis. NYL 0.45 µm, syringe filters were used for the filtration of the sample solution and 0.45 µm pore size, Millipore, membrane filters were used for filtration of the mobile phase.

CAMAG HPTLC, system was used for the experiments, which comprised of LINOMAT V automatic sample applicator, WINCATS software, scanning densitometer CAMAG scanner 3 and photo-documentation apparatus CAMAG reprostar 3. For the stationary phase 20 x 20 cm size, aluminum-based silica gel plate 60 F254 (Merck, Mumbai, India) in a particle size of 5-10 µm was used. Solvents used during the analysis were HPLC grade solvents. For sample application on HPTLC plates, 100µl syringe (Hamilton, Switzerland) was used. Empower 2 software programs were used for Quantitative estimation using the external standard calibration method. Methanol, glacial acetic acid, petroleum ether and ethyl acetate, toluene, formic acid and other solvents used were obtained from Merck and were of AR grade. The standard markers used were ferulic acid (>99%), quercetin (>98%), chlorogenic acid (>95%), were obtained from Sigma-Aldrich.

3.3.2. Preparation of standard and sample solutions

Standard stock solutions of all three biomarkers used i.e., quercetin, ferulic acid and chlorogenic acid, were prepared by dissolving 0.1 mg of standard in 1 ml methanol. To obtain calibrated samples in the range of 1-100 µg/ml, further dilution were carried out. For the preparation of sample solution, 10 mg of extract (*G. Glabra*, *M. fragrans*, *H. indicus* and *I. racemosa*) were dissolved in 1.0 ml of methanol and the solution was then filtered with the help of 0.45 µl syringe filter prior to injection.

3.3.3. RP-HPLC standardization of selected plants with respective phyto-markers

For RP-HPLC analysis different isocratic methods were developed, out of which the following gave the ideal separation thus chosen; Acetonitrile and 0.1 M orthophosphoric acid. Solvent A (Acetonitrile): solvent B (0.1 M orthophosphoric acid in water with pH 2.5) ratio 85:15 v/v for glycyrrhizin, Solvent A (Acetonitrile): solvent B (0.1 M orthophosphoric acid in water with pH 2.5) ratio 75:25 v/v for quercetin, Solvent A (Acetonitrile): solvent B (0.1 M orthophosphoric acid in water with pH 2.5) ratio 80:20 v/v for ferulic acid, Solvent A (Acetonitrile): solvent B (0.1 M orthophosphoric acid in water with pH 2.5) ratio 60:40 v/v for chlorogenic acid. The external standard calibration curve for all the three markers were prepared with calibration solutions in a concentration range of 80-200µg/ml. 20 µl of standard solution was injected in the rheodyne loop. The same method was applied for the extract. The flow was monitored at wavelength of 254nm.

3.3.4. HPTLC method of standardization for selected plants

The mobile phase was optimized for the HPTLC analysis. Out of many mobile phases that had been tried, following mobile phase gave the better separation thus optimized mobile phase was toluene: acetone: water (3:5:2, v/v/v) for quercetin. For ferulic acid toluene: ethyl acetate: formic acid (5.5:3.5:1, v/v/v), for chlorogenic acid, chloroform: ethyl acetate: methanol (3:6:1, v/v/v).Then 50 µl of standard solution was drawn into CAMAG LINOMAT syringe and put to linomat applicator to give the concentrations of standards required. The same method was applied for the extracts. The external standard calibration curves for all the three marker compounds were prepared in the range of 200 - 1000 ng/ml. The plate was pre-heated and developed in a CAMAG twin trough glass chamber with the same mobile phase. After development, the plate was air dried and scanned in camag TLC scanner 3 at a wavelength of 254 nm.

3.3.5. Validation of RP-HPLC and HPTLC methods

The RP-HPLC and HPTLC methods validation was carried out by determining linearity, specificity, accuracy and precision, limit of quantification and limit of detection on the basis of International Conference on Harmonization guideline (ICH, 2005). Method specificity was determined by comparing the retention time of both standard and test samples. Sensitivity was evaluated by determining the limit of detection (LOD) and limit of quantification (LOQ) and calculated based on the equation: LOD = 3.3 σ/S and LOQ = 10 σ/S, where σ is the standard deviation and S is the slope of the calibration curve. Intra-day and inter-day assay accuracy and precision for each analyte were determined at LQC (low quality control), MQC (medium quality control) and HQC (high quality control). Both the parameters were assessed by comparing data from within one run (n=6). Accuracy of the method was determined by standard addition technique and expressed in terms of % relative standard deviation (% RSD) for mean recovery of the theoretical concentrations. The samples were spiked with three different amounts of standard compounds in triplicate. Injecting six replicates at three different concentrations of the reference compounds assessed the precision of the analytical method. The values were represented as % RSD of intra-day and inter-day analysis. Using six replicates of test concentrations performed system suitability testing. Variations in number of theoretical plates, capacity factor, and tailing factor were also calculated as average of six replicates. Statistical analysis was performed using the Graph Pad Prism Version 5.0. The result has been represented as the mean ± % RSD.

3.4. Results

3.4.1. Extraction yield

The extracts prepared were weighed and the percentage yield was calculated. The percentage yield of the methanolic extracts were found to be 5.66% w/w, 4.32% w/w, 3.76% w/w, and 4.55% w/w, for G. glabra, *M. fragrans*, *H. indicus* and *I. racemosa*, respectively.

3.4.2. Standardization of phenolic compounds by RP-HPLC method

The RP-HPLC chromatograms are shown in the figure 3.14-3.21. Glycyrrhizin present in G. glabra was 1.22% (w/w) with Rt of 9.13 min, Quercetin present in *M. fragrans* was 0.62% (w/w) with Rt of 7.44 min, ferulic acid present in *H. indicus* was found out to be

1.39% (w/w) with Rt of 8.083min and chlorogenic acid present in *I. racemosa* was established to be 1.03 % (w/w) with Rt of 3.19 min.

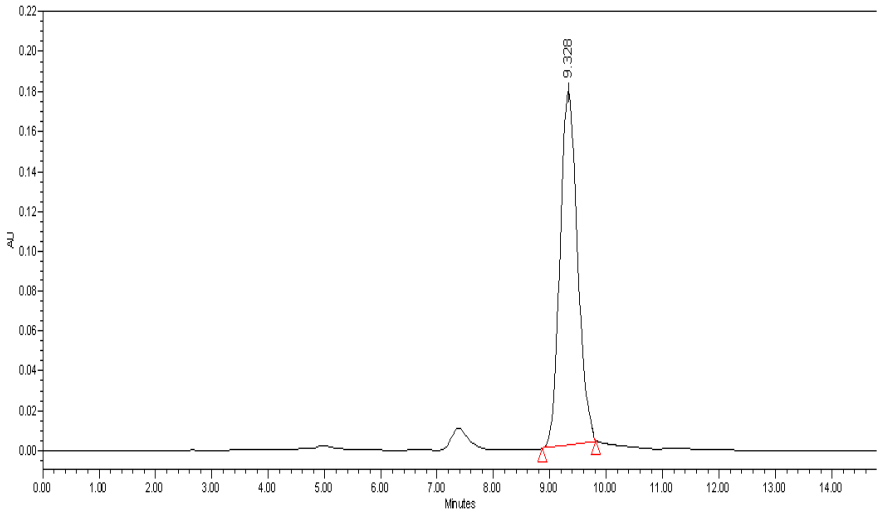


Fig 3.14. HPLC chromatogram of standard Glycyrrhizin

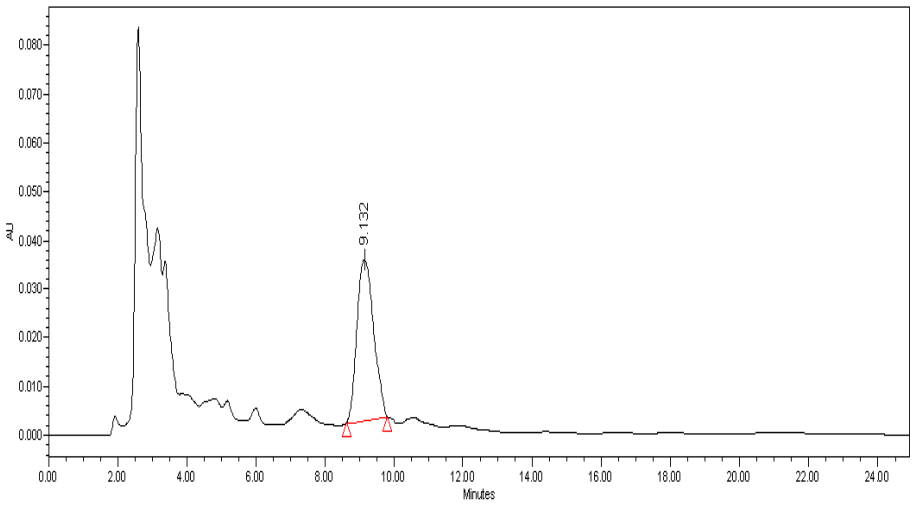


Fig 3.15. HPLC chromatogram of *G. glabra*

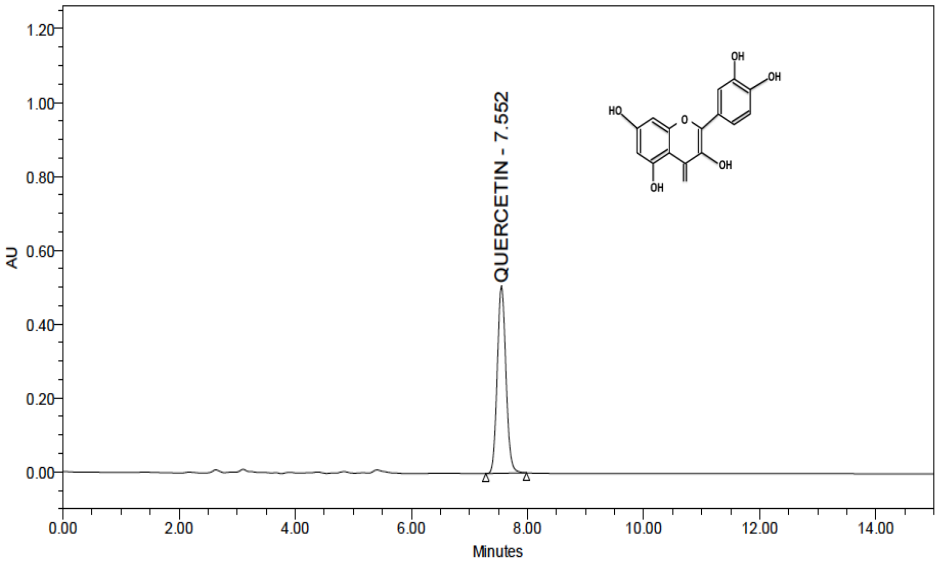


Fig 3.16. HPLC chromatogram of standard Quercetin

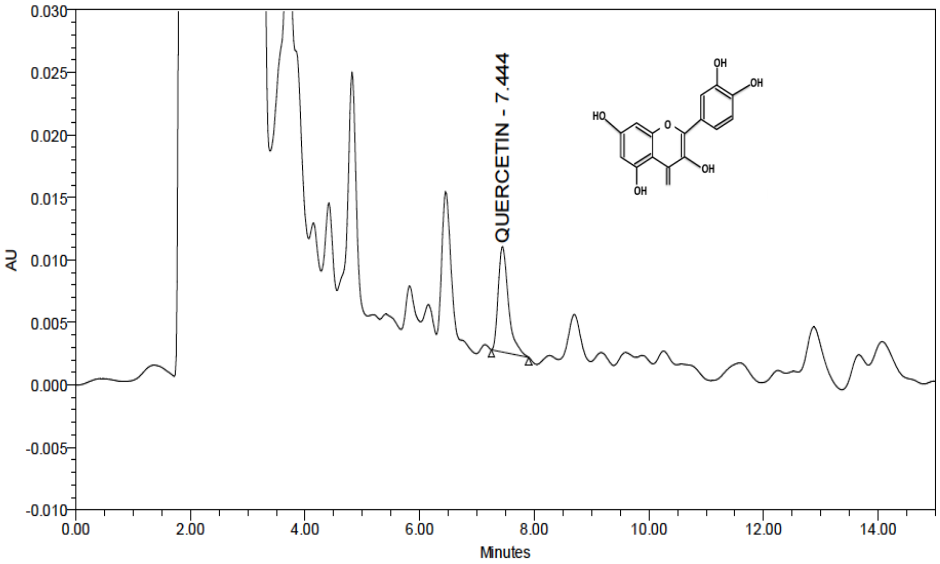


Fig 3.17. HPLC fingerprint of *M. fragrans* extract

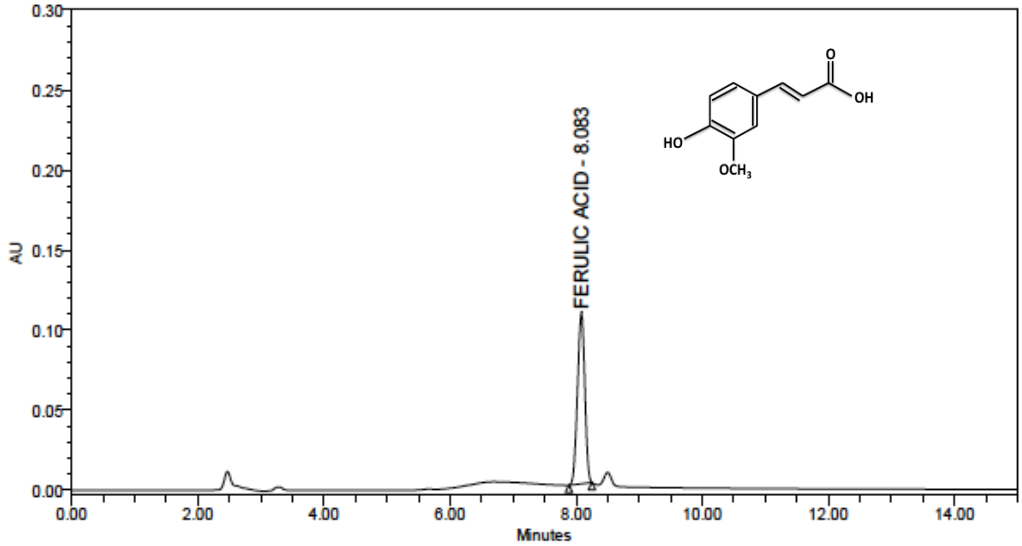


Figure 3.18. HPLC chromatogram of standard ferulic acid

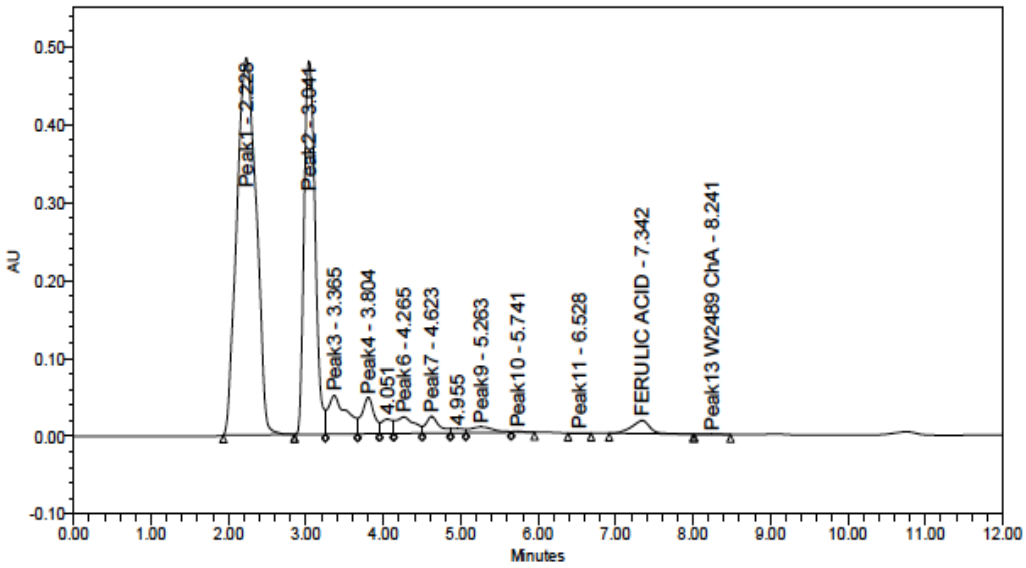


Figure 3.19. HPLC fingerprint of *H. indicus* extract

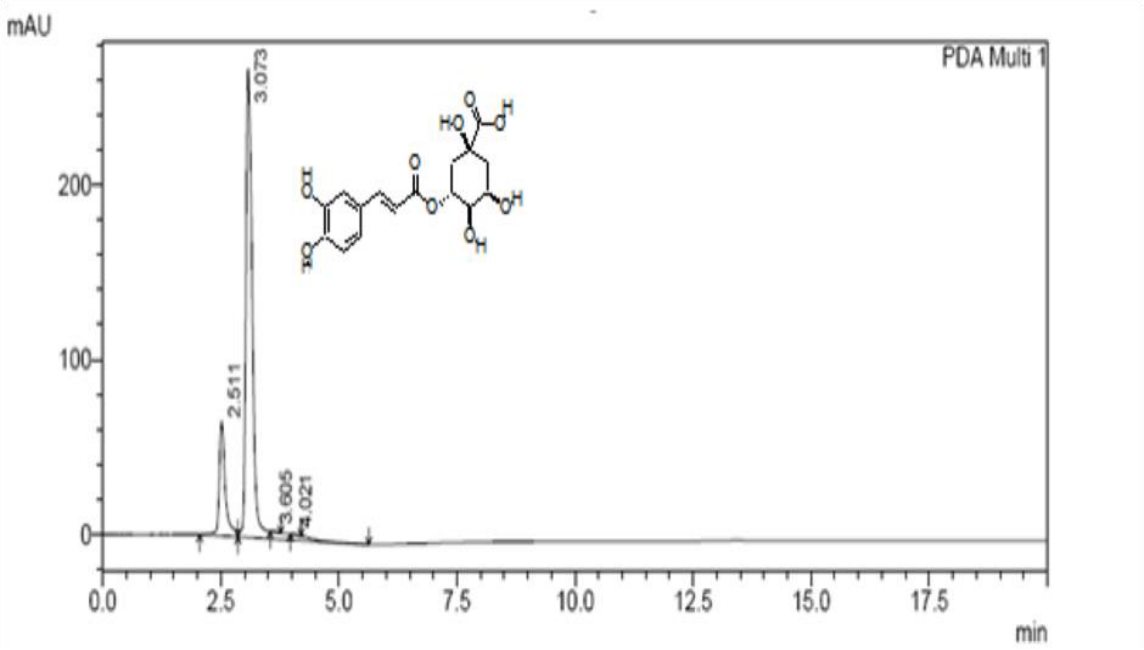


Figure 3.20. HPLC chromatogram of standard chlorogenic acid

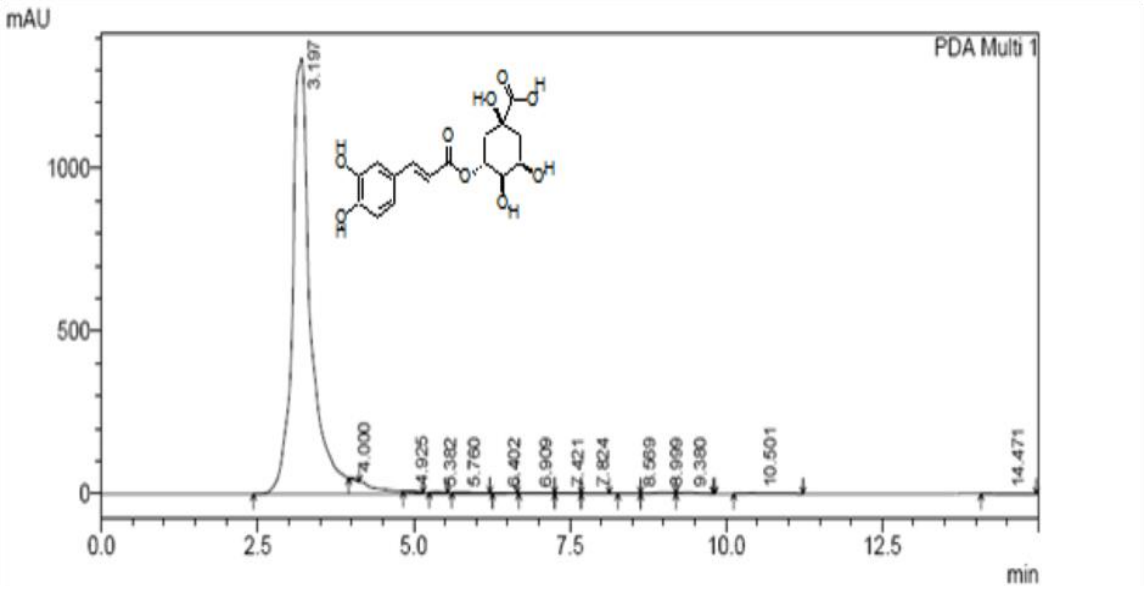


Figure 3.21. HPLC fingerprint of *I. racemosa* extract

3.4.3. HPTLC method

HPTLC chromatograms are shown in the figure 3.22-3.33. The study showed that percentage of glycyrrhizin present in *G. glabra* was 1.52% (w/w) with Rf value of 0.57, quercetin present in *M. fragrans* was found to be 1.23% (w/w) with Rf value of 0.48, ferulic acid present in *H. indicus* was found to be 1.52% (w/w) with Rf value of 0.44 and chlorogenic acid in *I. racemosa* was 1.09% (w/w) with Rf value of 0.52.

