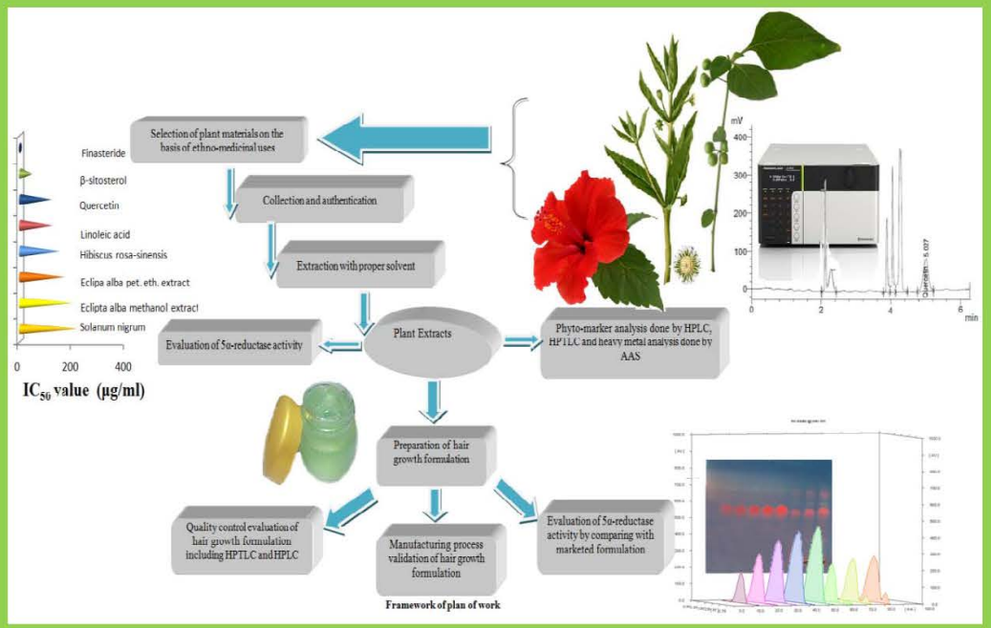
Natural products are rich sources used for treating a number of diseases. The present study deals with screening the extract of *Eclipta alba*, *Hibiscus rosa-sinensis*, *Solanum nigrum* to investigate the effect of 5α-reductase inhibitory activity. As a conclusion of this research work, it can be said that these plant materials can be used as raw materials as well as in cosmetic formulations for treatment and controlling alopecia disorder.



Development of Hair Growth Formulation with 5α – Reductase Inhibitory Activity

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ArpanChakraborty Development of Hair Growth Formulation with 5α - Reductase Inhibitory Activity 2016

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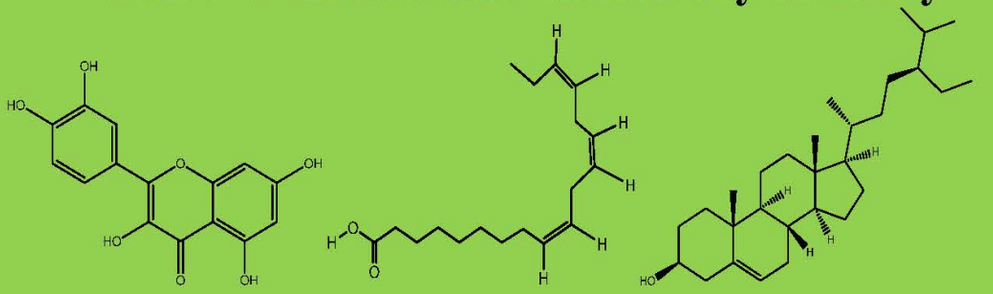

Thesis submitted in partial fulfillment of the requirements for the degree of

Master of Pharmacy

In the Faculty of Engineering & Technology
Jadavpur University
Kolkata - 700032, India

By

Arpan Chakraborty, B. Pharm.
Roll no:- M4PHA1611
Registration No:- 129109 of 14-15

DEVOLOPMENT OF HAIR GROWTH FORMULATION WITH 5α-REDUCTASE INHIBITORY ACTIVITY

Thesis submitted in partial fulfillment of the requirements for the degree of

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2016

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SCHOOL OF NATURAL PRODUCT STUDIES
DEPARTMENT OF PHARMACEUTICAL TECHNOLOGY
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CERTIFICATE

This is certify that Mr. Arpan Chakraborty (B. Pharm), bearing the Exam Roll No: M4PHA1611 and Reg. No: 129109 of 2014-2015, has successfully completed the research work on the subject entitled "DEVOLOPMENT OF HAIR GROWTH FORMULATION WITH 5α-REDUCTASE INHIBITORY ACTIVITY" under the supervision and guidance of Prof. Pulok K. Mukherjee, Director, School of Natural Product Studies, Department of Pharmaceutical Technology, Jadavpur University, Kolkata-700032. He has incorporated finding of his research work in this thesis of the same title being submitted by him, in partial fulfillment of requirements for the degree of Master of Pharmacy of Jadavpur University. He has carried out this research work independently and with proper diligence and attention to our entire satisfaction.

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DECLARATION OF ORIGINALITY AND COMPLIANCE OF ACADEMIC ETHICS

I hereby declare that this thesis contains literature survey and original research work by the undersigned candidate, as part of my "DEVOLOPMENT OF HAIR GROWTH FORMULATION WITH 5α-REDUCTASE INHIBITORY ACTIVITY" studies.

All information in this document have been obtained and presented in accordance with academic rules and ethical conduct.

I also declare that as required by these rules and conduct, I have fully cited and referenced all materials and results that are not original to this work.

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Arpan Chakraborty

Preface

This thesis was designed base on the 5α-reductase inhibition activity of selected medicinal plants. Natural products have been major sources of the various potential as well as life saving ingredients of the modern medicines. Finding a lead molecule from natural sources and to establish their 5α-reductase inhibitory potential is the main target of this work. It is well established that medicinal plants are being one of the major sources for newer drug development, available for the treatment of a variety of human diseases like prostate hyperplasia, alopecia so on.

In this thesis, three traditionally important Indian medicinal plants were screened for 5α-reductase inhibitory activity. They were standardized with respect to their major biomarkers through HPTLC and HPLC analysis. The 5α-reductase inhibitory potential of plant extracts have also been discovered by in-vitro inhibition assay model. All the plant extracts showed significant IC_{50} values which justify its pharmacological potential as 5α-reductase inhibitors. Herbal formulations were produced by using these three plants which can be useful in the management of alopecia disorder. Further, evaluation of herbal formulations was carried out by checking different critical parameters of formulations. Drug content of the formulations was determined by HPLC and HPTLC process. Finally, comparative evaluation of 5α-reductase inhibitory potential was performed by using some marketed product with prepared formulations. Standardization of formulation along with their quality control measurement may be useful in alopecia disorder.

Arpan Chakraborty

Abbreviations

Abb. form	Explanation
AAS	Atomic absorption spectroscopy
ANOVA	Analysis of variance
cm	Centimeter
T	Testosterone
5α-reductase	5 alpha reductase
DHT	Dihydrotestosterone
HPTLC	High performance thin layer chromatography
HPLC	High performances liquid chromatography
ICH	International conference for harmonization
ISM	Indian system of medicine
L-DOPA	3,4-di-hydroxyphenylalanine
ng	Nano-gram
µL	Micro liter
µg	Micro gram
µm	Micro meter
mm	Millimliter
µM	Micro mole
SD	Standard deviations
mL	Milli liter
% RSD	% Relative standard deviations
R_t	Retention time
R_f	Retardation factor
(r)	Correlation coefficient
UV	Ultraviolet
w/w	Weight/Weight
g	Gram

This thesis is dedicated to my parent, teachers and friends.

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5α-reductase and its importance

1.1. 5α-reductase

1.2. 5α-reductase Inhibitors

1.3. Application and importance of inhibitors

1.1. 5α-reductase

5α-reductase, also known as 3-oxo-5α-steroid 4-dehydrogenases involved in steroid metabolism. Over here the one of the principal role of (5α-reductase) i.e., conversion of testosterone (T) to dihydrotestosterone (DHT) and its inhibition is considered. Testosterone reduction by steroid 5α-reductase is shown in figure 1.1.