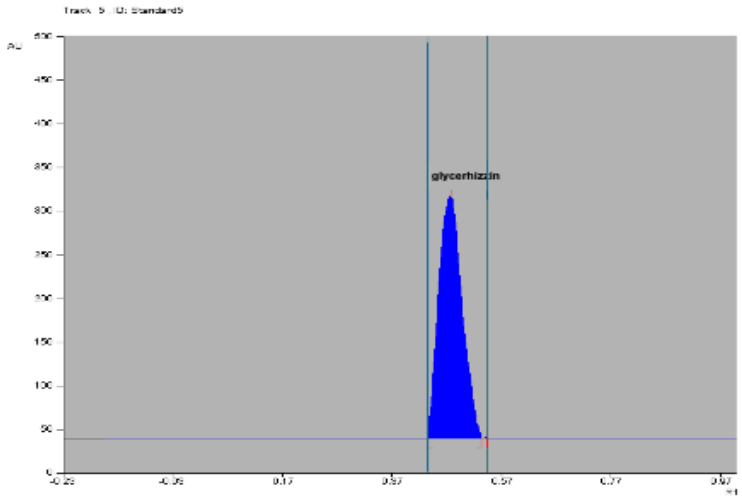


Figure 3.22. HPTLC chromatogram of *standard Glycyrrhizin*

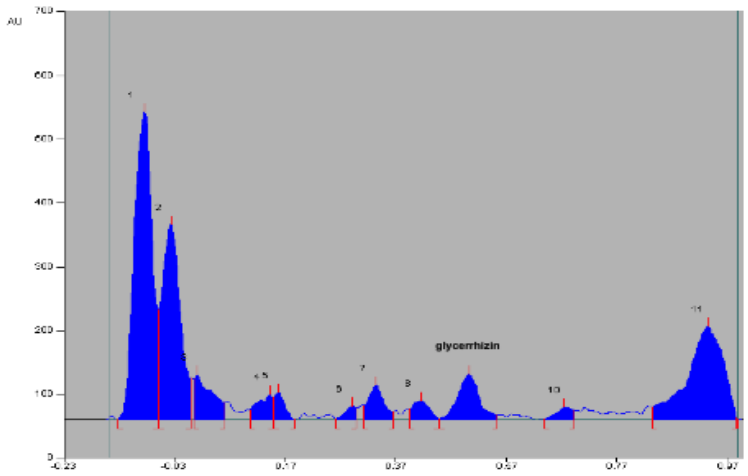


Figure 3.23. HPTLC fingerprint of *G. glabra*

Figure 3.24. Photo documentation of standard Glycyrrhizin and *G. glabra* extract

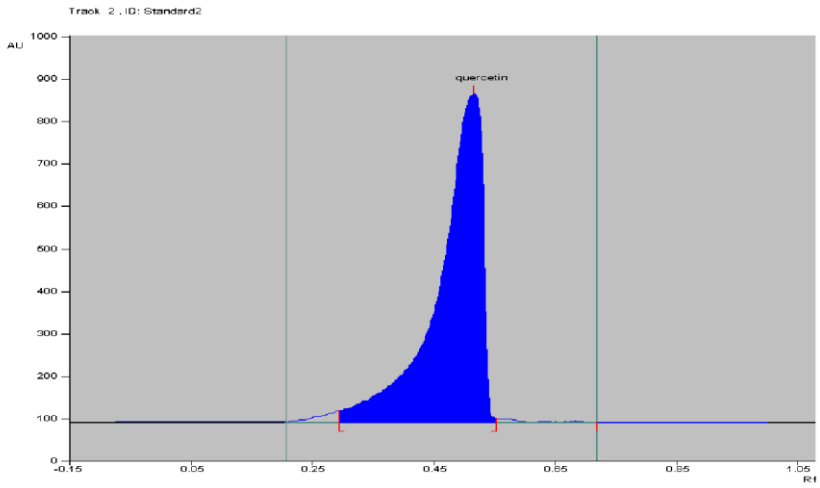


Figure 3.25. HPTLC chromatogram of standard Quercetin

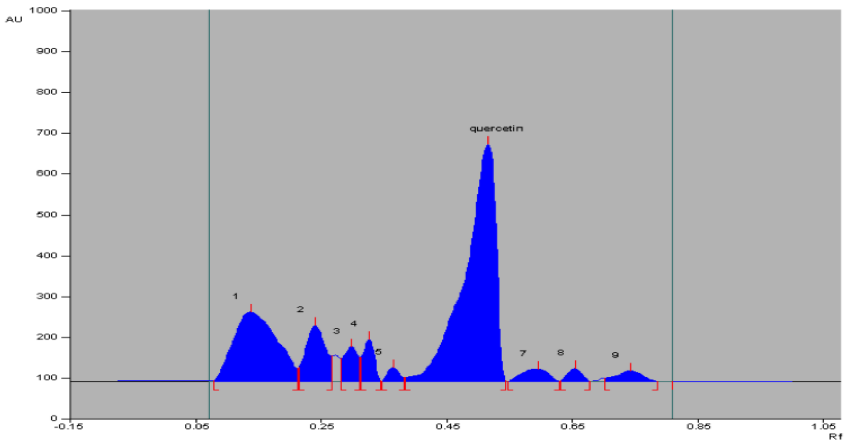
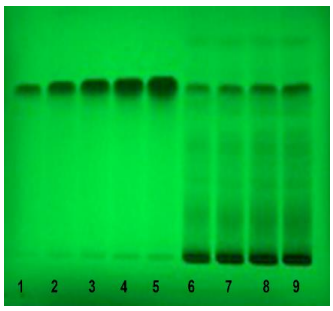


Figure 3.26. HPTLC fingerprint of *M. fragrans* extract



1-5 standard quercetin and 6-9 M. fragrans extract

Figure 3.27. Photo documentation of standard Quercetin and *M.fragrans* extract

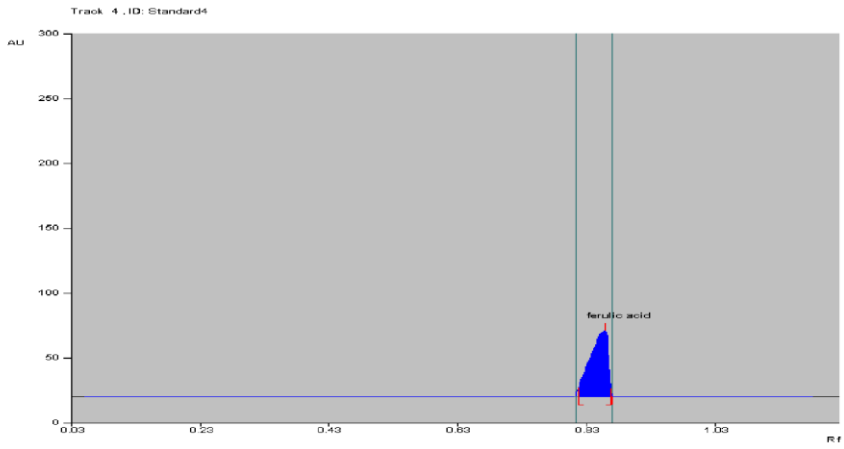


Figure 3.28. HPTLC chromatogram of standard Ferulic acid

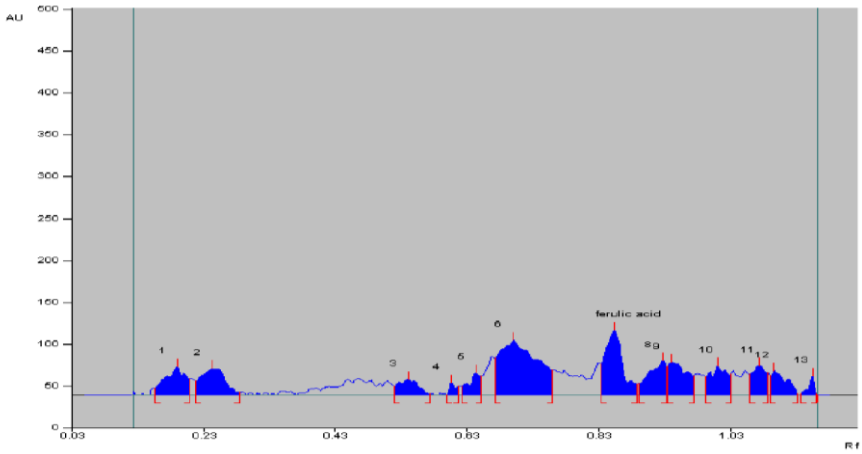


Figure 3.29. HPTLC fingerprint of *H. inducus*

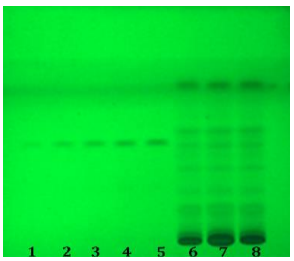


Figure 3.30. Photo documentation standard ferulic acid and *H.indicus* extract (1-5 standard ferulic acid and 6-8H. indicus extract)

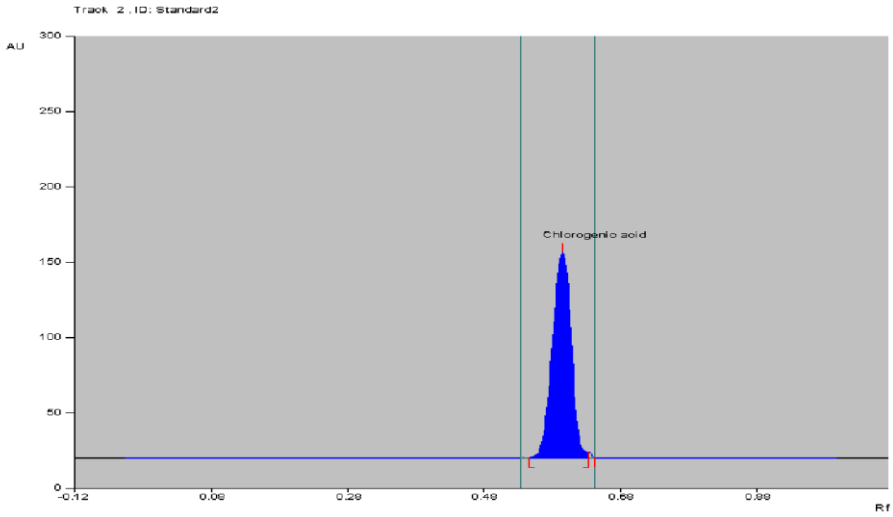


Figure 3.31. HPTLC chromatogram of standard chlorogenic acid

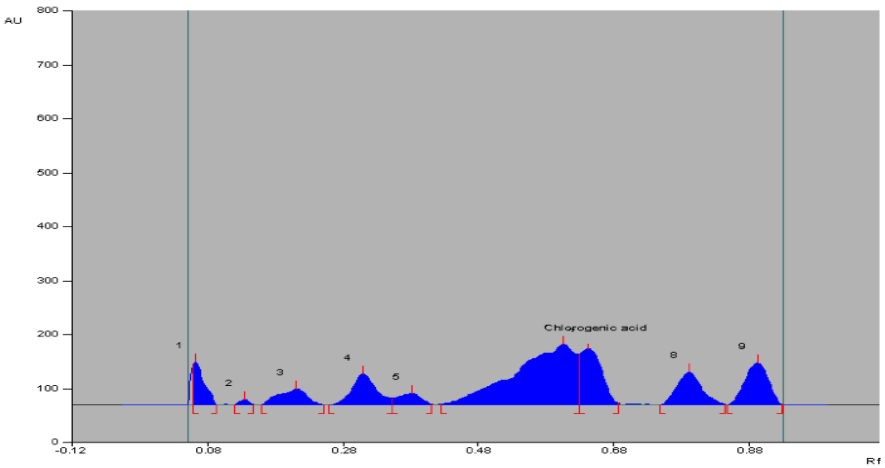


Figure 3.32. HPTLC fingerprint of *I. racemosa* extract

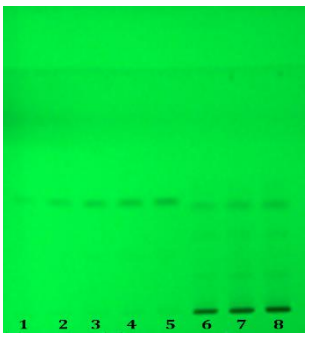


Figure 3.33. Photo documentation standard Chlorogenic acid and *I. racemosa* extract (1-5 standard chlorogenic acid and 6-8 I. racemosa extract)

3.4.4. Method validation for RP-HPLC

It was found that the linearity range of the quercetin method in RP-HPLC was 1-100 µg/ml. With the help of calibration curve, the correlation coefficient was found to be > 0.99, which shows that the data is closer to the line of best fit. The regression equation was found to be Y=19123X-55377. The LOD and LOQ shows the method sensitivity, which was found to be 1.41µg/mL and 6.54 µg/mL, respectively. Accuracy of the method was showed by the percent recovery value, which was found to be 94.65-98.14%. The method was highly repeatable as the % RSD of intra-day and inter-day precision was found to be < 2%. The % recovery value of 97.04-98.79% showed that method was accurate. From the mean of six determinations of test concentrations, the number of theoretical plates (desirable > 2000), capacity factor (desirable 2-10) and tailing factor (desirable < 1.5) were found to be 2908, 6.12 and 1.08 respectively.

It was seen that the linearity range of the chlorogenic acid method in RP-HPLC was found to be 1-100 µg/ml. The correlation coefficient was found to be> 0.99, and the obtained regression equation was Y=16653X-23567.The LOD and LOQ showed was found to be 2.03µg/mL and 5.98 µg/mL respectively. Accuracy of the method was determined in terms of percent recovery value, which was found to be 94.65-98.14%. The method was found to be highly repeatable with % RSD< 2% for intra-day and inter-day precision. The percent recovery value of 97.04-98.79% indicating that method was accurate. The number of theoretical plates (desirable > 2000), capacity factor (desirable 2-10) and tailing factor (desirable < 1.5) were found to be 2908, 6.12, 1.08 respectively.

The linearity range of ferulic acid was found to be 1-100 µg/ml. The correlation coefficient > 0.99 and the regression equation was found to be Y = 18145X-45687. The LOD and LOQ were found to be 1.56µg/mL and 6.25µg/mL respectively. Accuracy of the method was found to be 94.65-98.14% was in terms of percent recovery. The method was highly repeatable as the % RSD < 2%. The percent recovery value of 97.04-98.79% showing that method was accurate. The number of theoretical plates (desirable > 2000), capacity factor (desirable 2-10) and tailing factor (desirable < 1.5) were found to be 2908, 6.12 and 1.08 respectively.

3.4.5. Method validation for HPTLC

Validation of the method was performed as recommended by the International Conference on Harmonization guidelines defining the linearity, specificity, peak purity, LOD, precision, accuracy and robustness (ICH, 2005). The specificity of the method was determined by analyzing the standards and test samples. Peaks of glycyrrhizin in *G. Glabra*, quercetin in *M. fragrans*, ferulic acid in *H. indicus* and chlorogenic acid in *I. racemosa* were identified by comparison of Rf and spectrum of the spot of standard compounds with that of extracts. Peak specificity was determined by comparing the UV spectrum of the standard glycyrrhizin, quercetin, ferulic and chlorogenic acid with the test sample of *G. Glabra*, *M. fragrans*, *H. indicus*, and *I. racemosa* respectively. The calibration curve for glycyrrhizin was Y= 1453.784X+709.852 with standard deviation of 13.99%. For glycyrrhizin LOD and LOQ were found to be 0.03µg/mL and 0.06µg/mL respectively. For quercetin was Y= 10976.084X+2113.371 with standard deviation of 14.97%. For quercetin, the LOD and LOQ were found to be 0.02µg/mL and 0.07µg/mL respectively. The equation for straight line of ferulic acid was Y= 0.354X+ 226.466 with standard deviation as 11.99% and the LOD and LOQ were found to be 0.17µg/mL and 0.53µg/mL respectively. Whereas the equation of a calibration curve for chlorogenic acid was Y= 1113.371X+887.909 with standard deviation as 3.48%. The LOD and LOQ for chlorogenic acid were found to be 0.01µg/mL and 0.04µg/mL respectively. The accuracy of the method was determined by analyzing the percentage recovery of the glycyrrhizin, quercetin, ferulic and chlorogenic acid in the *G. glabra*, *M. fragrans*, *H. indicus* and *I. racemosa* extracts respectively. The method was studied by performing standard addition technique and was expressed in terms of %RSD from mean recovery of the theoretical concentrations. The repeatability of the analysis was calculated from

replicate spotting of one test sample solution with the calibration curve of the standards. The experiment was repeated for six times.

3.5. Discussion

The varnya herbs as mentioned in Ayurveda are important as they are widely used in different skin care products. For quality evaluation of these herbs and their standardization with the help of RP-HPLC and HPTLC method of analysis have proven to be very essential. It has been observed that phenolic compounds are present in all the three plants under study. Phenolic compounds are aromatic in nature, and they are widespread in the plant kingdom. Due to their roles in food quality and their organoleptic properties, phenolic compounds have been widely analyzed and studied. The developed RP-HPLC and HPTLC method has been proved to be accurate, precise and reproducible for quantification of the phenolic compounds with a narrow linear range. The HPLC and HPTLC methods can be used for quality control using the phenolic compounds quercetin, ferulic acid and chlorogenic acid as quality markers. As these methods are robust and reproducible, they can help in combating the major challenges for quality of herbal raw materials which varies on the basis of various factors like origin, time of collection, heavy metal contamination, drying method, microbial contamination (Mukherjee, et al., 2006). Although, marker profiling of the herbs is important to certify their quality, inadequate evidence of the markers present in the herbs remain an un-dealt issue. In this present study the method validation of RP-HPLC and HPTLC analysis of the herbs shows reproducibility of the method developed (Mukherjee, et al., 2015). For quantification of the amount of quercetin, ferulic acid and chlorogenic acid in M. fragrans, H. indicus, and I. racemosa respectively, the developed method was validated, and it was found to be specific, accurate, robust, precise, reproducible and falls under linearity range. The percentage yield of glycyrrhizin, quercetin, ferulic acid and chlorogenic acid found in *G. glabra, M. fragrans, H. indicus,* and *I. racemosa* are 5.66% w/w, 4.32% w/w, 3.76% w/w, and 4.55% w/w respectively. The result of the present HPLC and HPTLC analysis correlate with previous reports where it has been observed that the retention time for the marker compounds quercetin, ferulic acid, and chlorogenic acid are similar. In this study HPLC analysis showed the retention time (Rt) for quercetin, ferulic acid, and chlorogenic acid to be 9.13, 7.44 min, 7.34 min, and 3.19 min respectively.

Similarly, the HPTLC study of the herbs showed the Rf value for glycyrrhizin, quercetin, ferulic acid, and chlorogenic acid were found out to be 0.57, 0.48, 0.44, and 0.52 respectively. Therefore, it can be stated that the methods used for HPLC and HPTLC analysis of the herbs in this study are improved in robustness, reproducibility than the previously applied method.

3.6. Conclusion

In Ayurveda, the herbs used for skin whitening and brightening are stated as complexion promoters, which on further standardization can be observed as potent inhibitors for tyrosinase enzyme. These selected herbs are clinically useful for the treatment of dullness of the skin and also used as a main ingredient of such formulations. In Traditional system of medicine Liquorice (*G. glabra*), Nutmeg (*M. fragrans*), China rose (*H. indicus*) and Pushkarmool (*I. racemosa*) are considered as potent herbs for treatment of skin alignment. RP-HPLC and HPTLC methods are specific and precise methods, which are useful for the quality evaluation and quantitative determination of herbs from Ayurveda and Unani system of medicine. Further these methods can be employed for standardization of certain Ayurvedic formulations containing the above-mentioned herbs as one of the ingredients, such as Pushkarmool Churna, Sarivadyasavam, Mathala rasayanam, etc.

Publication

Sharma, A., Katiyar, C.K., Banerjee, S., Chanda, J., Kar, A., Biswas, S., Mukherjee P.K., 2020. RP-HPLC and HPTLC Methods for Analysis of Selected Herbs Used as Complexion Promoters in Ayurveda and Unani Systems of Medicine. ***Journal of AOAC international***. 103 (3), 2020.https://doi.org/10.5740/jaoacint.19-0290

Chapter - 4

4. *In-vitro* Tyrosinase inhibitory potential of Varnya herbs.

4.1. Tyrosinase Enzyme and Varnya herbs

4.2. Features of the tyrosinase enzyme

4.3. An overview of the reaction mechanism of tyrosinase

4.4. Tyrosinase inhibition assay

4.5. Results

4.6. Discussion

4.7. Conclusion

4.1. Tyrosinase Enzyme & Varnya herbs

Skin protects itself against harmful factors with the help of melanin, a pigment. Melanin helps protect the skin from UV damage. Even though melanin has many advantages, it also causes abnormal pigmentation and melanoma, so different approaches to the study of skin disorders have been developed (Riley, 2003). As a key enzyme in the first two steps of melanin biosynthesis, Tyrosinase catalyses 2,4-dihydroxyphenylalanine (DOPA) and its oxidation to dopaquinone (Di Petrillo et al., 2016).

There are several conditions associated with the overproduction and accumulation of melanin, such as solar melanosis, ephelides, melasma, senile lentigos, and post inflammatory hyperpigmentation. In hyperpigmentation disorders, tyrosinase inhibitors have become increasingly important as depigmenting agents because it is the limiting step enzyme in melanogenesis (Ortonne et al., 2008)

There is a constant search for better inhibitors that will be free of harmful side effects and are derived from natural sources as the currently available tyrosinase inhibitors are toxic and/or ineffective (Ortonne et al., 2008). Therefore, in this study, the concept of Varnya herbs has been used to show the efficiency and effective nature of the herbal extracts obtained from Ayurvedic importance. In Ayurvedic literature, Varnya herbs have been mentioned as the skin whitening and brightening agents. Hence, to understand their potential, tyrosinase inhibition assay was performed on the selected herbs belonging to the class of Varnya herbs.

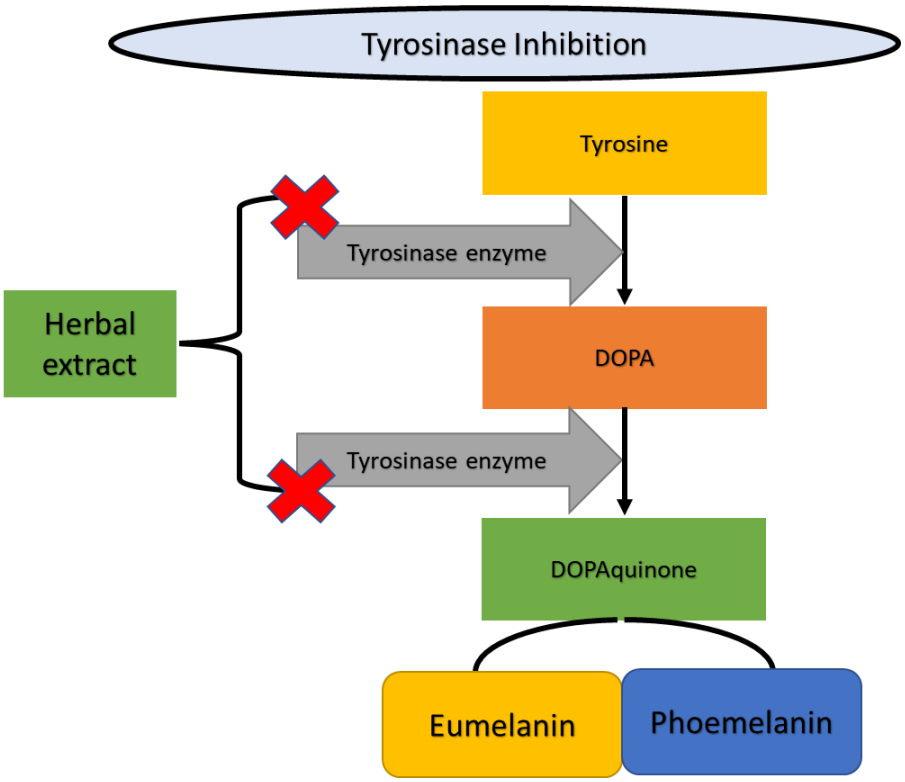


Figure 4.1. Diagrammatic representation of Tyrosinase inhibition of melanin formation

Mammals regulate melanogenesis in complex ways. At the cellular level, it is controlled by the formation and destruction of the melanocyte organelle, the melanosome. Tyrp-1, Tyrp-2, and tyrosinase are melanogenesis-related enzymes that are expressed subcellularly. Different hormones, growth factors, interleukin, prostaglandins, and interferons participate in the regulation of melanogenesis, which determines both the quantity and quality of the synthesized melanin. Melanogenesis is also affected by UV exposure and environmental influences (Sardana and Ghunawat, 2015; Solano, 2014).

Melanogenesis is down-regulated by two common signalling pathways. A transcription factor that regulates all signalling pathways, microphthalmia-associated transcription factor (MITF) is a class E basic helix-loop-helix transcription factor. Tyrosinase, Tyrp-1, and Tyrp-2 are all regulated by MITF, which contains an M-box as a promoter region. Up-regulation of MITF activity stimulates melanogenesis by up-regulating enzymes related to it. MITF activity is downregulated by downregulation of related enzymes, inhibiting melanogenesis in the opposite direction (Chang, 2012). Activated by UV or

other receptors, diacylglycerol (DAG) is found in cell membranes. Tyrosinase is activated by phosphorylating the serine/threonine residues of the protein kinase-C (PKC) enzyme (Bae-Harboe and Park, 2012).

The cyclic adenosine monophosphate (cAMP) pathway mediates mechanism which gets activated by adrenocorticoid Hormones (ACTH) and monomorphic melanocyte-stimulating hormones. Following cAMP activation, protein kinase A (PKA) activates MITF gene expression and stimulates melanogenesis. Another pathway targeting the expression of MITF is the Wnt signal pathway. It plays a key role in the synthesis of intracellular β-catenin. By contrast with melanogenesis being upregulated, melanogenesis can be downregulated via the mitogen-activated protein kinase (MEK) and extracellular signal-regulated kinase (ERK) pathway (Chang, 2012). The phosphatidylinositol 3-kinase (PI3K) also activates serine-threonine protein kinase (Akt). Melanogenesis is downregulated when Akt pathway is stimulated (Oka et al., 2000).

4.2. Features of the tyrosinase enzyme

The type-3 copper protein family is composed of tyrosinases, catechol oxidases and hemocyanins (the oxygen-carrying protein in arthropods and mollusks). A peroxy configuration exists between two Cu atoms that are coordinated by three histidine residues. Dioxygen is bound to each of these histidine residues in a peroxy configuration. A bridging ligand covalently overlaps with the two s = 1/2 metal ions, resulting in strong anti-ferromagnetic coupling (i.e. spin pairing). There is an unusual feature associated with the tyrosinase isolated from Streptomyce scastanglobisporus: a 'caddie' protein. The tyrosinase in Agaricus bisporus is a heterotetramer with two heavy chains about 48 kDa and two light chains (14 kDa). Light chains may have an unrelated function, or they may be fragments from the C-terminus of a latent (62 kDa) precursor form of the enzyme (Ismaya et al., 2011; Ramsden and Riley, 2014).

4.3. An overview of the reaction mechanism of tyrosinase

Tyrosinase contains type-3 (two) copper metals coordinated by three histidine residues bound to two oxygen atoms in a peroxy configuration. Both monooxygenase and monophenolase activities of tyrosinase can be explained by the binding of di-oxygen to the two copper ions in the active site (usually identified as CuA and CuB). It has been shown that catechols bind to CuB while monohydric phenols bind to CuA in mammalian tyrosinase. (Ismaya et al., 2011) describe oxy-, met-, and deoxy-tyrosinase as three types of tyrosinase involved in melanin biosynthesis. The oxidation reactions that tyrosinase catalyze.

Monophenolase & Diphenolase Activity

Monophenolase activity of enzyme begins when phenols are converted by L-tyrosinase to ortho-quinones by oxy-tyrosinase, then converted to dopaquinone by monophenolase activity. Initially, the phenolic oxygen is bound to the CuA of oxy-tyrosinase, then another copper ion is bound to the complex followed by homolytic dissociation to give orthoquinone and deoxy-tyrosinase. Until the substrates (phenol and oxygen) run out, this process continuously occurs. There is a characteristic feature of monophenolase activity called the 'lag phase.' In this stage, phenolic substrates are oxidized very slowly before reaching maximum oxidation velocity. In order to understand the lag period, it is necessary to understand the requirements for the conversion of met-tyrosinase to deoxy-tyrosinase. The stable form of the enzyme is met-tyrosinase (resting state), which contains Cu(II)2 in its oxidized state. This form can no longer bind to phenolic oxygen. Monophenolase activity therefore cannot be expressed (Ramsden and Riley, 2014).

Experimental evidence now shows that catechols (L-dopa) do not form from phenols (L-tyrosinase). Catechols are formed during the formation of dopachrome when leukodopachrome (cyclodopa) is produced. There is little oxy-tyrosinase in native tyrosinase, which produces minimal ortho-quinone. Ortho-quinones will produce catechols, which will activate met-tyrosinase. As a result, all enzymes will be converted into oxy forms. To generate more met-enzyme, L-dopa is clearly necessary in small amounts. Monophenolase activity lags as a result of this process (Ramsden and Riley, 2014).

In the active site, copper ions bind two adjacent hydroxyl groups of the catechols to produce diphenolase activity. Sterically, Catechol's bindings are more favorable in tyrosinase's hydrophobic active site pocket. The catechol binding pattern differs from the phenol (L-tyrosinase) binding pattern. Catechols initially bind through the CuB. Two steps are involved in catechol oxidation. To produce meta-tyrosinase, oxy-tyrosinase must first be converted. By reducing the peroxy-bridge, catechol increases oxy-tyrosinase activity. In this step, ortho-quinone and water are obtained. As a result, copper ions are in oxidized form as met-tyrosinase, probably in a protonated state. During the second phase, catechols again reduce the copper ions in the active site of met-tyrosinase to produce deoxy-tyrosinase [Cu(I)] and orthoquinone. By binding di-oxygen to the active site copper ions [Cu (II)] the oxidation states are restored (Ramsden and Riley, 2014).

4.4. Tyrosinase inhibition assay

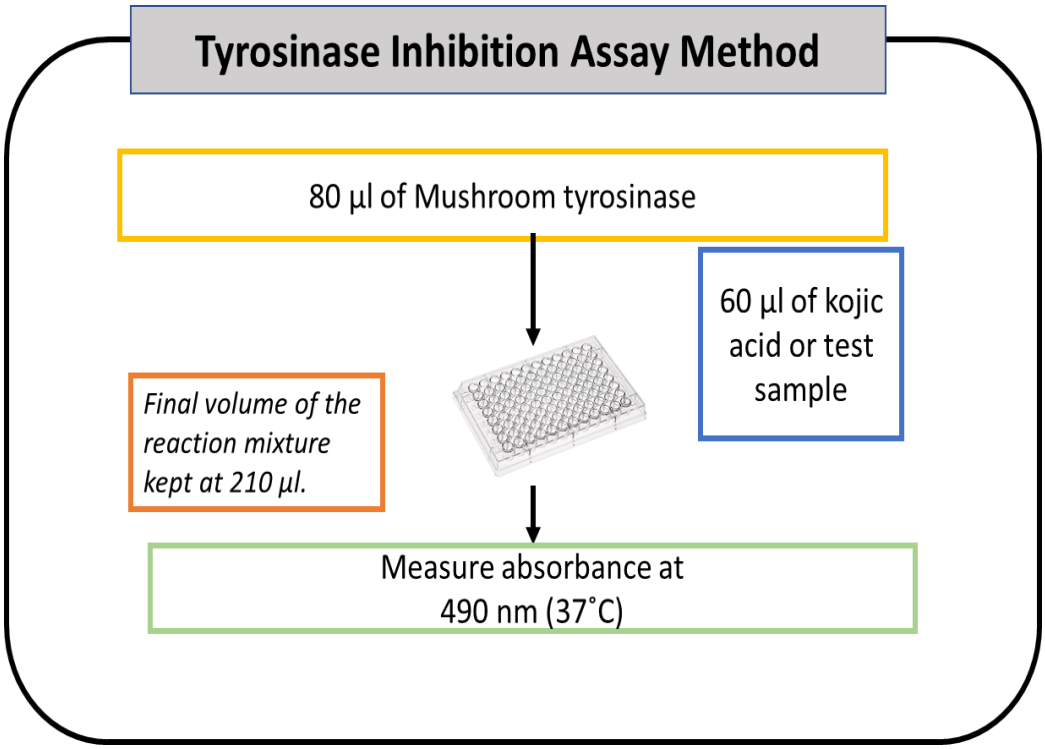


Figure 4.2: Flow diagram of tyrosinase inhibition assay

4.4.1. Reagents and chemicals

Betulinic acid (B8936-5MG), 1-Anilino-8-naphthalene sulfonate (A1028-5G), Tyrosinase (T3824-25KU), Kojic acid (K3125-5G), L-Tyrosine and L-dopa were purchased from Sigma-Aldrich, Inc (St. Louis, MO, USA). Spectrophotometric measurements were performed with 96 well microplate (Multiplate™ 96 well, BioRAD).

4.4.2. Mushroom tyrosinase inhibition assay

The method for enzyme inhibition assay mentioned by Biswas et. al., (2016) was followed with slight modifications. Herbal extract sample was prepared by dissolving in 2% Dimethyl sulfoxide (DMSO). Concisely, 80 µL of BFKO sample was added with 30µL of tyrosinase in a 96 well plate. The concentration of tyrosinase enzyme taken was 333 U/ml in phosphate buffer with pH 6.8. Different concentrations of Kojic acid and BFKO sample were prepared in the range of 0.5-10 µg/ml The above-mentioned 96 well plate was then kept aside for incubation at 25°C for 5 min. After incubation 100 µL of substrate was added to each well and incubated for same time and temperature before measurement of absorbance. The substrates used were 4mM l-tyrosine or 6 mM L-DOPA, substrate was dissolved in 50mM phosphate buffer pH 6.8. Using UV–visible spectroscopy (SpectraMax® Plus, United States), the optical density of the reaction mixture in each well was measured at 475 nm. Kojic acid was used as positive control (Jo et al., 2012). Calculation of the Tyrosinase activity was performed with the help of following formula:

$$\textit{Enzyme activity} = \frac{(\Delta A475nm/min_{test} - \Delta A475nm/min_{blank})(\text{Volume of Assay, ml})}{(\varepsilon_{dopachrome}\ 3500\ M^{-1}\ cm^{-1})(\text{Volume of Enzyme used, ml})}$$

ε Is the extinction coefficient dopachrome. Enzyme activity unit is M^{-1} s^{-1} (mole per second). For determining the IC50 values of inhibitors, graph was plotted with % tyrosinase inhibition on the Y-axis and inhibitor concentrations on the X-axis.

Relative activity% = 1- Enzyme activity

For all the four herbal extracts the method was used and results were obtained individually.

4.4.4 Statistical analysis

The IC_{50} values were expressed as relative activity (%). All calculations for tyrosinase kinetic analysis for non-competitive-type inhibition and fluorescence measurements were analyzed using Sigma Plot version 12.5 (Systat Software Inc, Chicago, USA) and Origin Pro 8.5 (Origin Lab; Northampton, MA).

4.5. Results

The tyrosinase inhibition assay was performed using mushroom tyrosinase, where Kojic acid was taken as a control in the study. The table 4.1 shown below represents the activity of the selected Varnya herbs on the tyrosinase enzyme at different concentrations. From the obtained values, the IC50 value was graphically derived.