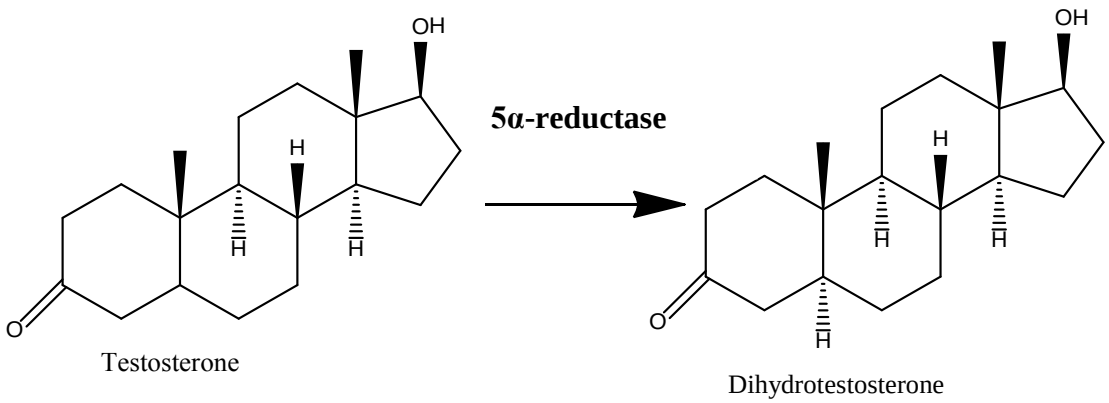


Figure 1.1. Testosterone reduction by steroid 5α-reductase.

Inhibition of DHT formation serves as a strategy for the treatment of alopecia by proposed mechanism as shown in figure 2 and prostate hyperplasia (Raynaud et al., 2002; Roh et al., 2002). There are synthetic medicines, such as e.g. finasteride, dutasteride, alfatradiol but these compounds have been reported to have their adverse effects which necessitate the interest in finding better 5α-reductase inhibitors from natural resources.

Alopecia (hair loss or baldness), is a health condition in which hair is lost from some or all areas of the body, usually from the scalp. Genetic tendencies, environmental triggers, exposure to chemicals, medicines, nutritional deficiency, extreme stress or long illness etc are the different causes of hair falls. Alopecia can be classified into several categories on the basis of hair loss pattern and causes, alopecia areata and androgenetic alopecia being the two major forms. At present a number of synthetic remedies are there for treatment of this disorder, which include corticosteroids, dithranol, tretinoin, minoxidil, zinc, systematic cortisone, immune- suppressive drugs, finasteride, azelaic acidetc., but not a single or multiple drug therapy is giving satisfactory and permanent results to the alopecia patients. Besides, a number of side effects are associated with the use of these synthetic compounds, including erythema, scaling, pruritis, dermatitis, itching, etc. (Kaushik et al., 2011).

Lower urinary tract symptoms are frequently associated with benign prostatic hyperplasia (BPH), a non-malignant enlargement of the prostate which predominantly occurs in men over 60 years of age. Since medical therapy offers an attractive

alternative to surgery, so the number of transurethral resections of the prostate has declined over the recent years and 5α-reductase inhibitors and α-blockers agents are assuming increasing importance in the treatment of lower urinary tract symptoms. However, the tolerability of this agent varies. In fact, 5α-reductase inhibitors are associated with sexual dysfunction and α_1-blockers are associated with postural hypotension; therefore, use of phytotherapy (plant extracts) to alleviate the symptoms of BPH is of growing interest (Debruyne et al., 2002). In traditional practices of numerous plants have been used to treat hair fall disorders, and prostrate disorders. Ethnopharmacological approach and bioassay-guided isolation have provided a lead in identifying potential 5α-reductase inhibitors from plant sources. This article highlights on the plants and/or their active constituents so far reported to have 5α-reductase inhibitory activity. Several methods for screening of 5α-reductase inhibitory activity from natural resources have been reported. Establishment of an in vitro screening model for steroid 5α-reductase inhibitors with the 96 well plate and microplate reader have been reported to be useful (Jh and Zy, 2013). HPLC method was established for detection of 5α-reductase inhibition (Kumar et al., 2011). GC-MS and LC-MS have also being used for identifying compounds in active constituents (Shimizu et al., 2000).

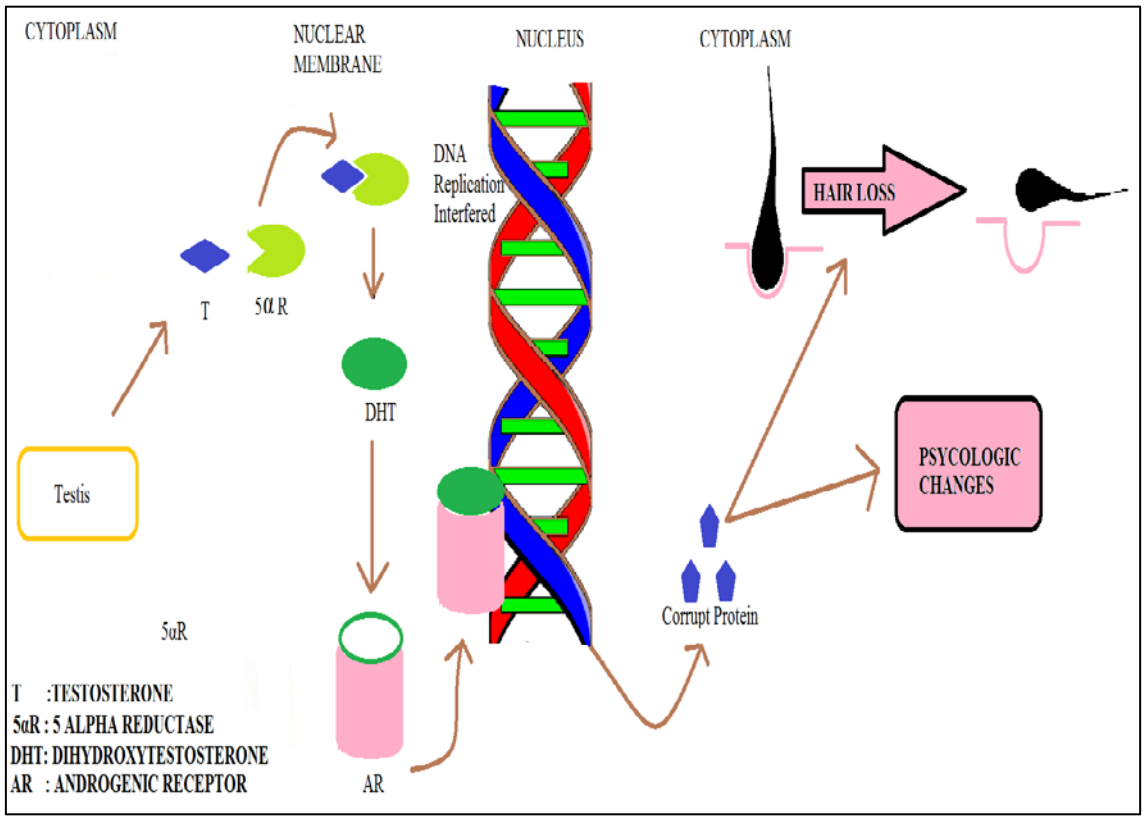


Figure 1.2. Proposed mechanism of action of DHT blockers and 5α-reductase blockers.

1.2. 5α-reductase Inhibitors

1.2.1. Synthetic 5α-reductase inhibitors

Synthetic 5α-reductase inhibitors are reported with antiandrogen effects and used primarily in the treatment of benign prostatic hyperplasia (BPH) and androgenic alopecia though there are a number of adverse reactions associated. Some of the 5α-reductase inhibitors are as follows:

1.2.1.1. *Finasteride*

Finasteride, a type 2-selective 5α-reductase inhibitor was approved in 1997 as the first oral pharmacologic therapy for the treatment of men with androgenetic alopecia. Originally it was developed for the treatment of men with benign prostatic hyperplasia at a dose of 5 mg/day (Shapiro and Kaufman 2003). Finasteride is associated with intraoperative floppy iris syndrome and cataract formation (Issa and Dagres, 2007; Wong and Mak, 2011).

1.2.1.2. *Dutasteride*

Dutasteride, an inhibitor of the type 1 and type 2 5α-reductase isoenzymes, reduces circulating DHT by more than 90% (Roehrborn et al., 2004).

1.2.1.3. *Alfatradiol*

Alfatradiol is a topical 5α-reductase used for androgenic alopecia in men and women (Artur and Helmut, 1998; Ernst et al., 2001).

1.2.2. Plants as Sources of 5α-reductase Inhibitors

A several plants have been reported to have 5α-reductase inhibitory activity and which may be relevant for the treatment of alopecia and prostate hyperplasia. A list of plants reported to have significant 5α-reductase inhibitory activity has been represented in Table 1.1.

Seneroa repen (family Arecaceae) is an exotic herb, berries of which are used increasingly in the prevention and treatment of androgenic alopecia with defines success. It inhibits both 5α-reductase type 1 and 2 (Chatterjee and Agrawala, 2003). An investigation was carried out to evaluate the hair growth promoting activity of *Abrus precatorius* leaf on Wistar albino rats. An aqueous extract of rosary pea *Abrus precatorius* **(**family Fabaceae**)** suggest that the plant is a potent hair growth promoter in preclinical screening, the effect of aqueous extract on hair growth promotion exhibited a promising activity in covering bald patch of treated (Sandhya et al., 2012). *Cuscuta reflex* Roxb. (Cuscutaceae a division of Convolvulaceae) is an extensive climber parasite. It occurs throughout the plains of India. Experiment was reported to evaluation

of 5α-reductase inhibitory activity. Petroleum ether extract of *C. Reflexa* was studied for its hair growth promoting activity in a alopecia induced albino mice and observed potential pharmacological activity on hair growth, useful in treatment of androgen-induced alopecia by inhibiting the enzyme 5α-reductase (Pandit et al., 2008). Ethanolic extract of *Carthamus tinctorius* is the most active 5α-reductase inhibitor and hair growth promoter, compared to finasteride and minoxidil, respectively. The plant extracts showed strong relationships between 5α-reductase inhibitory and hair growth promoting activity, and between 5α-reductase inhibition and the number of hair follicles (Kumar et al., 2012). *Eclipta alba* is traditionally known to potentiate hair growth. It is reported that that methanol extract of whole plant of *E. alba* may have potential as a hair growth promoter. The extract, exhibited dose dependent activity in C57BL6 mice, as assessed by studying the melanogenesis in resected skin, follicle count in the subcutis, skin thickness and surrogate markers in vehicle control and extract treated animals (Datta et al., 2009). *Cucurbita pepo* (pumpkin) seed oil (PSO) has been shown to block the actionof 5α-reductase and to have antiandrogenic effects on rats. In a randomized, placebo controlled, double-blind study in the efficacy and tolerability of PSO for treatment ofhair growth in male patients with mild to moderate androgenetic alopecia was investigated. The PSO treated group had more hair after treatment thanat baseline, compared to the placebo group (Cho et al., 2014). Most of the species of Piper is reported to have 5α-reductase inhibitory activity, *P. betel, P. cubeca, P. kadusra, P. longum, P. Methysticum* and *P. Nigrum* (Hirata et al., 2007).

Rhinacanthus nasutus, Salix rorida, Sapindus rarak, Serenoa repens, Sonneratia caseolaris, Terminali abellirica, Terminalia chebula, Thujae occidentalis, Thujopsisdo labrata var. homadae, Tinospora rumphii, Trichosanthes cucumerina, Zingiber officinale, Acacia mearnsii, Alpinia galangal, Andrographis paniculata, Averrhoa carambola, Boehmeria longispica, Boehmerian ipononivea, Boehmeria plantanifolia, Boehmeria sieboldiana, Carthamus tinctorius, Cassia siamea, Centella asiatica, Citrus hystrix, Myrica rubra, Rosa rugosa, Citrus reticulate, Lycopersicon esculentum, Artocarpus altilis are some of the plants reported to exhibit 5α-reductase inhibitory activity.

1.2.3. Phytoconstituents having 5α-reductase inhibitory activity

Work on new bioactive compounds from medicinal plants has led to the isolation and structure elucidation of a number of exciting new pharmacophores. A list of phytoconstituents having significant 5α-reductase inhibitory activity is provided in Table 1.2 and structures of these compounds are shown in figure 3. Phytoconstituents having 5α-reductase inhibitory activity include methysticin, curcumenol, dehydrocurdione, docosanol, elaidic acid, germacrone, isocurcumenol, lauric acid, lauric acid ethyl ester, linoleic acid, linolenic acid, myristic acid, oleic acid, oleic acid ethyl ester, palmitic acid, piperine, sitosterol, stearic acid, tocopherol, yangonin, zederone, α-linolenic acid,

epigallocatechingallate, epicatechin, epicatechingallate, epigallocatechin, epicatechingallate, zedoarondiol.

The active principal 5α-reductase inhibitor of the berries of S. repen has been found phytosterols (β-setosrerol, campesterol, stigmasterol) conjugated with certain esterified fatty acids. They are act through synergy to prevent androgenic alopecia by diverse mechanisms. These free fatty acids are present in the extract of ripe berries; the main components are oleic, lauric, linoleic, capraicin, caproic, caprylic, palmitic, stearic and myristic acid. It also contains fixed oil, β-carotene (Chatterjee and Agrawala, 2003). *Camellia sinensis* (Green Tea) may have an important anti-androgen effect since it contains epigallocatechins, which inhibit the 5α-reductase conversion of normal testosterone into DHT (Grant and Ramansay, 2012). Cactus flower extracts may prove beneficial in benign prostatic hyperplasia due to inhibition of 5α-reductase activity (Jonas et al, 1988). Lipid extract of the fruits of the *Roystonea regia* (royal palm fruit) is found to have on 5α-reductase inhibitory activity (Carabajal et al., 2004; Arruzazabala et al, 2006). A number of sesquiterpenes including curcumenol, dehydrocurdione, germacrone, isocurcumenol, zederone and zedoarondiol exhibiting 5α-reductase inhibitory activity are found in *Curcuma aeruginosa* belonging to family Zingiberaceae (Suphrom et al., 2012).

Lauric acid, linoleic acid, myristic acid, oleic acid, palmitic acid, stearic acid, elaidic acid and α-linolenic acid are the free fatty acids reported to exhibit 5α-reductase inhibitory activity (Raynaud et al., 2002; Shimizu et al., 2000; Hiipakka et al., 2002; Matsuda et al., 2002). *Camellia sinensis* belonging to the family of Theaceae is reported to have catechins including epigallocatechingallate, epicatechin, epicatechingallate and epigallocatechin, possessing 5α-reductase inhibitory activity (Hiipakka et al., 2002). Yangonin and (+)-methysticin, two kavalactones and piperine an alkaloid are found in *Piper nigrum* and are reported to show significant 5α-reductase inhibitory activity (Hirata et al., 2007). *Phellinus linteus* (is a white colored rotting fungi which belongs to the family hymenochaetaceae) water extracts may be an useful remedy for treating the benign prostatic hyperplasia by inhibiting 5α-reductase. They contain large amounts of β-glucan, oxyphenyl carbon and polyphenol. In addition the flavonoid which is present in it, found 5α-reductase inhibitory activity (Kim et al., 2013). *Epilobium parviflorum* the small flowered willow herb (family Onagraceae) of Central Europe, use for the treatment of prostate disorders. The aqueous extract of the plant inhibit 5α-reductase enzyme. The active fraction analaysed and purified. The active compound identified as macrocyclic tannin, oenothein B (Chodounska, H and Kasal, A., 1996). The diethyl ether extract of *Anemarrhenae Rhizoma* (rhizomes of *Anemarrhena asphodeloides*) showed testosterone 5α-reductase inhibitory activity. Two major constituents, cis-hinokiresinol and 2,6,4-trihydroxy-4-methoxybenzophenone were identified as the active principles (Matsuda et al., 2001). Acetone extract of leaves of *Artocarpus altilis* (family Moraceae) is found to have potent 5α-reductase inhibitory activity (Shimizu et al., 2000).