Table 4.1: Varnya herbs and their tyrosinase inhibition activity

Concentration (µg/ml)	**Relative activity (%)**			
	G. glabra	***M. fragrans***	***I. racemosa***	***H. indicus***
1.5	7.65	22.65	2.5	15.3
5	25.8	40.8	11.6	29.02
7.5	38.1	59.1	22.5	42.12
10	55.82	74.82	35.5	55.3
12.5	79.2	89.2	50.8	66.12
15	100	100	62	88.5

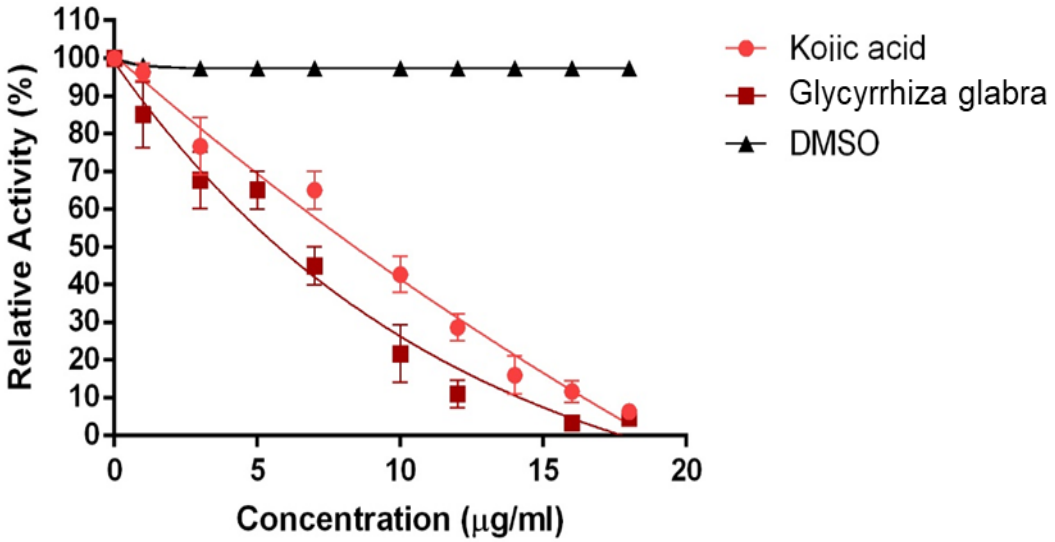


Figure 4.3: Graphical representation of IC_{50} value of *G. glabra*

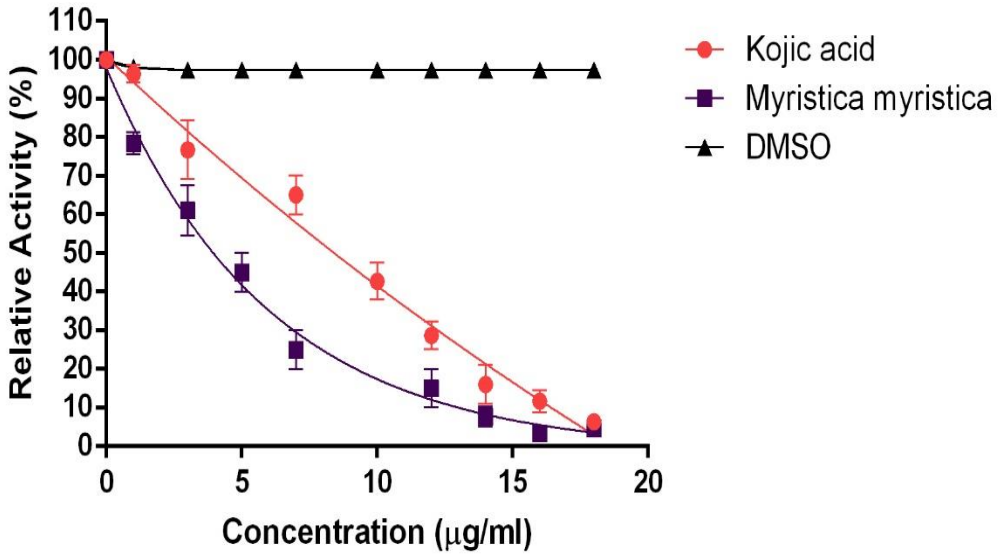


Figure 4.4: Graphical representation of IC_{50} value of *M. fragrans*

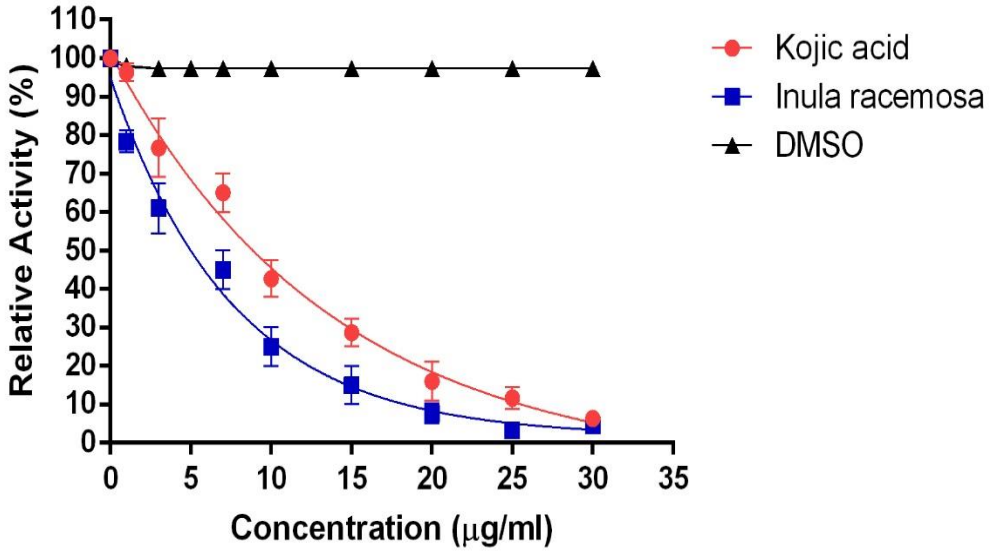


Figure 4.5: Graphical representation of IC50 value of *I. racemosa*

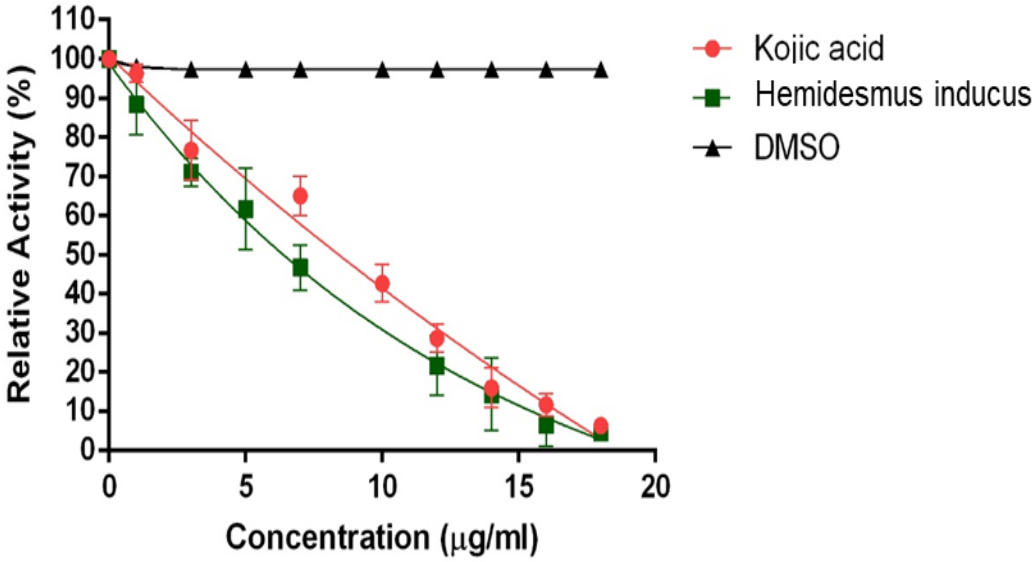


Figure 4.6: Graphical representation of IC_{50} value of *H. indicus*

Table 4.2: IC_{50} value of the Varnya herbs

Herbal extract	Tyrosinase inhibition (IC50) (µg/ml)
Glycerrhiza glabra	**8.42**
Myristica fragrans	**6.15**
Inula racemosa	**12.76**
Hemidesmus indicus	**8.67**

4.6. Discussion

All the four herbs mentioned in this study are classed under the Varnya herbs. According to Ayurvedic literature, the Varnya herbs should be used for skin brightening and whitening. In this modern pharmaceutical era, it has been observed that ingredient which can inhibit tyrosinase enzyme are classed under skin whitening agents. In this study these two concepts were combined by analyzing the tyrosinase inhibition potential of Varnya herbs. Hence, *G. glabra*, *M. fragrans*, *I. racemose*, *H. indicus* were selected and tyrosinase inhibition assay was performed by taking the dilutions of the herbal extracts in DMSO. The assay was performed using mushroom tyrosinase as the enzyme, L-DOPA as the substrate and Kojic acid as the control. The results obtained showed the enzyme activity obtained for the individual herbal extracts. From the enzyme activity, the IC50 value was graphically obtained, which showed good tyrosinase inhibition potential of the selected Varnya herbs.

4.7. Conclusion

The result of the study shows that all the herbs -*G. glabra*, *M. fragrans*, *I. racemose*, *H. indicus* have good tyrosinase inhibition potential. Based on the finding it can be suggested that the above-mentioned herbs have good skin whitening and brightening properties imparted by their ability to inhibit tyrosinase enzyme present in the skin. Furthermore, these herbs can be used for formulation development in the future. Although, more research work has to be performed on this topic for better development of the concept of Varnya and herbs present under this class, potentially inhibiting tyrosinase enzyme.

The herbs mentioned in this study are mentioned as Varnya herbs in traditional medicinal system, Ayurveda. Hence, it can be said that the Varnya herbs have tyrosinase inhibiting property which results in Skin whitening and brightening.

Presentation

Akanksha Sharma, Subhadip Banerjee, Prasant Maitra, Amit Kar, P.K Mukherjee, Evaluation of tyrosinase potential of standardized extract of Ayurvedic Varnya herb **Nagkeshar (*Messua ferrea*)**. 4th Convention of SFE-India, 2017 Jadavpur university; India, 09-10 September 2017.

Akanksha Sharma, Subhadip Banerjee, P.K Mukherjee, C.K Katiyar. Evaluation of tyrosinase potential of standardized extract of indigenous herb of ***Nelumbo nucifera*** at ISE-SFEC, 2018 Dhaka University; Bangladesh,13-15 January 2018.

Chapter - 5

5. Development and Evaluation of Varnya formulation.

5.1. Varnya Formulations- Introduction

5.2. Preparation of Varnya Formulation

5.3. Evaluation of Varnya formulations

5.4. Results

5.5. Discussion

5.6. Conclusion

5.1. Varnya Formulations- Introduction

Since ancient times, women have started to dress for themselves because they want to increase their beauty. Even today, people, especially those in rural areas, still choose natural remedies (plant extracts) for traditional cosmetics. Cosmetics are products used by to purify and beautify the skin. These products contain active ingredients that claim to have medical and drug-like benefits in the field of cosmetics (Gediya et al., 2011). Some women still use herbal cosmetics to beautify their skin. The best reason to use herbal cosmetics is that they are made entirely from herbs and shrubs. The natural ingredients of herbs have no side effects on the human body, but these herbs can provide nutrients and other useful minerals to the body. However, now more and more scientific evidence shows that plants have many complex active ingredients (photochemistry), these ingredients can soothe or soften the skin, but also actively restore and heal (Shivanand et al., 2010).

5.2. Preparation of Varnya Formulation

The formulation method used for this study has been based on method developed by Mishra et al., 2014 with some modifications.

5.2.1. Materials Used

Materials used for the preparation of the Varnya Cream formulation were Herbal extracts and Excipients. Stearic acid, Cetosteryl alcohol, Propylene Glycol, Glycerin, EDTA, and Propyl paraben were obtained from Merck, USA. Olive oil was obtained from KAZIMA, India. Lastly, the Triethanolamine and Silicon oil used were from Sigma Aldrich, USA.

A. Herbal extract

For development of Varnya formulations, herbal extracts were tested based on their tyrosinase inhibition potential. The extraction method of the herb has been mentioned in chapter 3. On performing Tyrosinase inhibition assay, herbal extracts of *G. glabra*, *M. fragrans*, *H. indicus* and *I. racemosa* were selected, showing good tyrosinase inhibition potential. The herbal extracts were used in the development of four formulations – Formulation F1, F2, F3, and F4. Here the formulations consist of combination of two herbal extracts. The selected herbs have shown good tyrosinase inhibition potential upon performing tyrosinase inhibition assay in previous chapter (chapter 4).

B. Excipients

(i) Stearic acid

Stearic acid is one of the useful types of saturated fatty acids that comes from many animal and vegetable fats and oils. It is a waxy solid, mainly used as an emollient, lubricant and emulsifier. As well as in the diet, it is used in hardening soaps, softening plastics and in making cosmetics, candles and plastics. It has a role as a plant metabolite, a human metabolite, a Daphnia magna metabolite and an algal metabolite. It is a long-chain fatty acid, a straight-chain saturated fatty acid and a saturated fatty acid. It is a conjugate acid of an octadecanoate. It derives from a hydride of an octadecane (Singh et al., 2011).

(ii) Cetosteryl alcohol

Cetostearyl Alcohol (CH3(CH2)nOH) is a mixture of cetyl and stearyl alcohols that can come from vegetable or synthetic sources. It is classified as a fatty alcohol. Cetostearyl alcohol is a Off- white, waxy, solid material in the form of flakes. It is oil soluble, but it is not water-soluble. In the pharmaceutical and cosmetics industry, cetostearyl alcohol functions as an emulsion stabilizer; opacifying agent; surfactant - foam booster; and viscosity increasing agent. It is often used in creams and lotions. It has a melting point of 122°F (50°C) and a boiling point: 480.2°F (249°C) (Aswal et al., 2013).

(iii) Olive oil

Olive oil is a liquid obtained from olives, the fruit of Olea europaea, a traditional tree crop of the Mediterranean Basin. It belongs to the family of Oleaceae. The oil is produced by pressing whole olives. It is commonly used in cooking, whether for frying or as a salad dressing. It is also used in cosmetics, pharmaceuticals, and soaps, and as a fuel for traditional oil lamps, and has additional uses in some religions (Pandey et al., 2013).

(iv) Triethanolamine

Triethanolamine is an amine produced by reacting ethylene oxide with ammonia. It is primarily used as a pH adjuster and it is used as a buffering agent, masking and fragrance ingredient, and surfactant (Singh et al., 2009).

(v) Moisturizing conditioner

These are generally used to impart moisturizing property in the cream (Rajvanshi et al., 2011, Jain et al., 2009). Mainly used moisturising conditioners are:

Propylene glycol: Propylene glycol (CH3CH(OH)CH2OH) is a chemical molecule having the formula CH3CH(OH)CH2OH. It's a viscous, colourless liquid with a pleasantly sweet taste that's practically odourless. It is classified as a diol since it has two alcohol groups. Water, acetone, and chloroform are among the solvents that it is miscible with. Glycols are generally non-irritating, have a low volatility, and have a low toxicity. It prevents freezing and melting of the formulation. It has a humectant effect.

Glycerine: It is a colorless, odorless, viscous non-toxic liquid. The glycerol backbone is found in those lipids known as glycerides. Due to having antimicrobial and antiviral properties it is widely used in FDA approved wound and burn treatments. It is also widely used as a humectant in pharmaceutical formulations. Owing to the presence of three hydroxyl groups, glycerol is miscible with water and is hygroscopic nature. It is used as an emollient and helps in making skin supple and soft.

(vi) Chelating agent- Ethylenediaminetetraacetic acid (EDTA)

It is a colorless aminopolycarboxylic acid, which is water-soluble solid. Its conjugate base is ethylenediaminetetraacetate. It is widely used to dissolve limescale. It is a hexadentate ("six-toothed") ligand and chelating agent, i.e., its ability to sequester metal ions such as Ca2+ and Fe3+. After being bound by EDTA into a metal complex, metal ions remain in solution but exhibit diminished reactivity. EDTA is produced as several salts, notably disodium EDTA, calcium disodium EDTA, and tetrasodium EDTA (typically as the hydrate). It is also used as preservative, stabilizer and help in foaming and cleaning (Jain et al., 2009).

(vii) Preservative- Propyl paraben

Propylparaben is the propyl ester of 4-hydroxybenzoic acid's benzoate ester. It's a benzoate ester, a paraben, and a member of the phenol family. It works as both an antifungal and antibacterial agent. It's utilised in topical formulations as a preservative (Matangi et al., 2014).

5.2.2. Method of Preparation of Varnya formulations

The aqueous phase and the oil phase were taken in two separate beakers. The aqueous phase consists of Titanium dioxide, Triethanolamine, Propylene glycol, Glycerin, EDTA, Propyl paraben and water. Whereas, the contents of the oil phase were Stearic acid, Cetosteryl alcohol, olive oil.

The cream was prepared by heating the aqueous phase and the oil phase separately at temperature around 70 ˚C to 75 ˚C. To obtain a oil in water formulation, the oil phase was added to the aqueous phase slowly. The mixing was done using overhead stirrer with propellor type paddle at 2000 rpm for 25 min until emulsion solidified. The formulation was further stirred at 1000 rpm for 5 min to accomplish the homogenization.

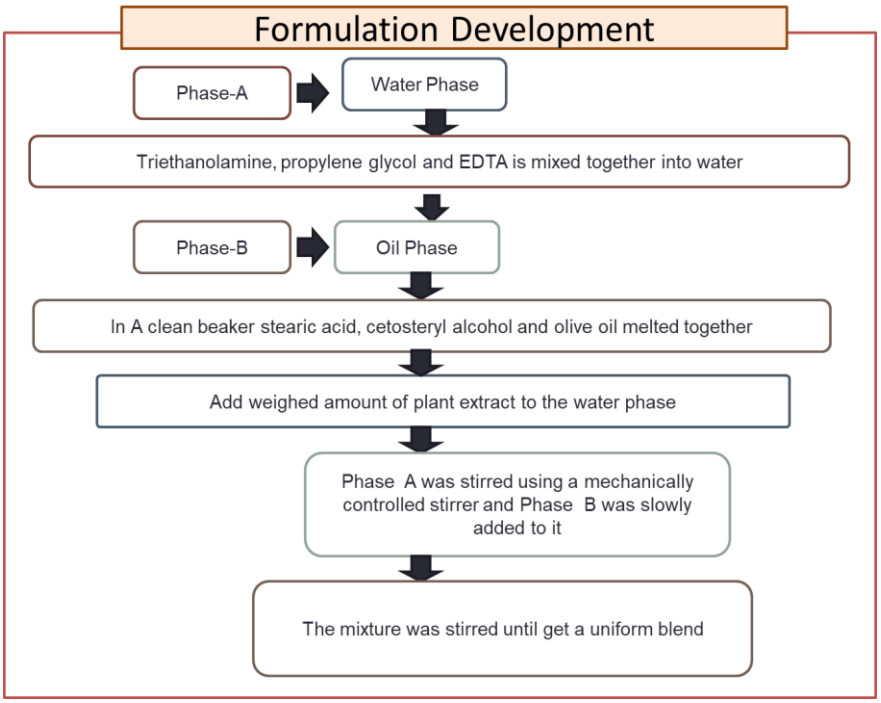


Fig 5.1: Flow diagram of formulation development

The extracts were dissolved in water and DMSO depending on the solubility. Formulations were prepared with combination of various herbs.