Table 1.1. List of natural sources exhibiting 5α-reductase inhibitory activity

Plant	Family	Parts used	Type of extract	Activity (% inhibition) (concentration)	Reference
Acacia concinna	Leguminosae	Plant	Ethanol	12.78 ± 0.87(FEA)	Kumar et al., 2012
Acacia mangium	Leguminosae	Bark	70 % acetone	80(50 µg/mL)	Liu et al., 2008
Acacia mearnsii	Leguminosae	Bark	70 % acetone	81(50 µg/mL)	Liu et al., 2008
Alpinia galanga	Zingiberaceae	Plant	Ethanol	18.54 ± 0.85(FEA)	Kumar et al., 2012
Andrographis paniculata	Acanthaceae	Plant	Ethanol	13.01 ± 0.81(FEA)	Kumar et al., 2012
Angelica sinensis	Umbelliferae	-	50% ethanol	2.8	Matsuda et al., 2001
Averrhoa carambola	Oxalidaceae	Plant	Ethanol	13.12 ± 0.87(FEA)	Kumar et al., 2012
Boehmeria longispica	Urticaceae	Leaves and aerial parts	Acetone	77.2 (480 µg/mL)	Shimizu et al., 2000
Boehmeria nipononivea	Urticaceae	Leaves and aerial parts	Acetone	70.0 (480 µg/mL)	Shimizu et al., 2000
Boehmeria plantanifolia	Urticaceae	Leaves and aerial parts	Acetone	89.1 (480 µg/mL)	Shimizu et al., 2000
Boehmeria sieboldiana	Urticaceae	Leaves and aerial parts	Acetone	81.5 (480 µg/mL)	Shimizu et al., 2000
Carthamus tinctorius	Asteraceae	Plant	Ethanol	24.30 ± 1.64(FEA)	Kumar et al., 2012
Carthamus tinctorius	Compositae	-	50% ethanol	9.9	Matsuda et al., 2001
Cassia siamea	Ceasalpiniaceae	Plant	Ethanol	12.87 ± 1.12(FEA)	Kumar et al., 2012
Centella asiatica	Apiaceae	Leaf	Ethanol	13.83 ± 1.03(FEA)	Kumar et al., 2012
Cinnamomum cassia	Lauraceae	-	50% ethanol	38.4	Matsuda et al., 2001
Citrus hystrix	Rutaceae	Plant	Ethanol	13.72± 0.79(FEA)	Kumar et al., 2012
Citrus reticulata	Rutaceae	Peel	Ethanol	5.56 ± 1.12(FEA)	Kumar et al., 2011
Clitorea ternatea	Fabaceae	Plant	Ethanol	15.39 ± 0.67(FEA)	Kumar et al., 2012
Coicis semen	Gramineae	-	-	47.6 (10 µg/mL) 73.3	Park et al., 2004
Corni fructus	Cornaceae	-	-	(10 µg/mL)	Park et al., 2004
Cryptomeria japonica	Taxodiaceae	Bark	70 % acetone	92(50 µg/mL)	Liu et al., 2008
Curcuma aeruginosa	Zingiberaceae	Rhizome	Hexane	72.8 ±2.6 (0.1 mg/mL)	Suphrom et al.,2012
Cuscuta reflexa	Cuscutaceae	Plant	Petroleum ether	50 (1.78 µg/mL)	Pandit et al, 2008

Cymbopogon citratus	Poaceae	Plant	Ethanol	18.55 ± 0.78(FEA)	Kumar et al., 2012
Dioscorea japonica	*Dioscoreaceae*	Leaves and aerial parts	Acetone	65.0 (480 µg/mL)	Shimizu et al., 2000
Epimedium grandiflorum	Berberidaceae	Whole plant	50% ethanolic	49.6(200 µg/mL)	Murata et al., 2012
Evodia bodinieri	Rutaceae	-	50% ethanol	25.9	Matsuda et al., 2001
Ganoderma lucidum	Ganodermataceae	Fruiting body	Methanol	50 (93 µg/mL)	Fujita et al., 2005
Garcinia mangostana	Guttiferae	Peel	Ethanol	11.62 ± 1.18(FEA)	Kumar et al., 2011
Glycyrrhizae radix	Fabaceae	-	-	93.1 (10 µg/mL)	Park et al., 2004
Houttuynia cordata	Saururaceae	Whole plant	Ethanol	15.37 ± 1.50(FEA)	Kumar et al., 2011
Ipomoea aquatica	Convovulaceae	Plant	Ethanol	13.16 ± 0.43(FEA)	Kumar et al., 2012
Kaempferia parviflora	Zingiberaceae	Plant	Ethanol	83.3(0.32 mg/mL)	Kim and Hun 2013
Kochia scoparia	Chenopodiaceae	-	50% ethanol	5.1	Matsuda et al., 2001
Larix leptolepis	Pinaceae	Bark	70 % acetone	82(50 µg/mL)	Liu et al., 2008
Lawsonia inermis	Lythraceae	Plant	Ethanol	12.58 ± 0.45(FEA)	Kumar et al., 2012
Lygodium japonicum	Schizaeaceae	Spore	70% ethanol	7.8 (50 µg/5 µL)	Matsuda et al., 2002
Mucuna birdwoodiana	Leguminosae	-	50% ethanol	58.4	Matsuda et al., 2001
Myrica rubra	Myricaceae	-	50% ethanol	80.7	Matsuda et al., 2001
Ocimum basilicum	Lamiaceae	Whole plant	Ethanol	17.59 ± 1.00(FEA)	Kumar et al., 2011
Oryza sativa	Poaceae	Grain	Ethanol	16.72 ± 0.95(FEA)	Kumar et al., 2011
Paeonia moutan	Paeoniaceae	-	50% ethanol	52.7	Matsuda et al., 2001
Panax japonicum	Araliaceae	-	50% ethanol	31.9	Matsuda et al., 2001
Pellionia minima	Urticaceae	Leaves and aerial parts	Acetone	40.9 (480 µg/mL)	Shimizu et al., 2000
Pellionia scabra	Urticaceae	Leaves and aerial parts	Acetone	30.8 (480 µg/mL)	Shimizu et al., 2000
Phyllanthus emblica	Euphorbiaceae	Plant	Ethanol	18.99 ± 0.40(FEA)	Kumar et al., 2012
Piper betel	Piperaceae	Whole plant	50% ethanol	24.3(1 mg/mL)	Hirata et al., 2007
Piper cubeca	Piperaceae	Fruit	50% ethanol	24.3(1 mg/mL)	Hirata et al., 2007
Piper kadusra	Piperaceae	Leaf	50% ethanol	16.7(1 mg/mL)	Hirata et al., 2007
Piper kadusra	Piperaceae	Stem	50% ethanol	15.9(1 mg/mL)	Hirata et al., 2007

Piper kadusra	Piperaceae	Root	50% ethanol	25.1(1 mg/mL)	Hirata et al., 2007
Piper kadusra	Piperaceae	Rhizome	50% ethanol	26.7(1 mg/mL)	Hirata et al., 2007
Piper longum	Piperaceae	Whole plant	50% ethanol	10.2(1 mg/mL)	Hirata et al., 2007
Piper methysticum	Piperaceae	Leaf	50% ethanol	14.0(1 mg/mL)	Hirata et al., 2007
Piper methysticum	Piperaceae	Stem	50% ethanol	14.8(1 mg/mL)	Hirata et al., 2007
Piper methysticum	Piperaceae	Rhizome	50% ethanol	40.7(1 mg/Ml)	Hirata et al., 2007
Piper nigrum	Piperaceae	Leaf	50% ethanol	39.3 (1 mg/mL)	Hirata et al., 2007
Piper nigrum	Piperaceae	Stem	50% ethanol	27.0(1 mg/mL)	Hirata et al., 2007
Piper nigrum	Piperaceae	fruit	50% ethanol	39.9(1 mg/mL)	Hirata et al., 2007
Piper nigrum	Piperaceae	Fruit	Ethanol	11.18 ± 0.81(FEA)	Kumar et al., 2011
Polygonum bistorta	Polygonaceae	-	50% ethanol	58.2	Matsuda et al., 2001
Polygonum multiflorum	Polygonaceae	Root tuber	50% ethanolic	15.3(200 µg/mL)	Murata et al., 2012
Polygonum multiflorum	Polygonaceae	Radix	50% ethanol	80.7 (500 µg/mL)	Cho et al., 2010
Polygonum multiflorum	Polygonaceae	-	50% ethanol	57.4	Matsuda et al., 2001
Prunus persica	Rosaceae	-	50% ethanol	2.1	Matsuda et al., 2001
Psoralea corylifolia	Leguminosae	-	50% ethanol	57.1	Matsuda et al., 2001
Pueraria thomsonii	Fabaceae	Flower	50% ethanolic	31.1(200 µg/mL)	Murata et al., 2012
Rhinacanthus nasutus	Acanthaceae	Plant	Ethanol	10.69 ± 0.96(FEA)	Kumar et al., 2012
Rosa rugosa	Rosaceae	-	50% ethanol	79.0	Matsuda et al., 2001
Rubus chingii	Rosaceae	-	50% ethanol	14.4	Matsuda et al., 2001

Salix rorida	Salicaceae	Bark	70 % acetone	87(50 µg/mL)	Liu et al., 2008
Salvia miltiorrhiza	Labiatae	-	50% ethanol	22.7	Matsuda et al., 2001
Sapindus rarak	Sapindaceae	Plant	Ethanol	12.81 ± 0.84(FEA)	Kumar et al., 2012
Scutellaria barbata	Labiatae	-	50% ethanol	20.2	Matsuda et al., 2001
Serenoa repens	Arecaceae	Dried ripe fruit	Hexane	50.0 (10 µg/mL)	Raynaud et al, 2002
Serenoa repens	Arecaceae	Leaf	Ethanol	50 (2.88 µg/mL)	Pais, 2010
Sonneratia caseolaris	Sonneratiaceae	Bark	70 % acetone	56(50 µg/mL)	Liu et al., 2008
Terminalia bellirica	Combretaceae	Fruit	Ethanol	11.58 ± 0.84(FEA)	Kumar et al., 2011
Terminalia chebula	Combretaceae	Fruit	Ethanol	12.74 ± 0.84(FEA)	Kumar et al., 2011
Thujae occidentalis	Cupressaceae	Fruits	95% ethanol	50(2.6 µg/mL)	Park et al., 2003
Thujopsis dolabrata	Cupressaceae	Bark	70 % acetone	96(50 µg/mL)	Liu et al., 2008
Tinospora rumphii	Menispermaceae	Plant	Ethanol	13.33 ± 0.30(FEA)	Kumar et al., 2012
Trichosanthes cucumerina	Curcubitaceae	Plant	Ethanol	13.37 ± 0.84(FEA)	Kumar et al., 2012
Zingiber officinale	Zingiberaceae	Plant	Ethanol	18.32 ± 0.82(FEA)	Matsuda et al., 2001

Finasteride equivalent 5α-reductase inhibition ability: FEA value (mg finasteride / 1 g crude extract).

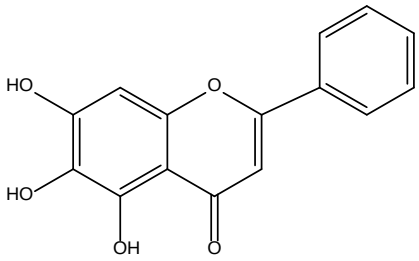
Table 1.2. Phytoconstituents having 5α-reductase inhibitory activity

Name	Type	Enzyme isoform	Sources	Plant family	% inhibition (conc.)	Reference
(+)-Methysticin (2)	Kavalactone	Not specified	*Piper nigrum*	Piperaceae	50 (>10 µg/ mL)	Hirata et al., 2007
Baicalein (1)	Flavone	5α-reductase -I	-	-	50(>29 µM)	Hiipakka et al., 2002
Biochanin A (4)	Isoflavone	5α-reductase -II	-	-	50(>17 µM)	Hiipakka et al., 2002
Curcumenol (3)	Sesquiterpenes	Not specified	*Curcuma aeruginosa*	Zingiberaceae	17.4 ±4.5 (0.1g/ mL)	Suphrom et al., 2012
Daidzein (5)	Isoflavone	5α-reductase -II	-	-	50(>29 µM)	Hiipakka et al., 2002
Dehydrocurdione (6)	Sesquiterpenes	Not specified	*Curcuma aeruginosa*	Zingiberaceae	45.2–48.1 (0.1g/mL)	Suphrom et al., 2012
Docosanol (7)	Fatty alcohol	5α-reductase -I	*Serenoa repens*	Arecaceae	50 (>100 µg/ mL)	Raynaud et al., 2002
Docosanol	Fatty alcohol	5α-reductase -II	*Serenoa repens*	Arecaceae	50(>100 µg/ mL)	Raynaud et al., 2002
Elaidic acid (8)	Free fatty acid	Not specified	*Boehmeria nipononivea*	Urticaceae	35.5 (31.3µg/ mL)	Shimizu et al., 2000
Epicatechin	Catechin	5α-reductase -I	*Camellia sinensis*	Theaceae	50(>100 µM)	Hiipakka et al., 2002
Epicatechin	Catechin	5α-reductase -II	*Camellia sinensis*	Theaceae	50(>100 µM)	Hiipakka et al., 2002
Epicatechingallate (9)	Catechin	5α-reductase -I	*Camellia sinensis*	Theaceae	50(>11 µM)	Hiipakka et al., 2002
Epicatechingallate	Catechin	5α-reductase -II	*Camellia sinensis*	Theaceae	50(>69 µM)	Hiipakka et al., 2002
Epigallocatechin (10)	Catechin	5α-reductase -I	*Camellia sinensis*	Theaceae	50(>100 µM)	Hiipakka et al., 2002
Epigallocatechin	Catechin	5α-reductase -II	*Camellia sinensis*	Theaceae	50(>100 µM)	Hiipakka et al., 2002
Epigallocatechingallate (11)	Catechin	5α-reductase -I	*Camellia sinensis*	Theaceae	50(>15 µM)	Hiipakka et al., 2002
Epigallocatechingallate	Catechin	5α-reductase -II	*Camellia sinensis*	Theaceae	50(>74 µM)	Hiipakka et al., 2002
Fisetin (12)	Flavanol	5α-reductase -I	-	-	50(>57 µM)	Hiipakka et al., 2002
Genistein (13)	Isoflavone	5α-reductase -II	-	-	50(>23 µM)	Hiipakka et al., 2002
Germacrone (14)	Sesquiterpenes	Not specified	*Curcuma aeruginosa*	Zingiberaceae	65.7 ±4.7 (0.1g/ mL)	Suphrom et al., 2012
Isocurcumenol (15)	Sesquiterpenes	Not specified	*Curcuma aeruginosa*	Zingiberaceae	4.7 ±8.5 (0.1g/ mL)	Suphrom et al., 2012
Kaempferol (16)	Flavanol	5α-reductase -II	-	-	50(>12 µM)	Hiipakka et al., 2002
Lauric acid (17)	Free fatty acid	5α-reductase -I	*Serenoa repens*	Arecaceae	50 (16.7±2.9 µg/mL)	Raynaud et al., 2002
Lauric acid	Free fatty acid	5α-reductase -II	*Serenoa repens*	Arecaceae	50(18.6±9.3 µg/ mL)	Raynaud et al., 2002
Lauric acid ethyl ester (18)	Esterified fatty acid	5α-reductase -I	*Serenoa repens*	Arecaceae	50 (>100 µg/ mL)	Raynaud et al., 2002