Table 5.1: The herbal extract combination used for formulation preparation

Formulation	Combination of Herbs
F1	*Glycerrhiza glabra* and *Hemidesmus indicus*
F2	*Glycerrhiza glabra* and *Inula racemosa.*
F3	*Myristica fragrans* and *Hemidesmus indicus*
F4	*Myristica fragrans* and *Inula racemosa*

Table 5.2: Composition table of herbal formulations

Composition	F1 (%)	F2 (%)	F3 (%)	F4 (%)
Extract combination	2	2	2	2
Stearic acid	10	10	10	10
Cetosteryl alcohol	3	3	3	3
Olive oil	2	-	2	-
Silicon oil	-	2	-	2
Triethanolamine	1.3	1.3	1.3	1.3
Propylene Glycol	5	5	5	5
Glycerine	5	5	5	5
EDTA	0.1	0.1	0.1	0.1
Propyl paraben	0.02	0.02	0.02	0.02
Water	Q.S	Q.S	Q.S	Q.S

5.3. Evaluation of Varnya formulations

The prepared Varnya cream formulations were evaluated for various specifications in accordance with the Indian Pharmacopoeia.

5.3.1. Macroscopic evaluation of the formulations

Macroscopic evaluation was performed by observing the formulations and inferring the results basing upon the visual analysis of the formulations. The macroscopic evaluation includes visual observation of the creams including overall appearance, color, and texture.

5.3.2. Microscopic evaluation of the formulations

The microscopic evaluation was performed using the microscopic examination method. Microscopic evaluation of the creams helps in understanding the internal structure of the formulation which was impossible to examine macroscopically. Upon performing

microscopic evaluation of the formulations, the characteristic of the dispersed phase, particle size of the droplets, and presence of drug particulates were observed and noted

5.3.3. pH determination of the formulation

The pH of the formulations was observed after preparation of the formulations at t=0 time point. pH evaluation of the formulations performed to check the acidic or basic nature of the formulations. Most importantly, the pH of the formulation should be in accordance with the pH of the skin. Application of the cream should not cause any temporary or permanent damage to the skin.

5.3.4. Viscosity measurement of the formulations

Viscosity was determined using Brookfield viscometer. The viscometer helps in identifying the fluid nature of the formulation. As the formulations were creams therefore the viscosity should be high. For viscosity measurement, the temperature of the formulations was maintained at 25˚C. A 'T' spindle was used for the test, using a Heli-path which helps in the upward and downward movement of the 'T' spindle while it rotates. Upon performing the test, torque and viscosity were noted.

5.3.5. Rheological characterization of the formulations

For Rheological characterization a DHR-1 Rheometer (TA Instrument, West Sussex, England) was used. The rheological characterization of the formulations was performed using flat plate 20 mm geometry. Four tests, Oscillation amplitude, Oscillation frequency, Steady state sweep flow and Creep test were performed on the creams. To perform all the four tests on the formulations, methods were developed using combination of parameters like stress, temperature, torque, etc. (Dai et. al., 2014). Tables 5.2 to 5.6 show the developed methods for the tests and the parameters used.

Table 5.3: Description of Instrument and geometry for Rheological methods

Geometry Description	
Name	20.0 mm parallel plate, Peltier plate Steel
Diameter & Material	20.0 mm, Steel
Gap	1000.0 µm
Loading gap	60000 µm
Trim gap offset	50.0 µm

(i) Oscillation Amplitude

Table 5.4: Method development for Oscillation Amplitude test

Parameters	
Instrument Type	Discovery HR-1
Temperature	25 °C
Inherit Set Point	Off
Soak Time	60.0 s
Wait For Temperature	On
Frequency	1Hz
Stress	1 to 1000 Pa
Points per decade	5
Time	3s
Save waveform (point display)	On Number of points in waveform 64
Torque	0µN.m

Table 5.5: Method development for Oscillation Frequency test

Parameters	
Instrument Type	Discovery HR-1
Temperature	25 °C
Inherit Set Point	Off
Soak Time	60.0 s
Wait For Temperature	On
Frequency	1Hz
Stress	15.0 Pa
Frequency 1 to 100Hz	1 to 100Hz
Time	3s
Save waveform (point display)	On Number of points in waveform 64
Torque	0µN.m

Table 5.6: Method development for Steady state sweep flow test

Parameters	
Instrument Type	Discovery HR-1
Temperature	25 °C
Inherit Set Point	Off
Soak Time	60.0 s
Wait For Temperature	On
Frequency	1Hz
Stress	10 to 700 Pa
Points per decade	7
Time	3s
Save waveform (point display)	On Number of points in waveform 64
Torque	0µN.m

Table 5.7: Method development for Creep test

Parameters	
Instrument Type	Discovery HR-1
Temperature	25 °C
Inherit Set Point	Off
Soak Time	60.0 s
Wait For Temperature	On
Frequency Duration	300.0 s
Stress	15Pa
Steady state sensing	On % Tolerance 5.0
Points per decade	5
Time	3s
Save waveform (point display)	On Number of points in waveform 64
Torque	0µN.m

5.3.6. Microbial count of the Varnya formulations

According to the Ayurvedic pharmacopoeia of India, Varnya formulations were analyzed for their microbiological limit. Tests included in the microbial growth count included the total microbial plate count (TMPC), the total yeast & mould count, tests for Staphylococcus aureus, Salmonella, Pseudomonas aeruginosa, and Escherichia coli. The growth patterns and types of organisms differ. To express the total number of viable organisms, colony-forming units (CFU) have been used (Lohar et al., 2007b).

5.3.7. Heavy metal determination of the Varnya formulations

Thermofisher AA303 atomic absorption spectrometer (AAS) was used for heavy metal analysis. We used mercury, lead, cadmium, and arsenic as standard heavy metals in order to determine the heavy metal content of Varnya cream formulations. In order to remove moisture from the creams at a controlled environment, the samples were dried

at 55-70°C for 6-8 hours. Three milligrams of cream samples were placed in a flask with four milliliters of concentrated HNO3, and they were kept aside for five hours for the removal of fumes. Afterward, the mixture was treated with a 2:1 mixture of HNO3 and HClO4 and heated for 6 hours at 120-130°C. After adding 10 ml of milli-Q water, the mixture was boiled for 10-15 minutes to reduce the volume to half. A Whatman filter paper no. 42 was then used to filter the solution. In a flask, 50ml of milli-Q water was added to the filtrate. Similar to the blank solution, three replicate analyses were performed (Neema et al., 2014).

5.3.8. Accelerated stability testing

The formulations were kept at two temperature points 25°C/60%RH and 40°C/75% RH (for 28 days). The formulation was kept at formula at room temperature and high temperature, and observe the following properties on the 0th, 5th, 10th, 15th and 28th days) Sahu et al., 2012; Rajvanshi et al., 2011)

5.4. Results

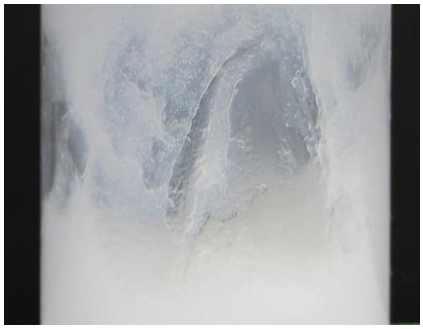
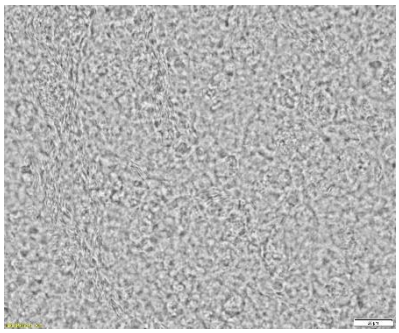
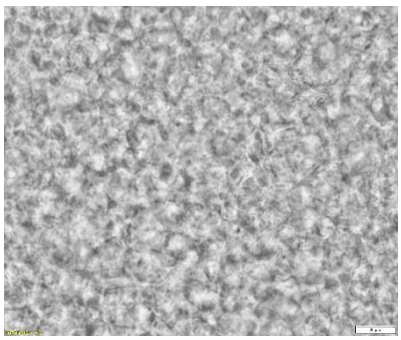
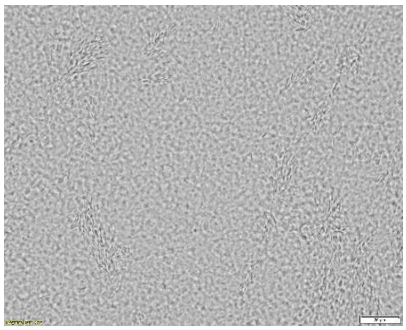
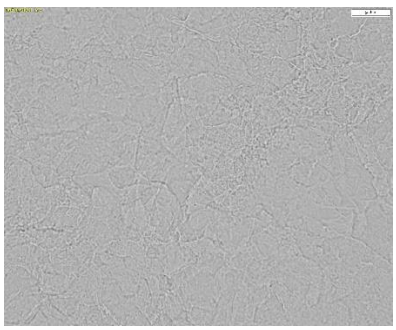
The results obtained upon subjecting the formulations through various descriptive tests showing the characteristics of the cream formulations. The tests performed highlighted the nature of the formulations under harsh conditions like temperature, pressure, and external force.

Table 5.8: pH, Macroscopy and Microscopy analysis

Formulation name	pH	Macroscopy		Microscopy	
		Characteristic	Observation	Characteristic	Observation
Formulation F1	5.94	Colour	Off- white	Drug particulate	Not observed
		Clarity	Opaque	Disperse phase distribution	Uniform
		Visual viscosity	High	Excipient particulates	Not observed
Formulation F2	5.89	Colour	Off- white	Drug particulate	Not observed
		Clarity	Opaque	Disperse phase distribution	Uniform
		Visual viscosity	High	Excipient particulates	Not observed
Formulation F3	6.00	Colour	Off- white	Drug particulate	Not observed
		Clarity	Opaque	Disperse phase distribution	Uniform
		Visual viscosity	High	Excipient particulates	Not observed
Formulation F4	5.98	Colour	Off- white	Drug particulate	Not observed
		Clarity	Opaque	Disperse phase distribution	Uniform
		Visual viscosity	High	Excipient particulates	Not observed

5.4.1. Macroscopic and Microscopic evaluation

Table 5.9: Pictorial representation of Macroscopy and Microscopy

Formulation	Macroscopic evaluation	Microscopic evaluation
F1		
F2		
F3		
F4		

5.4.2. Viscosity measurement

Table 5.10: Viscosity measurement of herbal formulations

Sample Details	Speed (rpm)	Spindle No.	% Torque	Viscosity (mPas)
F1	0.6	96	16.38	33559
F2	0.6	96	17.23	35547
F3	0.6	96	17.58	34658
F4	0.6	96	16.44	35568

5.4.3. Rheological evaluation of the formulations

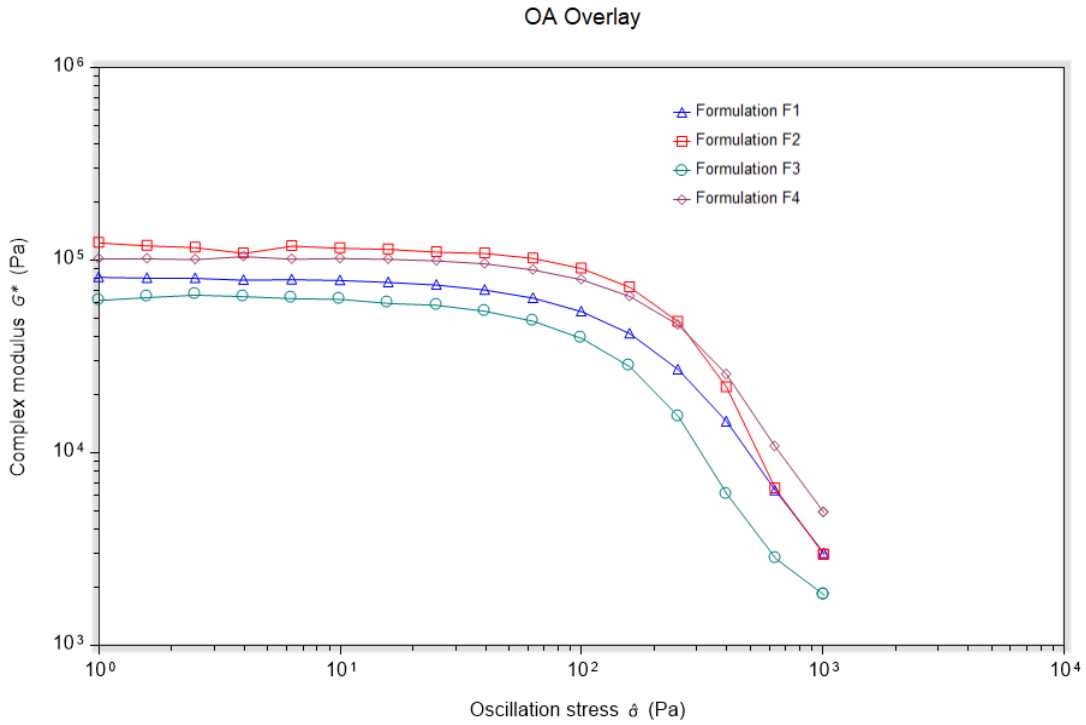


Fig 5.2: Overlay of oscillation amplitude test

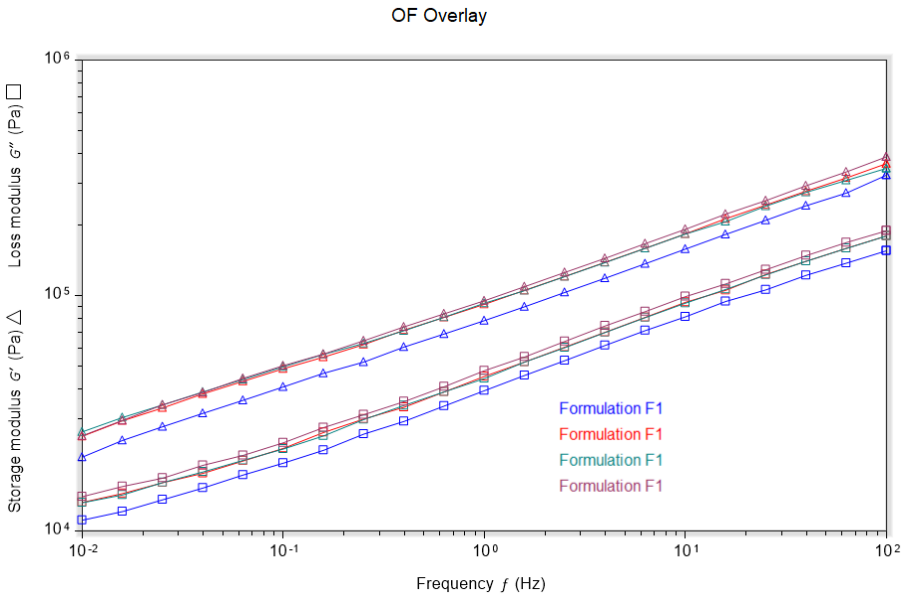


Fig 5.3: Overlay of oscillation frequency test

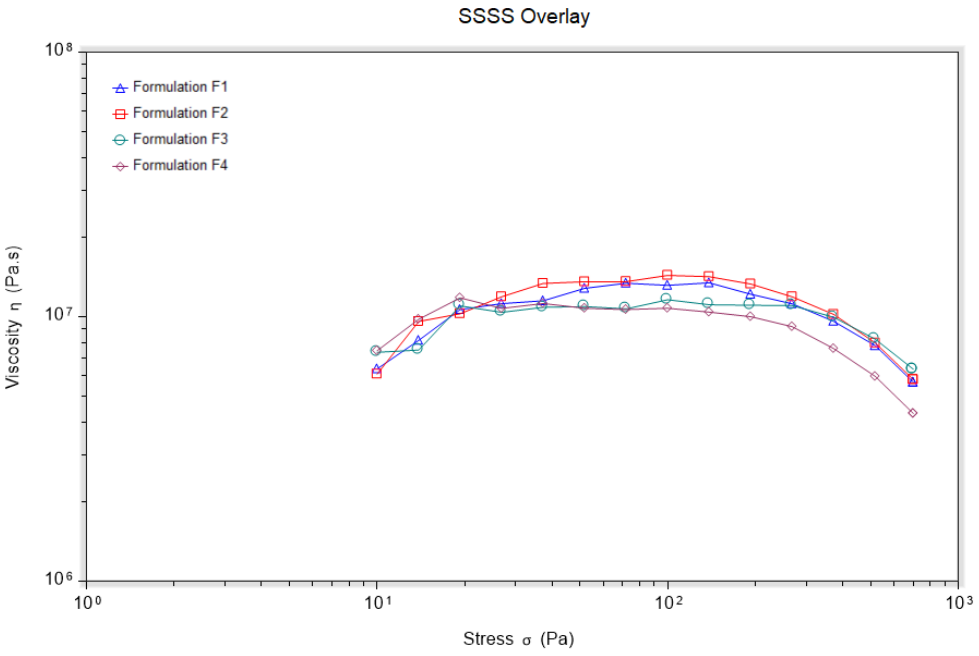


Fig 5.4: Overlay of steady state sweep flow test

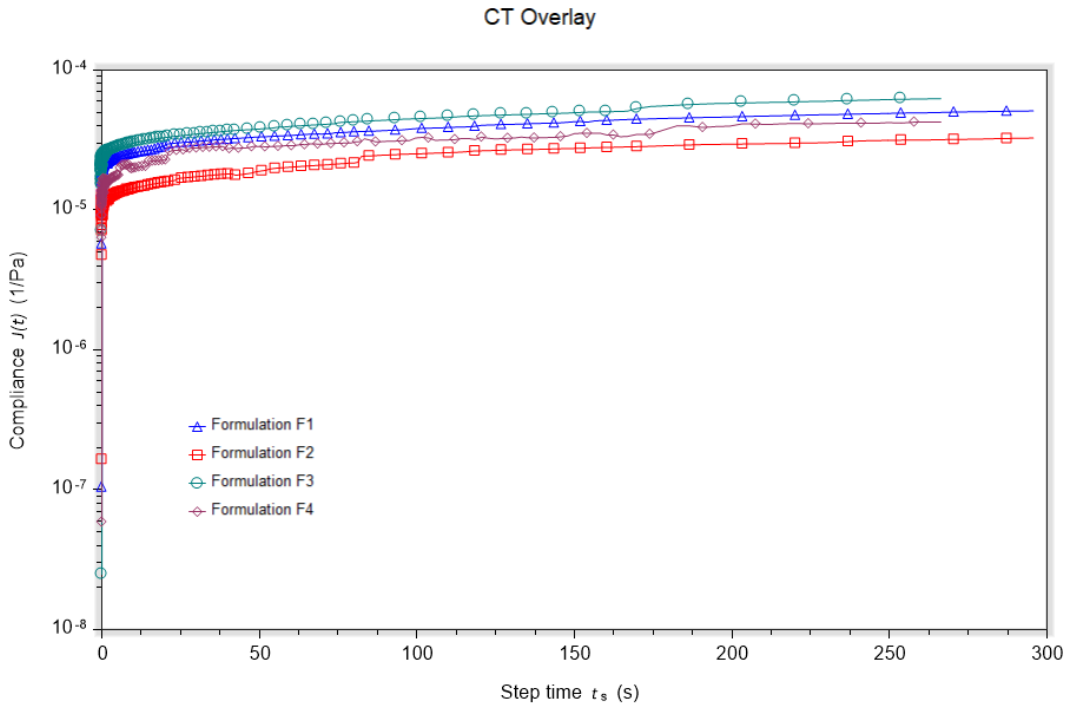


Fig 5.5: Overlay of creep test

5.4.4. Microbial Growth Analysis

The microbial growth analysis reports have been shown in Table 5.11. It was observed that the formulated Varnya cream formulations contained 1.0X100 -1.2X103 CFU of total bacterial count and 0.1- 0.5 CFU of total fungal count. Nevertheless, presence of microbes like E. coli, Salmonella, S. aureus and P. aeruginosa were not observed in the formulated creams.