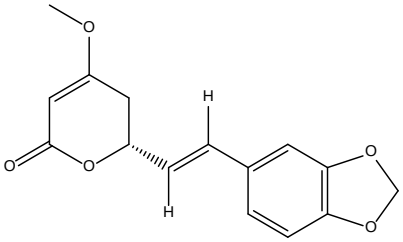
Lauric acid ethyl ester	Esterified fatty acid	5α-reductase -II	*Serenoa repens*	Arecaceae	50(>100 µg/ mL)	Raynaud et al., 2002
Linoleic acid	Free fatty acid	Not specified	*Lygodium japonicum*	Lygodiaceae	50(0.37±0.01µg/mM)	Matsuda et al., 2002
Linoleic acid	Free fatty acid	5α-reductase -I	*Serenoa repens*	Arecaceae	50(9 13±3 µg/ mL)	Raynaud et al., 2002
Linoleic acid	Free fatty acid	5α-reductase -II	*Serenoa repens*	Arecaceae	50(35±21 µg/ mL)	Raynaud et al., 2002
Linolenic acid (32)	Free fatty acid	Not specified	*Boehmeria nipononivea*	Urticaceae	70.7 (31.3µg/ mL)	Shimizu et al., 2000
Myricetin (19)	Flavonoid	5α-reductase -I	-	-	50(>23 µM)	Hiipakka et al., 2002
Myristic acid (31)	Free fatty acid	5α-reductase -I	*Serenoa repens*	Arecaceae	50 (ND)	Raynaud et al., 2002
Myristic acid	Free fatty acid	5α-reductase -II	*Serenoa repens*	Arecaceae	50(4.3±2.3 µg/ mL)	Raynaud et al., 2002
Oleic acid (20)	Free fatty acid	Not specified	*Lygodium japonicum*	Lygodiaceae	50(0.446±.02µg/ mL)	Matsuda et al., 2002
Oleic acid	Free fatty acid	Not specified	*Boehmeria nipononivea*	Urticaceae	50.6 (31.3µg/ mL)	Shimizu et al., 2000
Oleic acid	Free fatty acid	5α-reductase -I	*Serenoa repens*	Arecaceae	50(4±2 µg/ mL)	Raynaud et al., 2002
Oleic acid	Free fatty acid	5α-reductase -II	*Serenoa repens*	Arecaceae	50(>100 µg/ mL)	Raynaud et al., 2002
Oleic acid ethyl ester (21)	Esterified fatty acid	5α-reductase -I	*Serenoa repens*	Arecaceae	50 (>100 µg/ mL)	Raynaud et al., 2002
Oleic acid ethyl ester	Esterified fatty acid	5α-reductase -II	*Serenoa repens*	Arecaceae	50(>100 µg/ mL)	Raynaud et al., 2002
Palmitic acid (23)	Free fatty acid	Not specified	*Lygodium japonicum*	Lygodiaceae	50(1.35±03 µg/ mM)	Matsuda et al., 2002
Palmitic acid	Free fatty acid	Not specified	*Boehmeria nipononivea*	Urticaceae	23.6 (31.3µg/ mL)	Shimizu et al., 2000
Palmitic acid	Free fatty acid	5α-reductase -I	*Serenoa repens*	Arecaceae	50 (>100 µg/ mL)	Raynaud et al., 2002
Palmitic acid	Free fatty acid	5α-reductase -II	*Serenoa repens*	Arecaceae	50(>100 µg/ mL)	Raynaud et al., 2002
Piperine (24)	Alkaloid	Not specified	*Piper nigrum*	Piperaceae	50 (0.48 µg/ mL)	Hirata et al., 2007
Quercetin (25)	Flavonol	5α-reductase -I	-	-	50(>23 µM)	Hiipakka et al., 2002
Sitosterol (26)	Miscellaneous	5α-reductase -I	*Serenoa repens*	Arecaceae	50 (>100 µg/ mL)	Raynaud et al., 2002
Sitosterol	Phytosterols	5α-reductase -II	*Serenoa repens*	Arecaceae	50(>100 µg/ mL)	Raynaud et al., 2002
Stearic acid (27)	Free fatty acid	Not specified	*Boehmeria nipononivea*	Urticaceae	32.4 (31.3µg/ mL)	Shimizu et al., 2000
Stearic acid	Free fatty acid	5α-reductase -I	*Serenoa repens*	Arecaceae	50(>100 µg/ mL)	Raynaud et al., 2002
Stearic acid	Free fatty acid	5α-reductase -II	*Serenoa repens*	Arecaceae	50(>100 µg/ mL)	Raynaud et al., 2002
Tocopherol (28)	Miscellaneous	5α-reductase -I	*Serenoa repens*	Arecaceae	50 (>100 µg/ mL)	Raynaud et al., 2002
Tocopherol	Vitamin	5α-reductase -II	*Serenoa repens*	Arecaceae	50(>100 µg/ mL)	Raynaud et al., 2002

Yangonin (29)	Kavalactone	Not specified	*Piper nigrum*	Piperaceae	50 (>10 µg/ mL)	Hirata et al., 2007
Zederone (33)	Sesquiterpenes	Not specified	*Curcuma aeruginosa*	Zingiberaceae	46.0 ±12.9 (0.1g/mL)	Suphrom et al., 2012
Zedoarondiol (30)	Sesquiterpenes	Not specified	*Curcuma aeruginosa*	Zingiberaceae	16.4 ±5.1 (0.5g/ mL)	Suphrom et al., 2012
α-linolenic acid	Free fatty acid	Not specified	*Boehmeria nipononivea*	Urticaceae	88.0 (31.3µg/ mL)	Shimizu et al., 2000
Myricanone	Miscellaneous	Not specified	*Myricarubra*	Myricaceae	50(3.8mM)	Matsuda et al., 2001
Myricanol	Miscellaneous	Not specified	*Myricarubra*	Myricaceae	50(3.7mM)	Matsuda et al., 2001
Myricetin	Kavalactone	Not specified	*Myricarubra*	Myricaceae	50(8mM)	Matsuda et al., 2001
(–)-cubebin	Flavonoid	Not specified	*Piper nigram*	Piperaceae	50(0.44mM)	Hirata et al., 2007
Compound 2	Lignan	Not specified	*Piper nigram*	Piperaceae	50(1.03mM)	Hirata et al., 2007

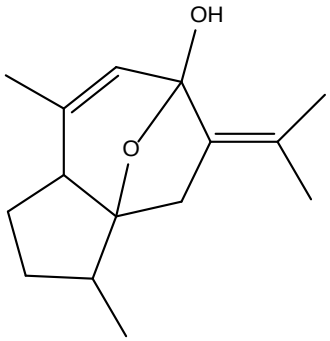
ND:Not Done. Compound2:(-)-3,4-dimethoxy-3,4-desmethylenedioxycubebin.



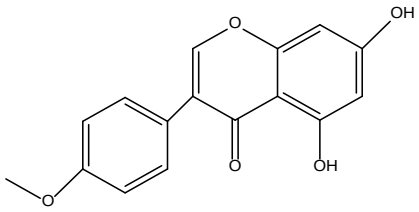
Baicalein (1)



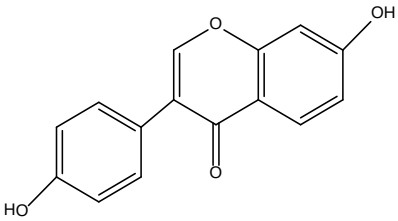
(+)-Methysticin (2)



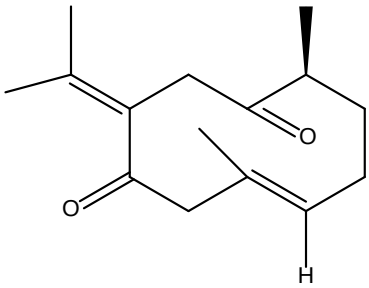
Curcumenol (3)



Biochanin A (4)



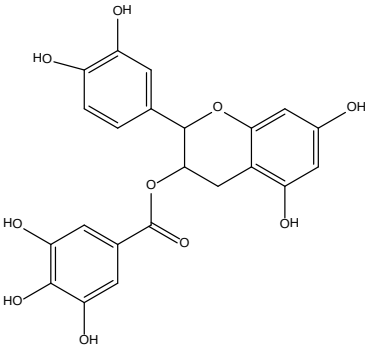
Daidzein (5)



Dehydrocurdione (6)

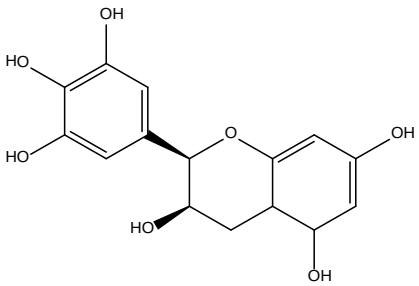
Docosanol (7)

Elaidic acid (8)

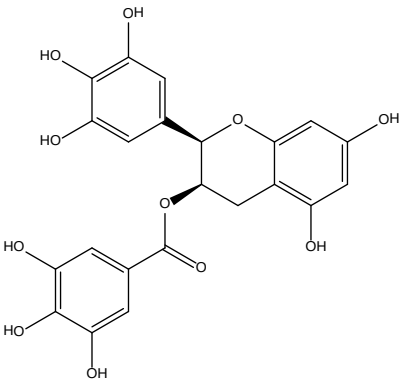


Epicatechingallate (9)

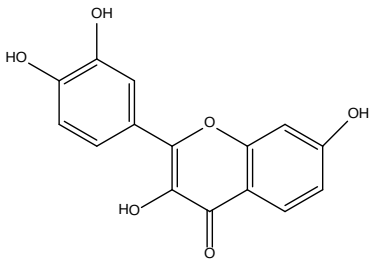
Figure 1.3. Structures of some 5α-reductase inhibitors obtained from plants.



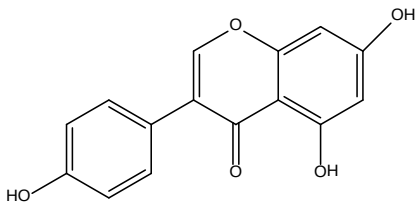
Epigallocatechin (10)



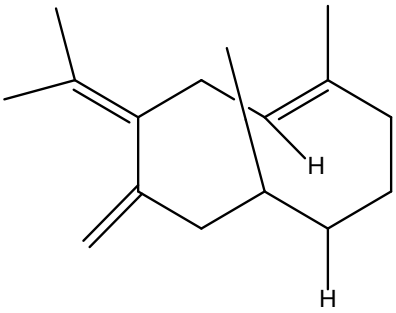
Epigallocatechingallate (11)



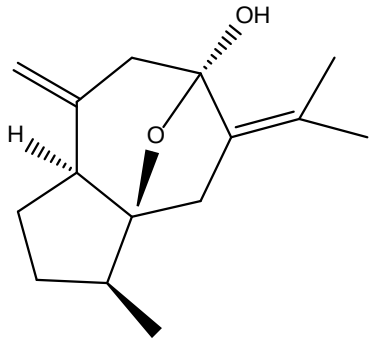
Fisetin (12)



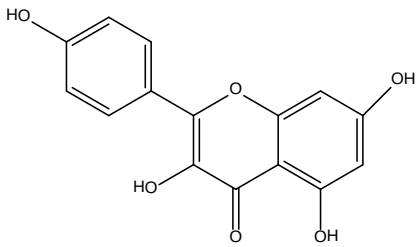
Genistein (13)



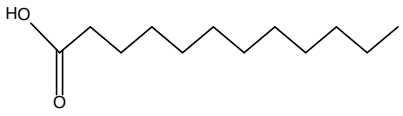
Germacrone (14)



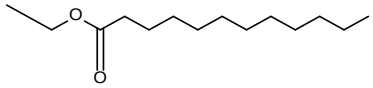
Isocurcumenol (15)



Kaempferol (16)

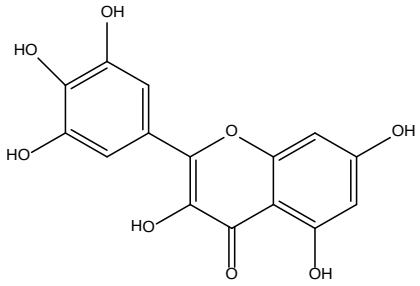


Lauric acid (17)

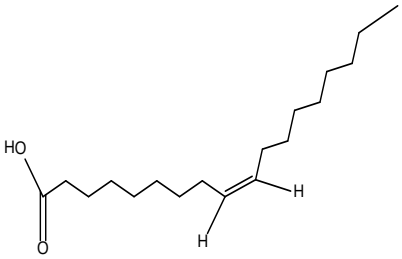


Lauric acid ethyl ester (18)

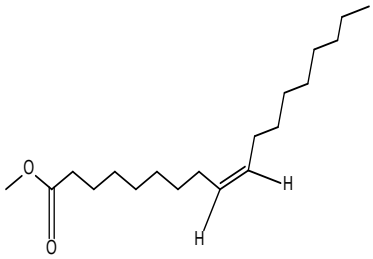
Figure 1.3. Structures of some 5α-reductase inhibitors obtained from plants.



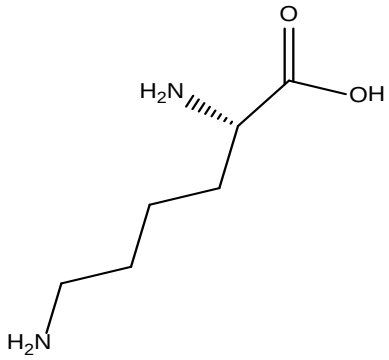
Myricetin (19)



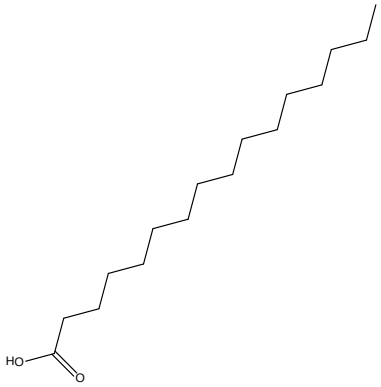
Oleic acid (20)



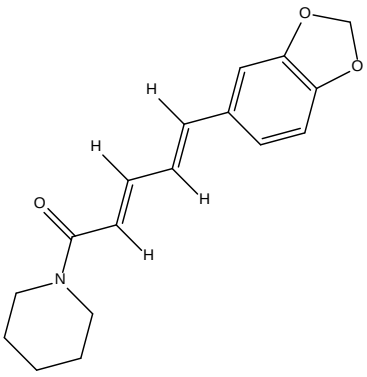
Oleic acid ethyl ester (21)



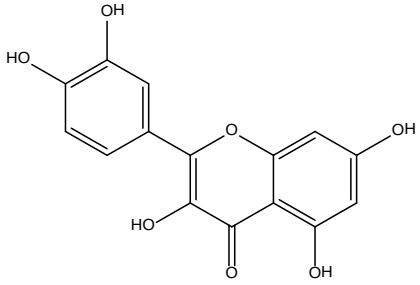
L-lysine (22)



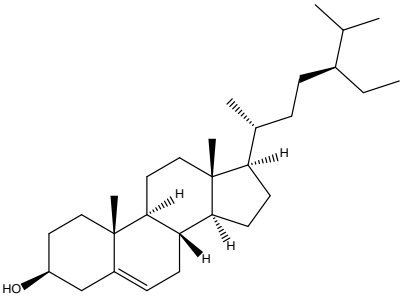
Palmitic acid (23)



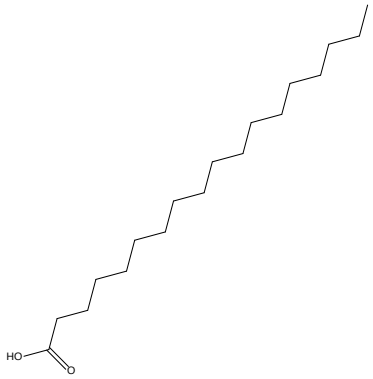
Piperine (24)



Quercetin (25)

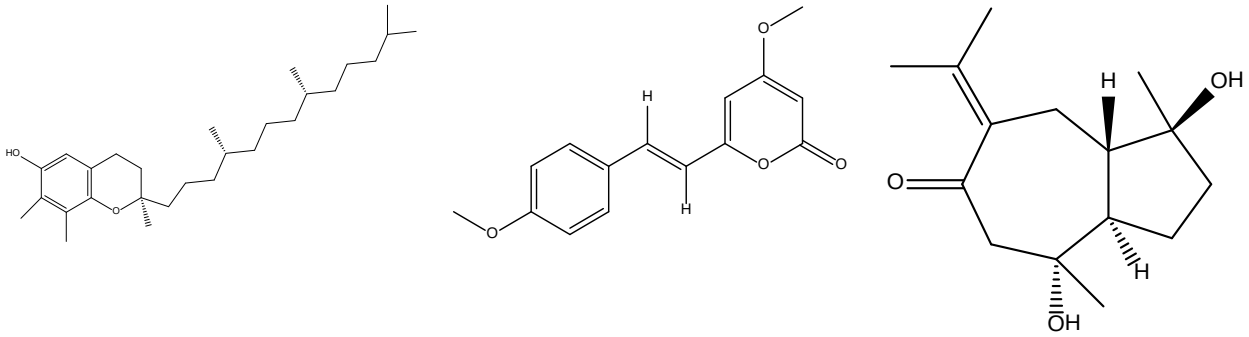


Sitosterol (26)