Table 5.11. Microbial growth analysis in the Varnya cream formulation

Factors	**#Pharmacopoeial laboratory for Indian medicines specifications (CFU/gm)**	**Kumkumadi oil (CFU/gm)**			
		F1	**F2**	**F3**	**F4**
Total Bacterial count	1×10^5	1.0×10^2	1.3×10^2	1.2×10^2	1.3×10^2
Total Fungal count	1×10^3	0.1	0.2	0.2	0.3
Escherichia coli	Absent	N.C	N.C	N.C	N.C
Salmonella	Absent	N.C	N.C	N.C	N.C
Staphylococcus aureus	Absent	N.C	N.C	N.C	N.C
Pseudomonas aeruginosa	Absent	N.C	N.C	N.C	N.C

[#Pharmacopoeial Laboratory for Indian Medicines, govt. of India, protocol for testing ayurvedic, siddha & unani medicines]

5.4.5. Heavy metal identification

For the assay, a standard solution of heavy metals at various concentrations was prepared. The amount of heavy metal found in the formulations were expressed in parts per million (ppm) based on the data obtained by performing AAS Study of the heavy metals. The cream samples were found to contain low levels of heavy metals (Lohar, 2007b). According to the Bureau of Indian Standards (BIS), the heavy metals found in the oil were within acceptable limits, as shown in Table 5.11.

Table 5.12. Heavy metal content of Varnya creams.

Formulation	Heavy metal			
	Pb	**Hg**	**Cd**	**As**
Pharmacopeial laboratory for Indian Medicines [#]	10	01	0.3	3
F1	3.139 ±1.032	0.092 ±0.072	0.11± 0.028	ND
F2	4.145 ±0.056	0.125 ±0.120	0.11± 0.056	ND
F3	3.499 ±1.745	0.142 ±0.125	0.12± 0.194	ND
F4	4.587 ±1.255	0.187 ±0.074	0.11± 0.103	ND

Results are means ± SD of three replicates (in ppm), [#]Pharmacopoeial Laboratory for Indian Medicines, govt. of India, protocol for testing ayurvedic, siddha & unani medicines.

5.4.6. Accelerated stability testing

Accelerated stability testing was performed for a short period of time to understand the nature of the cream formulation under the temperature and pressure conditions of 25°C/60%RH and 40°C/75%RH.

Table 5.13: Accelerated stability testing of herbal formulation F1 at 25°C/60%RH

Parameter	**Fresh**	**24 hr.**	**3 days**	**7 days**	**14 days**	**21 days**	**28 days**
Color	**Off-white**	**Off-white**	**Off-white**	**Off-white**	**Off-white**	**Off-white**	**Off-white**
Phase Separation	**-**	**No change**	**No change**	**No change**	**No change**	**No change**	**No change**
pH	**5.94**	**5.87**	**5.85**	**5.74**	**5.72**	**5.67**	**5.64**
Centrifugation	**No change**	**No change**	**No change**	**No change**	**No change**	**No change**	**No change**

Table 5.14: Accelerated stability testing of herbal formulation F1 at 40°C/75%RH

Parameter	Fresh	24 hr.	3 days	7 days	14 days	21 days	28 days
Color	Off-white	Off-white	Off-white	Off-white	Off-white	Off-white	Off-white
Phase Separation	-	No change	No change	No change	No change	No change	No change
pH	5.94	5.78	5.75	5.74	5.72	5.69	5.63
Centrifugation	No change	No change	No change	No change	No change	No change	No change

Table 5.15: Accelerated stability testing of herbal formulation F2 at 25°C/60%RH

Parameter	Fresh	24 hr.	3 days	7 days	14 days	21 days	28 days
Color	Off-white	Off-white	Off-white	Off-white	Off-white	Off- white	Off- white
Phase Separation	-	No change	No change	No change	No change	No change	No change
pH	5.89	5.77	5.54	5.52	5.38	5.36	5.31
Centrifugation	No change	No change	No change	No change	No change	No change	No change

Table 5.16: Accelerated stability testing of herbal formulation F2 at 40°C/75%RH

Parameter	Fresh	24 hr.	3 days	7 days	14 days	21 days	28 days
Color	Off- white	Off- white	Off- white	Off- white	Off- white	Off- white	Off- white
Phase Separation	-	No change	No change	No change	No change	No change	No change
pH	5.89	5.71	5.54	5.51	5.27	5.25	5.23
Centrifugation	No change	No change	No change	No change	No change	No change	No change

Table 5.17: Accelerated stability testing of herbal formulation F3 at 25°C/60%RH

Parameter	Fresh	24 hr.	3 days	7 days	14 days	21 days	28 days
Color	Off-white	Off-white	Off-white	Off-white	Off-white	Off-white	Off-white
Phase Separation	-	No change	No change	No change	No change	No change	No change
pH	6.00	5.81	5.82	5.75	5.73	5.60	5.49
Centrifugation	No change	No change	No change	No change	No change	No change	No change

Table 5.18: Accelerated stability testing of herbal formulation F3 at 40°C/75%RH

Parameter	Fresh	24 hrs	3 days	7 days	14 days	21 days	28 days
Color	Off- white	Off- white	Off- white	Off- white	Off- white	Off- white	Off- white
Phase Separation	-	No change	No change	No change	No change	No change	No change
pH	6.00	5.78	5.75	5.69	5.56	5.52	5.48
Centrifugation	No change	No change	No change	No change	No change	No change	No change

Table 5.19: Accelerated stability testing of herbal formulation F4 at 25°C/60%RH

Parameter	Fresh	24 hrs	3 days	7 days	14 days	21 days	28 days
Color	Off- white	Off- white	Off- white	Off- white	Off- white	Off- white	Off- white
Phase Separation	-	No change	No change	No change	No change	No change	No change
pH	5.98	5.88	5.87	5.82	5.77	5.69	5.55
Centrifugation	No change	No change	No change	No change	No change	No change	No change

Table 5.20: Accelerated stability testing of herbal formulation F4 at 40°C/75%RH

Parameter	Fresh	24 hrs	3 days	7 days	14 days	21 days	28 days
Color	Off-white	Off- white	Off- white	Off- white	Off- white	Off- white	Off- white
Phase Separation	-	No change	No change	No change	No change	No change	No change
pH	5.98	5.88	5.87	5.68	5.66	5.65	5.49
Centrifugation	No change	No change	No change	No change	No change	No change	No change

5.5. Discussion

In Ayurveda, various herbs have skin lightening and Off- whitening properties which are mentioned in the antient literatures. The formulations prepared by antient practitioners were mainly concoctions, pastes and oils. Formulations like creams, gels and lotions were not usually prepared in the traditional ayurvedic medicinal system. The major concept behind the development of herbal formulation in this study was to combine the traditional ayurvedic medicinal knowledge with modern pharmaceutical understanding of excipients. Application of herbal extract directly to the skin can have various limitations, therefore, excipients help in easy application and penetration of the herbal extracts into the skin. The formulations were prepared by mixing two phases-Aqueous (water) and non-aqueous (oil) phases. Both the phases were heated at the same temperature to avoid phasing or cracking of the formulation. While mixing the formulation the oil phase was poured into the water phase making the formulation oil in water type (o/w). Oil in water type formulations have water as the base which gives a vanishing effect to the formulation upon application.

Evaluation of the formulations ensures that the prepared formulation can withstand various temperature, pressure fluctuations. The evaluation characterization of formulations determines its stability and shelf life. The physical and structural properties of the creams were examined for better understanding of their nature.

5.6. Conclusion

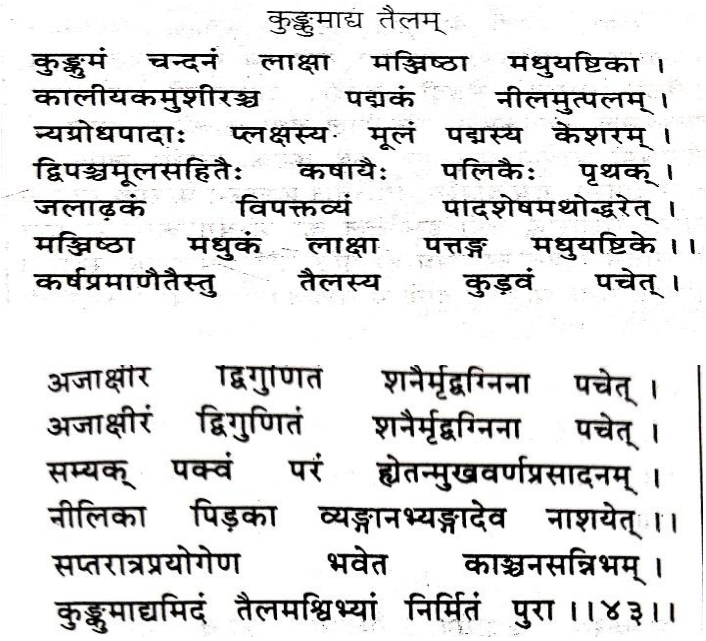
In this study the link between modern herbal cosmetic and traditional system of medicine 'Ayurveda' was explored. The Varnya herbs showing good tyrosinase inhibiting property were used for formulating skin Off- whitening and brightening creams. The Varnya formulation developed contains combination of two herbal extracts and various excipients as enlisted in table 5.2. The excipients used have their own importance in the formulation development method as mentioned in section 5.2.1. The developed formulations were evaluated, and characteristic based on their nature and appearance. The evaluation parameters used were macroscopic, microscopic, pH, viscosity, Rheology Microbial count, Heavy metal and stability. The colour of all the formulations were observed to be slightly Off- white, because of herbal extracts and their pigmentations. Upon performing the evaluation tests was observed that out of the four formulations prepared, Formulation F1 was more stable and has shown good viscosity and rheological properties.

Chapter - 6

6. Quality Evaluation and Validation of Traditional *Varnya* Formulation- Kumkumadi tailam

6.1 Kumkumadi tailam

Kumkumadi tailam is an Ayurvedic oil formulation, which has been used since ages for beautiful glowing and lustrous skin by women in India and other southern parts of Asia (Parihar, 2017). Ayurveda is age-old medicinal system practiced in various parts of world. Ayurveda describes thousands of herbs and herbal preparation. Ancient texts like Charak Samhita, Bhaisajyaratnavali, Sushruta samhita, Bhavprakash mentioned the concept of "varnya" (complexion promoters), which deals with skin brightening and whitening (Servesh et al., 2013). In reference to the varnya herbs used in the formulation of kumkumadi tailam, verses in Sanskrit have been mentioned in Bhaisajyaratnavali explaining the herbs and method of preparation of the formulation from ancient time as follows.



कुङ्कुमाद्य तैलम्

कुङ्कुमं चन्दनं लाक्षा मञ्जिष्ठा मधुयष्टिका ।
कालीयकमुशीरञ्च पद्मकं नीलमुत्पलम् ।
न्यग्रोधपादाः प्लक्षस्य मूलं पद्मस्य केशरम् ।
द्विपञ्चमूलसहितैः कषायैः पलिकैः पृथक् ।
जलाढ़कं विपक्तव्यं पादशेषमथोद्धरेत् ।
मञ्जिष्ठा मधुकं लाक्षा पत्तङ्ग मधुयष्टिके ।।
कर्षप्रमाणैतैस्तु तैलस्य कुड़वं पचेत् ।

अजाक्षीर द्विगुणात शनैर्मृद्वग्निना पचेत् ।
अजाक्षीरं द्विगुणितं शनैर्मृद्वग्निना पचेत् ।
सम्यक् पक्वं परं ह्येतन्मुखवर्णप्रसादनम् ।
नीलिका पिड़का व्यङ्गानभ्यङ्गादेव नाशयेत् ।।
सप्तरात्रप्रयोगेण भवेत काञ्चनसन्निभम् ।
कुङ्कुमाद्यमिदं तैलमश्विभ्यां निर्मितं पुरा ।।४३।।

Figure 6.1: *Kumkumadi tailam* mention in Ayurvedic book Bhaisajya Ratnavali

Dialectical-

Kumkuma chandana laksha manjishtha madhuyashthika
Kaliyakamukhshirchya padmaka nilutpalam
Nyagrodhpada plakshasya mulang padyasya kisharam
Dwipashchmulsahitey kashaya palikya pruthak
Jalaadhakan vipattivyang paadsheshmathodhwaret

Manjishtha madhukan lakkha patang madhuyashtika
Karnapramanestetu tailasya kudavan pachet
Ajakshir dwigunit shanaymrudhagni pachet
Samayak pakwa parng hwetanmukhawarnaprasadanam
Neelika peedikavyagna bhadyadev nashayat
Saptaratraprayogen bhavet kashchasanibham
Kumkumadhimid tailamswibham nirmitam pura
(Bhaisajya Ratnavali verse 43)

This formulation includes *Crocus sativus*, *Santalum album*, *Laccifer lacca*, *Rubia cordifolia*, *Glycerrhiza glabra*, *Pterocarpus santalinus*, *Vetiveria zinzanioides*, *Prunu scerasoides*, *Nymphae astellata*, *Ficus benghalensis*, *Inula recemosa*, *Nelumbo nucifera* and Dashmool (roots of ten plants) (table 1), *Madhuca longiforia*, *Pterocarpus santalinus*, *Glycerrhiza glabra.*

It has been observed that Crocus sativus present in Kumkumadi tailam enhances the skin brightening and whitening. The women in India use it for healthy and glowing skin. It is applied on the body by massing thoroughly during Ayurvedic body massage on the daily basis for long-term effect. It is used as the moisturizing agent in many Asian Countries and its preparation, distribution and sales is governed under drug and cosmetic act 1945 (Javed, et al., 2009; Lohar, et al., 2007a). Although the major concern is that the official standards for quality and safety has not been developed yet for Kumkumadi tailam.

6.2 Preparation of Kumkumadi tailam

Kumkumadi tailam (oil) was prepared according to the processes mentioned by Sen 2015. Ingredients used in the preparation of Kumkumadi oil are listed in table 1. Primarily, *Crocus sativus*, *Santalum album*, *Laccifer lacca*, *Rubia cordifolia*, *Glycerrhiza glabra*, *Pterocarpus santalinus*, *Vetiveria zinzanioides*, *Prunu scerasoides*, *Nymphae astellata*, *Ficus benghalensis*, *Inula recemosa*, *Nelumbo nucifera* and Dashmool (*Desmodium gangeticum*, *Uraria picta*, *Solanum indicum*, *Solanum xanthocarpum*, *Tribulu sterrestris*, *Aegle marmelos*, *Premna mucronata*, *Oroxylum indicum*, *Stereospermum suaveolens*, *Gmelina arborea*) were taken in equal amounts and boiled to form decoction. Equal amounts of *Rubia cordifolia*, *Madhuca longifolia*, *Laccifer lacca*, *Pterocarpus santalinus*, *Glycerrhiza glabra* were made into paste and added to

the decoction and heated on medium flame. Before addition of water, goat milk and sesame oil, bioactive fraction of kumkumadi oil (BFKO) was taken for tyrosinase inhibition assay, anti-oxidant assay, total phenolic content analysis and total flavonoid content analysis. Lastly, the decoction was boiled till the oil is formed.

Table 6.1. Ingredients used in the formulation of kumkumadi oil

Sr. no.	Plant & other ingredients	Plant parts used	Amount
1.	*Crocus sativus*	Stemen	0.33%
2.	*Santalum album*	Bark	0.33%
3.	*Laccifer lacca*	Bark	0.46%
4.	*Rubia cordifolia*	Root	0.46%
5.	*Glycerrhiza glabra*	Root	0.46%
6.	*Pterocarpus santalinus*	Root	0.46%
7.	*Vetiveria zinzanioides*	Root	0.33%
8.	*Prunu scerasoides*	Root	0.33%
9.	*Nymphae astellata*	Root	0.33%
10.	*Ficus benghalensis*	Root	0.33%
11.	*Inula recemosa*	Fruit	0.33%
12.	*Nelumbo nucifera*	Stemen	0.33%
13.	*Aegle marmelos*	Root	0.33%
14.	*Premna mucronata*	Leaves	0.33%
15.	*Oroxylum indicum*	Root	0.33%
16.	*Stereospermum suaveolens*	Leaves	0.33%

17.	*Gmelina arborea*	Root	0.33%
18.	*Desmodium gangeticum*	Leaves	0.33%
19.	*Uraria picta*	Leaves	0.33%
20.	*Solanum indicum*	Bark	0.33%
21.	*Solanum xanthocarpum*	Bark	0.33%
22.	*Tribulu sterrestris*	Root	0.33%
23.	*Madhuca longiforia*	Bark	0.13%
24.	Sesame oil	-	33.33%
25.	Goat milk	-	16.66%
26.	Water	-	50%

6.3 Total phenolic content (TPC) and total flavonoid content (TFC)

Using Folin-Ciocalteu reagent, The Total Phenolic and flavonoid Content of Kumkumadi oil was determined. 20 µg of BFKO was mixed with 110 µL of the Folin-Ciocalteu reagent, which was diluted to 1:10 with de-ionized water and 70 µL of 10%, w/v solution of sodium carbonate was used for neutralization. For 30 minutes the reaction mixture was incubated at room temperature and absorbance was measured at 765 nm. To determine TPC The standard curve of gallic acid was used and expressed as gallic acid equivalent per gram of extract (GAE/g dwf) Singleton et al. (1999), Liyanaarachchi et al., 2018.

To determine the total flavonoid content (TFC) of kumkumadi oil, method cited by Gursoy et al. (2009) was followed with some modifications. 100 µl of BFKO (50–500 µg/mL) was added with 100 µL of 2% AlCl3 dissolved in methanol. The Absorbance of the reaction mixture was measured at 415 nm wavelength after incubation for 10 min. TFC was expressed as mg quercetin equivalent per gram (mg QE/g dwf) of oil by using standard curve of quercetin i.e, y =0.045x + 0.028 (r2 =0.996).

6.4 Antioxidant activity of Kumkumadi Tailam

The antioxidant study was presented on the prepared Kumkumadi tailam by performing Diphenyl-1-picrylhydrazyl (DPPH)assay and Reducing power assay, as mentioned below.

6.4.1. Diphenyl-1-picrylhydrazyl (DPPH)assay

In a 96-wellmicro-plate (VersaMax™ ELISA Microplate), 100µl of BFKO sample was taken with 150µl of 0.20 µg/ml DPPH solution and the mixture was incubated in the dark for 20 min. Butylated hydroxyl anisole (BHA) was taken as positive control. UV-visible spectrophotometer was used for measurement of absorbance at the wavelength if 517 nm and IC50 value was calculated for the sample and positive control. IC50 value here implies as the concentration of the sample required for scavenging 50% of DPPH radicals, it was assessed by interpolation from linear regression analysis.

DPPH radical scavenging activity (%)= [(Acontrol – Asample) /Acontrol] X 100

Here Acontrol is absorbance of Butylated hydroxyl anisole (BHA) taken as positive control and Asample is the absorbance of the BFKO (Nema et. al., 2011; Biswas et al., 2016).

6.4.2. Reducing power assay

The reducing power assay reduces the ferric cyanide complex to the ferrous form and helps in estimation of the antioxidant capacity of the BFKO. Different concentrations of BFKO sample and positive control were prepared in the range of 1.5-15 µg/ml in 0.2M phosphate buffer (pH 6.5). Here Butylated hydroxyl anisole (BHA) was taken as positive control. In 96 well microplate 50µl of sample and 25µl of 1% potassium ferricyanide were taken and incubated at 45°C for 25 min. 20µl of 10% trichloro-acetic acid with 30 µl of 0.1% FeCl3 and 100µl distilled water were added to the above mixture after incubation. UV-visible spectrophotometer was used to determine the change in color at the wavelength of 618 nm. IC50 value was calculated by linear regression analysis, showing the concentration at which the absorbance was equal to 0.5.

Reducing activity = (AS-AB) X100/ (AS- AB)

Here AS refers to the absorbance of the BFKO, AB refers to the absorbance of the sample without BFKO, potassium ferri-cyanide, tri- chloro acetic acid and FeCl3 and AC denotes to the control (Carneiro et al., 2013).

6.5 GCMS Analysis of Kumkumadi Tailam

GCMS analysis was performed to identify the compounds present in the Kumkumadi tailam sample. The Chromatographic separation technique was performed using 7890A GC System with 7000 GC/MS Triple Quad (Agilent Technologies, Santa Clara, CA, USA). The separation was performed on column HP-5 MS (30 m x 0250 mm x 025 µm) with ionization voltage of 70ev for a total run time of 27 min. The carrier gas used was Helium at a flow rate of 1.2 ml/min. Initially the temperature was maintained at 70°C, then linearly increased 150°C for 10 min then the increase in temperature was 200°C for 1 min followed by 300°C for 4 min. Kumkumadi tailam. The obtained Retention time (Rt) was compared to the mass spectra present in the National Institute of Standards and Technology spectral data library already stored in the computer software of GCMS instrument. The peak area normalization was used to express the relative percentage constituent of each compound identified in the oil sample (Phillips et al, 2015). The compounds were identified basing on the retention indices and mass spectra data of the compounds obtained by maintaining the same experimental conditions using two-dimensional search algorithm from a point of similarity in the retention index and mass spectral (Adams., 2001; Konig et al., 2006). The obtained Retention time (Rt) was compared to the mass spectra present in the National Institute of Standards and Technology spectral data library already stored in the computer software of GCMS instrument.

6.6 Tyrosinase inhibition activity assay

The method for enzyme inhibition assay mentioned by Biswas et. al., (2016) was followed with slight modifications. BFKO sample was prepared by dissolving in 2% Dimethyl sulfoxide (DMSO). Concisely, 80 µL of BFKO sample was added with 30µL of tyrosinase in a 96 well plate. The concentration of tyrosinase enzyme taken was 333 U/ml in phosphate buffer with pH 6.8. Different concentrations of Kojic acid and BFKO sample were prepared in the range of 0.5-10 µg/ml The above-mentioned 96 well plate was then kept aside for incubation at 25°C for 5 min. After incubation 100 µL of

substrate was added to each well and incubated for same time and temperature before measurement of absorbance. The substrates used were 4mM l-tyrosine or 6 mM L-DOPA, substrate was dissolved in 50mM phosphate buffer pH 6.8. Using UV–visible spectroscopy (SpectraMax® Plus, United States), the optical density of the reaction mixture in each well was measured at 475 nm. Kojic acid was used as positive control (Jo et al., 2012). Calculation of the Tyrosinase activity was performed with the help of following formula:

$$\textit{Enzyme activity} = \frac{(\Delta A475nm/min_{test} - \Delta A475nm/min_{blank})(\text{Volume of Assay, ml})}{(\varepsilon_{dopachrome}\ 3500\ M^{-1}\ cm^{-1})(\text{Volume of Enzyme used, ml})}$$

ε Is the extinction coefficient dopachrome. Enzyme activity unit is M^{-1} s^{-1} (mole per second). For determining the IC50 values of inhibitors, graph was plotted with % tyrosinase inhibition on the Y-axis and inhibitor concentrations on the X-axis.

6.7 Physiochemical properties of Kumkumadi tailam

Various analytical parameters like color, odor, organoleptic property, taste, pH, viscosity and boiling point were observed for the prepared kumkumadi oil. The rancidity of the prepared kumkumadi oil was analyzed by observing the acid value of the oil. Acid value was estimated by the amount of potassium hydroxide required to neutralize the free acid in 1g of fat. Saponification value was observed for analysis of average molecular weight of a fat or oil present in kumkumadi oil. Further Peroxide value was observed to estimate the amount of peroxide oxygen present in the oil (Anonymous, 2016).

Stability study was performed by means of long-term stability study (40 72 °C) and accelerated stability study (75 75% RH). The prepared oil was kept in airtight container and assay was performed over the time period of 1, 3, and 6 months for analyzing changes in appearance, phenolic and flavonoid content, viscosity, saponification value, iodine value, peroxide value.

6.8 Microbial limit test

Microbial limit for the prepared kumkumadi oil was analyzed as per the guideline set by the Ayurvedic pharmacopoeia of India. The microbial growth count assay included total microbial plate count (TPC), Total yeast & mould count, tests for Staphylococcus aureus, Salmonella, Pseudomonas Aeruginosa, Escherichia coli. Organisms have their specific growth pattern and type. Therefore, colony-forming units (CFU) have been used for expressing the total number of viable organisms (Lohar et al., 2007b).

6.9 Determination of Heavy metal

Heavy metal analysis was performed using Thermofisher AA303 atomic absorption spectrometer (AAS). For analyzing the heavy metal content in the prepared kumkumadi oil, standard heavy metals like mercury, lead, cadmium and arsenic were used and calibration curve was drawn. To remove moisture at thoroughly controlled environment the sample of the prepared kumkumadi oil was dried at 55–70°C for 6-8 hrs. After sufficient removal of moisture from the oil, 3 ml of oil sample was taken in a flask with 4 ml concentrated HNO3 and kept aside for 5 hrs for removing the fumes. The mixture was then treated with 2:1 ratio of HNO3 and HClO4 and heated for 6 hrs at 120-130°C. the mixture was boiled for 10-15 min after addition of 10 ml of milli-Q water to reduce the volume to half of the initial volume. The solution was then cooled and filtered using Whatman filter paper no.42. The filtrate was taken in a flask and volume was made upto 50ml with milli-Q water. Similarly, blank solution was prepared and the analysis was performed in replicate of three (Neema et al., 2014).

6.10 Statistical analysis

All the analysis and assays were performed in triplicate. The IC50 value obtained have been represented as the mean ± standard deviation. Graphical representation was shown by plotting curve as a percentage of inhibition versus concentrations of the individual experiments measured. Using one-way ANOVA test the substantial mean difference between the samples of oil taken was determine with the help of Graph-Pad Prism version 6 (Graph Pad Software, USA).

6.11 Results

6.11.1. Estimation of total phenolic content and total flavonoid content

To find the total phenolic content (TPC), the most preferred method is Folin–Ciocalteu method. For total flavonoid content (TFC) 2% of AlCl3 solution in methanol was used. The TPC was stated as mg of gallic acid equivalents (GAE) in per g of Weight formulation (dwf) and TFC was expressed as mg quercetin equivalent per ml (mg Qr/g dwf) of the herbs. The total phenolic and flavonoid content of the herbs used in the formulation of Kumkumadi oil were observed to be 72.01 6.12 mgGAE/g dwf and 54.16 8.50 mg Qr/g dwf respectively (Table 2).

6.11.2. Antioxidant activity of the formulation

DPPH assay revealed the IC50 values of the kumkumadi oil and the positive control BHA were observed to be 8.42 ± 2.98 µg/ml and 6.15 ± 3.54 µg/ml respectively. While on performing the reducing power assay, the IC50 value obtained for kumkumadi oil and the positive control BHA were estimated to be 12.76± 6.08 µg/ml and 8.67 ± 1.46 µg/ml respectively.

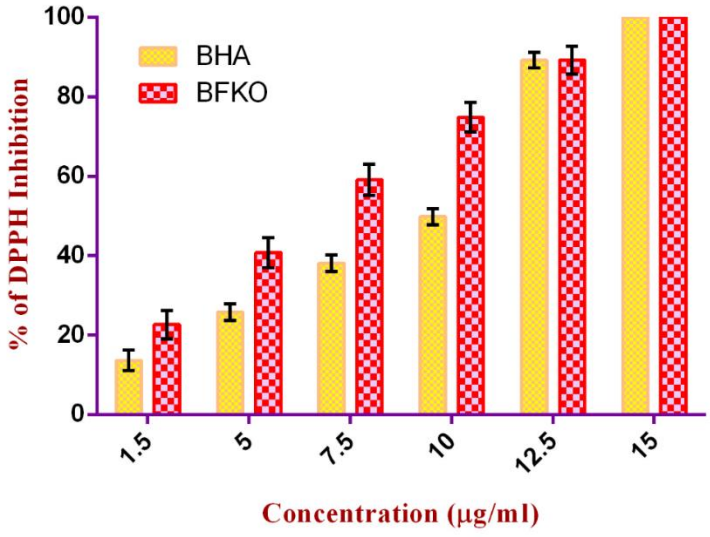


Figure 6.2: Free radical DPPH scavenging activity of Kumkumadi oil formulation under different concentrations. BFKO- Bioactive fraction of kukmkumadi oil; positive control- BHA-Butylated hydroxyl anisole. The represented results are mean±SD (n=3) of three replications.

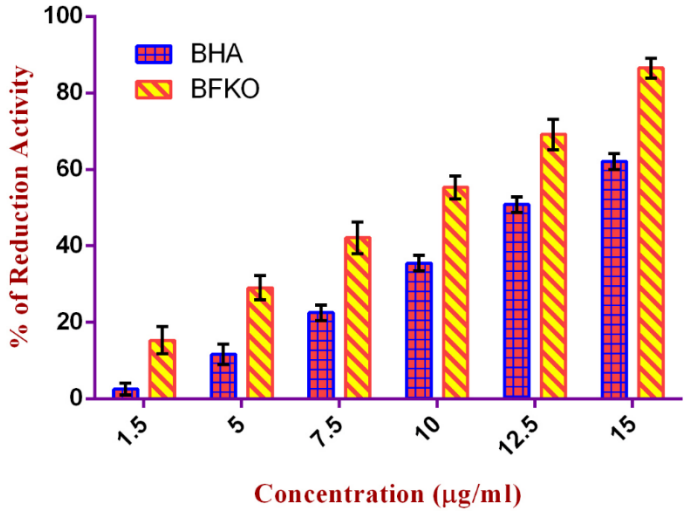


Figure 6.3: Ferric reducing antioxidant power of Kumkumadi oil formulations with under various concentrations. BFKO- Bioactive fraction of kukmkumadi oil; positive control- BHA-Butylated hydroxyl anisole. The represented results are mean±SD (n=3) of three replications.

6.11.3. Anti-tyrosinase potential of kumkumadi tailam

The tyrosinase inhibition potential of kumkumadi oil was observed in reference to the standard kojic acid. Kumkumdi oil showed considerable amount of tyrosinase enzyme inhibition potential. Tyrosinase inhibition assay of kumkumadi oil and kojic acid were performed at different concentrations. The relative activity of the oil and standard were estimated as IC50 values. Kojic acid was identified as the slow binding inhibitor as the inhibition expressed was slow in nature (Cabanes et al., 1994). Kojic acid showed mixed inhibitory activity on mushroom tyrosinase. The inhibitory activity of kumkumadi oil formulation was observed to be directly proportional to the concentration. The calculated IC50 values obtained for kojic acid and kumkumadi oil were found out to be 5.79±0.05 µg/ml and 7.13±0.21 µg/ml respectively. Results has been shown in the Figure no. 4.

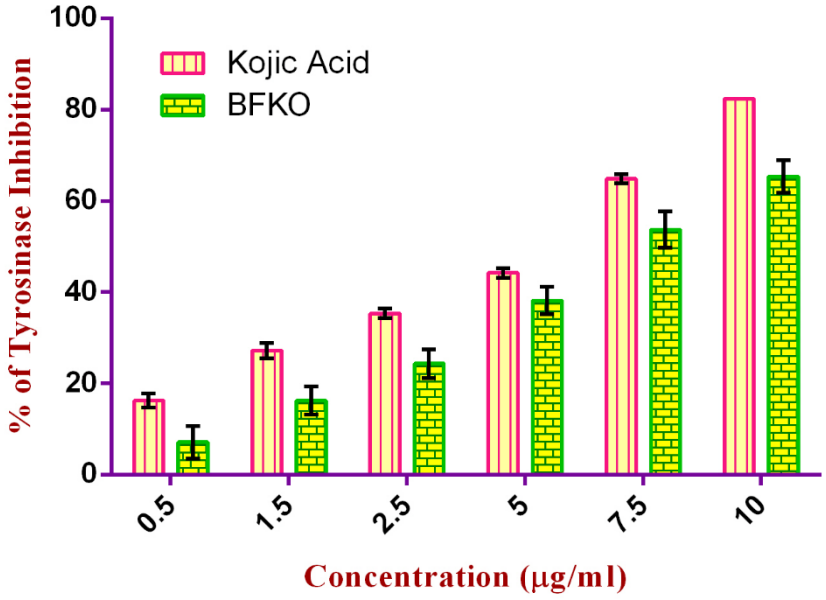


Figure 6.4: Dose dependent inhibition activity of kojic acid and kumkumadi oil on tyrosinase enzyme. Positive control- Kojic acid; mean±SD (n=3) of three repeatation of the assay.

Table 6.2. TPC, TFC, Tyrosinase inhibition assay, Antioxidant activity of herbs

Formulation code	Total Phenolic Content (TPC) (GAE/ml)	Total Flavonoid Content (TFC) (µl Qr/ml)	Tyrosinase inhibition (IC50) (µg/ml)	Antioxidant activity (IC50)	
				Diphenyl-1-picrylhydrazyl (DPPH) assay (IC50)	Reducing power assay (IC50)
BFKO	72.01±6.12	54.16±8.50	180.54±0.21	7.65±2.98	15.33± 6.08

Results are means ± SD of three replicates; BFKO- Bioactive Fraction of Kumkumadi oil

6.11.4. GCMS analysis of Kumkumadi tailam

The GCMS analysis of Kumkumadi oil facilitated identification of compounds by analyzing their accurate mass, MS fragmentation patterns which are matched within 25 ppm error tolerance, experimental and calculated m/z, molecular formula, error present in ppm, isotopic ratio. Table 3 briefly reviews 23 identified compounds with their individual retention time, molecular formulae, molecular weight, Area, Mass to charge ratio m/z, and MS/MS fragments. Figure 1 represents the chromatogram and the representative MS/MS spectra of the identified compounds. All the compounds identified in the oil belong to different classes and the mass spectrum of the compounds were analyzed and compared with the National Intitute of Standards and Technology (NIST) mass spectrum library. Basing on the retention time of the compounds present in the oil, the compounds were identified with the help GCMS analysis. It was observed that the identified compounds are found in different plants as used in preparation of the Kumkumadi oil have been previously reported to be used in the treatment of Acne vulgaris, Migrane, and Skin disorders (Prajapati et al., 2014; Rais et. al., 2017; Sharma et al., 2017). Safranal have been reported to have good antioxidant property, helps in protection from indomethacin-induced gastric ulcers and epilepticus (Rezaee et. al., 2013). Benzoic acid has been considered one of the potent tyrosinase inhibitor (Kubo et al., 1998). It has been reported that n-hexadecanoic acid are anti-inflammatory and potent antioxidant (Abubakar et al., 2016). Therefore the chemical constituents identified in the Kumkumadi tailam may play major therapeutical and pharmaceutical role in the skin care.

Table 6.3. Compounds identified through GCMS analysis.

Peak no.	Retention Time	Molecular Formulae	Molecular Weight (g/mol)	Area (%)	m/z	MS/MS Fragments	Identified Compound Name
1.	2.072	C3H7NO2	89.09	48.43	89	61.00, 90.10, 89.10	Sarcosine
2.	4.123	C7H6O2	122.12	22.15	105	50.00, 51.00, 60.90, 76.90, 104.90, 105.90, 121.80, 122.90	Benzoic acid
3.	4.221	C10H14O	150.21	1.1	91	60.90, 70.00, 76.90, 78.90, 90.90, 104.80, 106.80, 120.80, 121.80, 149.80	Safranal
4.	5.426	C16H32O2	256.42	0.79	55	55.00, 56.00, 57.00, 60.90, 68.90, 69.90, 71.00, 82.90, 84.00, 96.90	1-Tetradecyl acetate
5.	5.486	C14H30	198.39	0.28	57	55.00, 56.00, 57.00, 60.90, 69.90, 70.90, 72.90, 84.90, 87.90, 121.80	Tetradecane
6.	6.595	C6H5OH	94.11	1.16	191	57.00, 60.90, 70.00, 73.00, 73.90, 87.90	Phenol
7.	7.448	C16H34O	242.44	0.95	55	55.00, 56.00,	1-Hexadecanol

						57.00, 60.90, 68.90, 70.00, 71.00, 82.90, 84.00, 96.90	
8.	8.431	C11H10O	158.20	0.95	59	58.90, 60.90, 80.90, 90.90, 92.80, 104.80, 107.80, 108.80, 121.80, 148.80	2-Naphthalenemethanol
9.	10.062	C12H26O	186.34	1.41	57	55.00, 56.00, 57.00, 68.90, 69.90, 70.90, 82.90, 83.90, 96.90, 110.90	1-Dodecanol
10	11.187	C8H6O4	166.14	2.37	149	59.00, 90.90, 92.90, 104.90, 106.90, 118.90, 120.90, 146.80, 158.90, 186.80	Phthalic acid
11	11.625	C15H24O	220.35	32.57	59	55.00, 57.00, 59.00, 66.90, 67.90, 69.00, 72.90, 80.90, 81.90, 94.90	Tricyclo[4.4.0.0(2,7)]dec-8-ene-3-methanol
12	14.881	C21H3802	322.50	1.89	55	55.00, 57.00, 59.90, 68.90, 72.90, 82.90, 83.90, 86.90, 96.90, 128.80	1,1'-Bicyclopropyl]-2-octanoic acid
13	14.966	C18H34O	282.47	1.42	55	55.00, 67.00,	Oleic Acid

		2				68.00, 69.00, 78.90, 79.90, 80.90, 81.90, 94.90, 95.90	
14	18.681	C18H32O2	280.44	4.43	67	55.00, 67.00, 68.00, 69.00, 78.90, 79.90, 80.90, 81.90, 94.90, 95.90	Linoleic acid
15	18.711	C18H34O2	282.50	4.26	55	55.00, 67.00, 69.00, 80.90, 81.90, 82.90, 94.90, 96.90, 97.90, 128.80	9-Octadecenoic acid (Z)
16	18.996	C16H32O2	256.40	13.47	98	55.00, 57.00, 69.00, 71.00, 73.90, 82.90, 83.90, 97.90, 111.90, 133.90	Hexadecanoic acid
17	19.597	C16H17	271.311	5.99	256	127.90, 151.80, 164.70, 180.70, 209.70, 223.70, 224.70, 239.70, 255.70, 256.70	4-t-Butyl-2-[4-nitrophenyl]phenol
18	21.796	C27H46O2	402.70	6.34	137	55.00, 57.00, 69.00, 83.00, 135.80, 136.80, 137.80, 149.80, 176.80, 401.60	δ-Tocopherol
19	22.548	C28H48O	416.7	16.26	15	149.80, 150.80,	γ-Tocopherol

		2			1	151.90, 190.80, 415.70	
20	23.214	C12H5Cl6N3O2	435.9	17.69	149	121.90, 130.80, 134.80, 147.80, 148.80, 49.80, 160.80, 177.80, 202.80, 353.60	2,6-Bis (3,4-methylenedioxyphenyl)
21	24.22	C28H48O	400.68	11.67	55	80.90, 92.90, 94.90, 104.90, 106.90, 132.80, 134.80, 144.80, 158.80, 160.80	Campesterol
22	24.507	C29H48O	412.69	9.46	55	55.00, 69.00, 80.90, 82.90, 94.90, 96.90, 132.80, 144.80, 146.80, 158.80	Stigmasterol
23	25.162	C29H50O	414.71	25.35	55	71.00, 80.90, 92.90, 94.90, 104.90, 106.90, 144.80, 158.80, 160.80, 212.80	β-Sitosterol

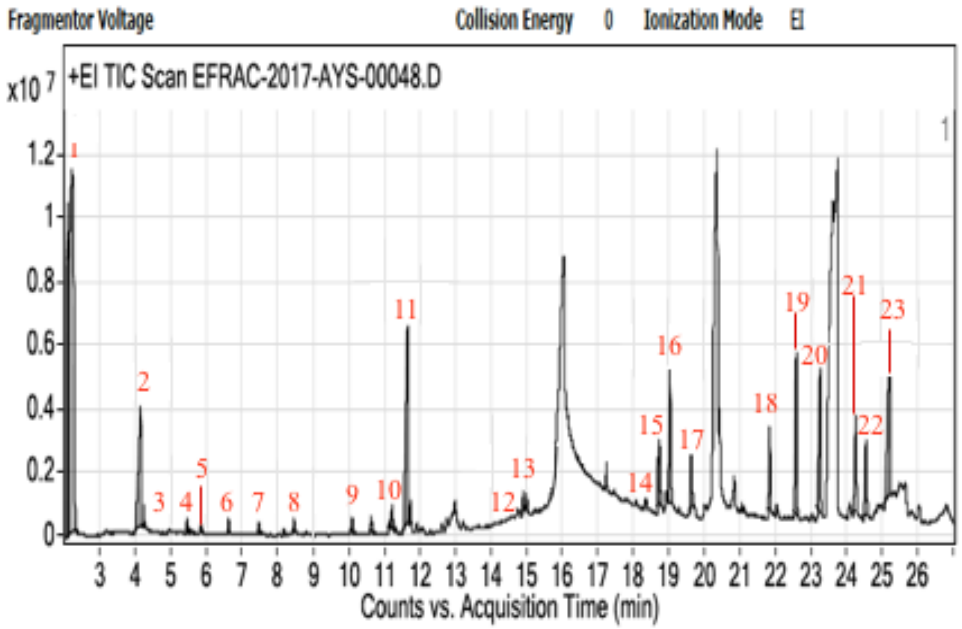


Figure 6.5: GCMS analysis of Kumkumadi oil showing the major compounds present in the formulated oil sample.

6.11.5. Physiochemical properties of prepared oil formulation

The physiochemical properties of kumkumadi oil have been shown in Table 4. It was observed that the color of the prepared oil was brown with very distinct smell and tastes slightly bitter. Upon keeping Kumkumadi oil in a sealed glass airtight container for long term, no microbial growth was observed. The viscosity and pH of the oil was observed to be 1.09 and 6.3 respectively. Acid value and saponification value were found out to be 205.26 and 0.1068 respectively.

For accelerated stability study analysis the oil preparation was evaluated during the time period of 1, 3 and 6 months. During the time period of 6 months the oil showed no alterations in the appearance, smell, phenolic and flavonoid content, pH as shown in table 5. However it can be inferred from the stability study that the prepared kumkumadi oil was stable for a prolonged period of time.

Table 6.4. Physicochemical properties of kumkumadi oil

Properties	Kumkumadi oil
Appearance	Brown color
pH	6.3
Density (g/mL)	0.9212 g/ml
Viscosity (millipoise)	1.09
Acid value (mg)	205.26 mg
Smoke Point (°C)	210°C
Saponification Value (mg)	0.1068 mg
Iodine Value (g)	0.12g
Peroxide value (meq/kg)	2.9 meq/kg

Table 6.5. Stability study analysis of the formulation

Time (month)	Temperature (°C)	Viscosity (millipoise)	Saponification Value (mg)	Peroxide value (meq/kg)	Iodine Value (g)	Total phenolic content (%)	Total flavonoid content (%)
0	40 ± 2 (75 ± 5% RH)	1.04	1.1062	0.9212	0.12	100	100
1		1.06	1.1059	0.9210	0.11	99.92	99.99
3		1.08	1.1059	0.9207	0.11	99.65	99.70
6		1.05	1.1058	0.9206	0.11	99.01	98.96

6.11.6. Microbial growth analysis

The microbial growth analysis reports have been shown in Table 6.6. It was observed that the prepared kumkumadi oil contained 1.2X102 CFU of total bacterial count and 0.5 CFU of total fungal count. However, presence of microbes like E. coli, Salmonella, S. aureus and P. aeruginosa were not observed in the prepared kumkumadi oil.

Table 6.6. Microbial growth analysis in the kumkumadi oil formulation

Factors	**[#]Pharmacopoeial laboratory for Indian medicines specifications (CFU/gm)**	**Kumkumadi oil (CFU/gm)**
Total Bacterial count	1 X 10^5	1.2 X 10^2
Total Fungal count	1 X 10^3	0.5
Escherichia coli	Absent	N.C
Salmonella	Absent	N.C
Staphylococcus aureus	Absent	N.C
Pseudomonas aeruginosa	Absent	N.C

[[#]Pharmacopoeial Laboratory for Indian Medicines, govt. of India, protocol for testing ayurvedic, siddha & unani medicines]

6.11.7. Heavy metal identification

The standard solution of heavy metals in different concentration ranges was prepared for the assay. From the data obtained by performing AAS Study of the heavy metals a standard calibration curve was drawn and the amount of heavy metal obtained in the kumkumadi oil was expressed as parts per million (ppm). The heavy metal content in the kumkumadi oil sample was found to be under the permissible limits (Lohar, 2007b). As per the Bureau of Indian Standards (BIS) the heavy metals found in the oil was in acceptable limit (Table 6.7).

Table 6.7. Heavy metal content of individual herbs and kumkumadi oil.

Formulation	Heavy metal			
	Pb	**Hg**	**Cd**	**As**
Pharmacopoeial laboratory for Indian Medicines [#]	10	01	0.3	3
Kumkumadi tailam	4.239 ±1.032	0.152 ±0.082	0.13± 0.098	ND

Results are means ± SD of three replicates (in ppm), [#]Pharmacopoeial Laboratory for Indian Medicines, govt. of India, protocol for testing ayurvedic, siddha & unani medicines.

6.12 Discussion

Kumkumadi oil is an Ayurvedic preparation used for healthy and glowing skin. The preparation methods used were collected from different Ayurvedic medicine practitioners and Bhaishajya ratnavali book as reference. All the ingredients used in the formulations are mentioned in the vedic literature as Varnya herbs which have skin brightening and whitening property. Kumkumadi oil is widely used as aromatic massage oil in India and other south Asian Countries since ages. This study helps in revealing the important aspects related to the Kumkumadi oil used since vedic period. The GCMS analysis showed that the major compounds present in the oil has significant positive effect on the skin. GCMS analysis showed 23 major compounds having different activities on the skin. A proof of the claims of the oil having skin whitening and brightening property was established by observing the tyrosinase inhibition potential of the oil which showed considerably good result. Hence it was observed that the IC50 value of Kumkumadi oil was found be 180.54±0.21 µg/ml. Kojic acid was used as the standard reference of tyrosinase inhibition, which showed IC50 value to be 2.28±0.05 µg/ml. Phytochemical screening of the prepared oil showed the brown color and distinctive smell with slightly bitter taste. Upon prolonged keeping at 40 2□C (75 5% RH) temperature, it was observed that the Kumkumadi oil did not deteriorate in appearance, a slight change in TPC and TFC were observed. Heavy metal analysis and microbial count were observed to be under permissible limit as per the pharmacopoeial laboratory guidelines. Further, creams were formulated from the kumkumadi tailam,

which then evaluated for its appearance, texture and stability. pH and viscosity were observed to be in the range as mentioned in the Indian pharmacopoeia guideline. For the stability testing of the cream phase separation and centrifugation were observed.

6.13 Conclusion

The present study focuses on the Kumkumadi oil, an Ayurvedic preparation, which has been used for ages for improving health and appearance of the skin. Kumkumadi oil ingredients and preparation method have been mentioned in the ancient traditional book like Charak Samhita, Bhaisajyaratnavali, Sushruta samhita, Bhavprakash. GCMS analysis showed the major compounds like Sarcosine, Benzoic acid, Safranal present in the oil. These compounds have been reported as good tyrosinase inhibitors and also have antioxidant potential. Herbs like *Crocus sativus*, *Santalum album*, *Rubia cordifolia*, *Glycerrhiza glabra* used in the preparation of oil are considered to be potent tyrosinase inhibitors and also considered as good antioxidants. In this study prepared kumkumadi oil formulation has shown promising tyrosinase inhibitory potential, anti-oxidant potential and total phenolic content. The formulated creams have shown good stability potential. This scientific exploration may be useful for further investigation and eventually curing skin pigmentation.

Presentation

Akanksha Sharma, P.K Mukherjee, C.K Katiyar, Subhadip Banerjee, Joydeb Chanda, Amit Kar, Rajarshi Biswas. Evaluation of tyrosinase inhibition potential of **Kumkumadi tailam – An Ayurvedic preparation** at 6th Convention of SFE-India, 2019 Jadavpur university; India, 7-8 September 2019.

Chapter - 7

7. Summary and conclusion

7.1 Summary

7.2 Conclusion

7.1 Summary

The effectiveness of drugs and the principles of traditional medicine are increasingly being supported by scientific evidence. Using natural products can rejuvenate and protect the skin against environmental pollution, chemicals, temperature fluctuations, ultraviolet A and ultraviolet B radiation, hyperpigmentation, and inflammation. The ingredients are still used in cosmetics today because they were useful to ancient civilizations. Using cosmetic formulations containing herbal components, the skin is less likely to be irritated and more easily adjusted. Conventional treatments irritate the skin with symptoms such as itching, redness, drying, and allergic reactions. Chemical medications are known to produce side effects. Natural treatments are gaining popularity. Traditional medicine, therefore, is becoming more popular today.

Melanin is synthesized by a copper-containing enzyme called tyrosinase. Melanin determines the color of the skin. Melasma and age spots are caused by excessive melanin accumulation in the skin. Moreover, tyrosinase also causes enzymatic browning reactions in damaged fruits and vegetables. Loss of nutritional quality occurs as a result of deterioration of the color clarity of plant-derived products. However, despite recent developments in tyrosinase inhibitors, their safety concerns still need to be studied due to their undesirable side effects. In order to find new tyrosinase inhibitors from natural products, continuous research has been happening. The use of tyrosinase inhibitors for cosmetic purposes is available on the market. In addition to being used in the treatment of hyperpigmentation disorders, they are also applicable to the anti-browning industry in the food industry.

The study involved evaluating the tyrosinase inhibition activity of four Indian medicinal plants and their metabolites. Four different plant extracts were screened for their ability to inhibit tyrosinase in this thesis. Furthermore, phytoconstituents with tyrosinase inhibitory activity were identified by extractions. The results can be used for evaluating skin whitening activity, such as hyperpigmentation.

A detailed analysis of various tyrosinase inhibitory medicinal plants and their phytoconstituents is discussed in the introductory chapter (Chapter 1). In this chapter, we present an overview of the literature on tyrosinase inhibitors derived from medicinal plants, including the plant extracts and isolated constituents. We have investigated more than 2000 articles using the keywords 'tyrosinase', 'Plants inhibiting tyrosinase,' 'melanogenesis,' and 'skin whitening agents' from several search engines, such as

PubMed, SciFinder, Scopus, Science Direct and Google Scholar up to April 2021. Furthermore, tyrosinase contributes to melanin synthesis. This pathway also explains how its expression is regulated through cellular signaling. The study of tyrosinase inhibition is an active field of research in dermatological, biomedical, food and agricultural science, and also has potential relevance to insect physiology. Tyrosinase inhibitors have been developed several times, but their safety concerns are still in need of investigation due to their undesirable side effects. Researchers are researching medicinal plants to find potent and safe tyrosinase inhibitors.

The study's scope and rationale are described in chapter 2, as well as its objective. Considering all the experiments performed, a framework has been designed for the study.

Chapter 3 deals with standardization of the extracts obtained from Varnya herbs. These herbs used for skin whitening and brightening are described as complexion promoters, which, upon standardization, are found to be potent inhibitors of the tyrosinase enzyme. In addition to their clinical benefits, these herbs are also used as an ingredient in formulations for the treatment of dull skin. Several herbs are considered potent herbs for treating skin alignment by Traditional system of medicine such as Licorice (*G. glabra*) Nutmeg (*M. fragrans*), China rose (*H. indicus*), and Pushkarmool (*I. racemosa*). In Ayurvedia, herbs can be quantified and evaluated with RP-HPLC and HPTLC methods, which are specific and precise. Further these methods can be employed for standardization of certain Ayurvedic formulations containing the above-mentioned herbs as one of the ingredients, such as Pushkarmool Churna, Sarivadyasavam, Mathala rasayanam, etc. Using phenolic compounds in the Varnya herbs like glycyrrhizin, quercetin, ferulic acid, and chlorogenic acid as quality markers, HPLC and HPTLC methods can be used for quality control. They are robust and reproducible, which can help in combating the major challenges for the quality of herbal raw materials because they vary based on various factors including origin, time of collection, heavy metal contamination, drying method, and microbial contamination. Despite the importance of determining the markers present in herbs to verify their quality, insufficient evidence exists concerning the markers. As shown in the present study, RP-HPLC and HPTLC method validation for herb analysis shows reproducibility of the developed method.

In Chapter 4 the tyrosinase inhibition potential of the selected Varnya herbs were

observed. Tyrosinase inhibition assay was performed on the individual herbal extracts,

where Kojic acid was taken as the control. Upon performing the assay the IC50 values of the herbs were derived, showing potential of the herbs as good tyrosinase inhibitors.

Chapter 5 deals with the formulation development and evaluation of Varnya cream. These formulations were prepared by using the four herbs in combinations. The herbs used have been observed to be good tyrosinase inhibitors. The formulations prepared contain various excipients like olive oil, propylene glycol, glycerin have their own properties providing a synergistic effect on application. The formulations were evaluated based on their physical and chemical characteristics. The tests performed on the formulations were Rheology, Viscosity, microscopic and macroscopic characterization which shows positive results. Stability parameters were observed, and the formulations were found to be stable after prolonged exposure to high temperature.

Kumkumadi oil is widely used as aromatic massage oil in India and other south Asian Countries since ages as described in chapter 6. Kumkumadi oil is an Ayurvedic preparation used for healthy and glowing skin. The preparation methods used were collected from different Ayurvedic medicine practitioners and ayurvedic book as reference. All the ingredients used in the formulations are mentioned in the vedic literature as Varnya herbs which have skin brightening and whitening property. This study helps in revealing the important aspects related to the Kumkumadi oil used since vedic period. The GCMS analysis showed that the major compounds present in the oil have significant positive effect on the skin. Upon performing tyrosinase inhibition study it was found that kumkumadi tailam can be used as an potent tyrosinase inhibitory formulation. The phytochemical screening of the prepared oil revealed a brown color and distinctive smell as well as a mild bitter taste. After prolonged storage, Kumkumadi oil did not deteriorate in appearance. However, a slight change in TPC and TFC were observed. According to the pharmacopeial laboratory guidelines, the heavy metal analysis and microbiological count were within permissible limits. pH and viscosity were observed to be in compliance with the Indian Pharmacopoeia guidelines.

7.2 Conclusion

A key phenotypic characteristic of humans is skin pigmentation. Melanin, the pigment's main constituent, is produced by the melanocyte during a complex process known as melanogenesis. In any case, hyperpigmented spots caused by the accumulation of unusual melanin in particular parts of the skin can be esthetically problematic. Melanin synthesis is regulated by a regulator enzyme called tyrosinase. Thus, tyrosinase inhibitors prevent melanin synthesis. The use of tyrosinase inhibitors in dermatology is used to treat Hyperpigmentation. In addition, these inhibitors are used in cosmetic formulations. In India, Kumkumadi tailam is a popular cosmeceutical that uses traditional knowledge to beauty. Kumkumadi tailam has developed a scientific standard based on its phytochemical content and therapeutic benefits. The results of our study revealed that Kumkumadi tailam formulations possess potent free radical scavenging and UV protection abilities. Also, it inhibits tyrosinase the most effectively.

Our study evaluated the tyrosinase inhibitory activity of four traditionally used skin lighting medicinal herbs (varnya). The evaluation revealed that plants (*G. glabra* and *H. indicus*) and their phytoconstituents glycerrhizin and Ferulic acid were potentially tyrosinase inhibitory when compared to reference standard kojic acid. In addition, *M. fragrans* and *I. racemosa* extracts exhibited potent tyrosinase inhibitory effects. In this context, these four herbs were chosen for development of cream formulations. using combinations of these tyrosinase inhibitory herbs, (F1 and F2) formulations were developed. The cream F1 was superior among others based on physicochemical, biological and stability assessments. Its higher tyrosinase inhibition may be due to synergistic inhibition at the active site of the enzyme.

In addition to cream formulation, an ancient Ayurvedic skin brightening and lightening oil, Kumkumadi tailam was also prepared. Upon performing GCMS analysis, on Kumkumadi tailam, the key components were identified. It was also phytochemically standardized through atomic adsorption spectroscopy analysis it was found that heavy metal content (Pd, As, Hg and Cd) within the officially prescribed limit. Microbial load calculation and stability study support its safer uses without changes of any product quality. Efforts are being made to explore this advanced formulation for commercial exploitation through any industry. Hence new formulations and processes should be developed for formulation of more advanced formulations with herbal extracts.

Chapter – 8

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