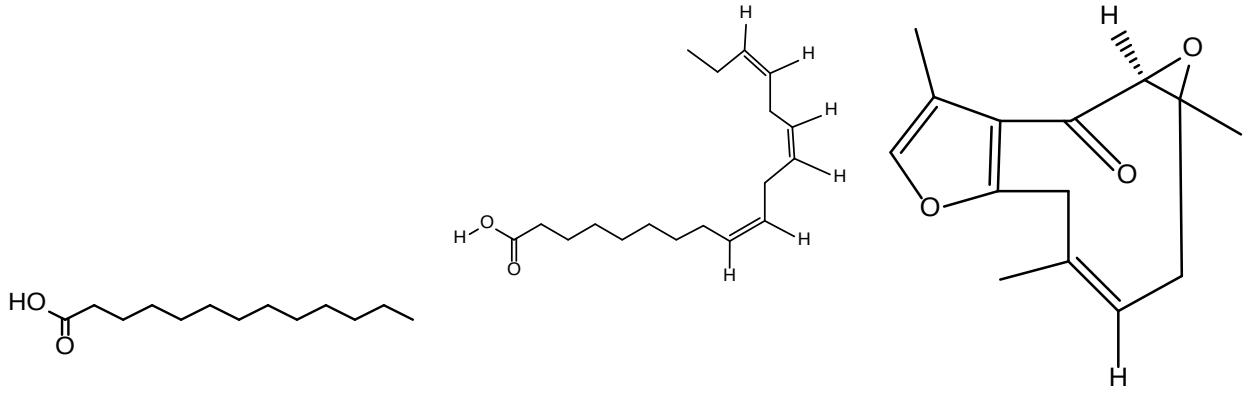


Stearic acid (27)

Figure 1.3. Structures of some 5α-reductase inhibitors obtained from plants.



α-Tocopherol (28) Yangonin (29) Zedoarondiol (30)



Myristic acid (31) α-Linolenic acid (32) Zedoarone (33)

Figure 1.3. Structures of some 5α-reductase inhibitors obtained from plants.

1.3. Application and importance of inhibitors

5α-reductase inhibitor is clinically used in the treatment of conditions that are exacerbated by DHT.

- Mild-to-moderate benign prostatic hyperplasia and lower urinary tract symptoms
- Androgenic alopecia in both men and women

They have also been explored in the treatment and prevention of prostate cancer. So, the natural inhibitors are very much useful for treating diseases. So it is very important to make pharmaceutical formulation by using natural inhibitors for more acceptability.

1.3.1. *Cosmetic and medicinal fields*

The therapeutic uses of 5α-reductase inhibitor are prevailing now a day in the cosmetic and medicinal industries due to their preventive effect on alopecia disorder. 5α-reductase inhibitors reduce the dihydrotestosterone (DHT) biosynthesis, thus helping to reduce alopecia. Till date a large number of 5α-reductase inhibitors have been screened from natural sources. Among them,

saw palmetto barriers lipophilic extract is most widely used in treatment for baldness. Oral formulations of saw palmetto barriers and β-*sitosterol* were administered *in vivo* to patients twice daily for around 4.6 months and were assessed for improvements in hair growth; six out of ten patients showed a significant positive response (Prager et al., 2002). *Asiasari radix, Aloe barbadensis, Ginkgo biloba* leaf extract, Proanthocyanidins, Citrus *Aurantium Bergamia* (Bergamot) Fruit Oil and Leaf Oil, Chinese herb extract "*Dabao*", *Ginseng radix* in the form of a 70% extract of red ginseng (steamed and dried roots of *Panax ginseng*), *Lawsonia alba,Hibiscus rosa-sinensis, Eclipta alba, Hydrangea macrophylla, Illicium anisatum* extracts use in hair growth products in cosmetic industry. *Salvia officinalis, Rosmarinus officinalis, Thymus vulgaris, Allium sativum, Juglans regia,* the leaves of *Melaleuca alternifolia,* anti-inflammatory, antifungal, and exfoliative, anti-dandraff and to treat alopecia. Some of the over-the-counter botanical (natural) hair promoters incorporate minoxidil as an active ingredient. An internet search reveals that many of these products are available, such as NioxinR Intensive Therapy Follicle Booster, and Scalp MedR, Vitadil-5A and Vitadil-2A. The majority of plant DHT inhibitors contain saw palmetto, liposterolic extract of Serenoa repens and beta-sitosterol, azelaic acid, zinc, B6, linoleic acid, and polyphenols. Some of these available internet products are AvacorR, ProvillusR and RivivogenR (Blume-Peytavi et al., 2008).

Chapter - 2

Plants used for 5α-reductase inhibitory activity

2.1. *Eclipta alba* - plant profile

2.2. *Hibiscus rosa-sinensis* - plant profile

2.3. *Solanum nigrum* - plant profile

2.1. *Eclipta alba* - plant profile

2.1.1. *Botanical taxonomy*

Scientific classification			Vernacular names		
Kingdom	:	Plantae	English	:	Bhringaraj
Division	:	Magnoliophyta	Sanskrit	:	Bhringaraj
Class	:	Magnoliopsida	Hindi	:	Bhangara, Bhangaraiya
Order	:	Asterales	Bengali	:	Kesuriya, Kesari
Family	:	Asteraceae	Tamil	:	Karisalai
Genus	:	Eclipta L.	Marathi	:	Maka
Species	:	Eclipta prostrata	Telugu	:	Guntagalagara

2.1.2. *Plant description*

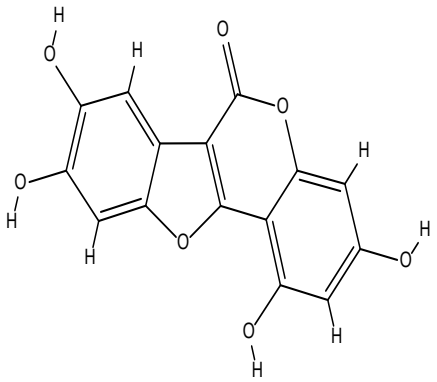
E. alba is a small branched perennial herbaceous plant along with a history of traditional medicines uses in various countries especially in tropical and subtropical regions of the world. Throughout India, it commonly grows as a natural weed, in Himalayas arises to 1800 m, commonly found in regions of upper northern plains, in grazing lands, Chota Nagpur roadsides and in territories of Orissa and Bihar, Punjab, Western India, South India. It is perpendicular or prone, many are branched, perennial, almost hairy, rooting at the buds, opposite leaves, stalkless and simple leaves. It is commonly found in India, China, Taiwan, Philippines, Japan and Indonesia. Leaves of this plant are 2.5-7.5 cm long. On a long stalk, it has small white daisy like flowers and short, prostrate or circular, brown stem. It has been reported that *E. alba* grows in India, Bengal, Sri Lanka, Myanmar, Malaysia, Japan, China, Korea, Hong Kong and Pakistan (Mahmood et al., 2013).

Figure 2.1A. *Eclipta alba* plant.

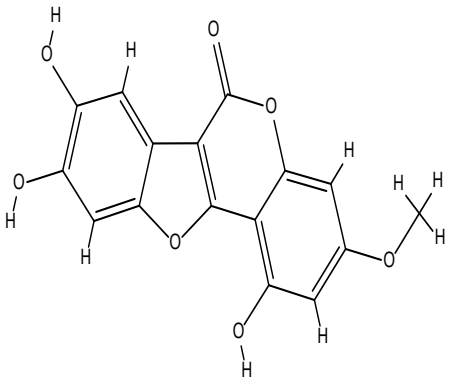
Figure 2.1B. *Eclipta alba* flower.

2.1.3. *Chemical constituents of Eclipta alba*

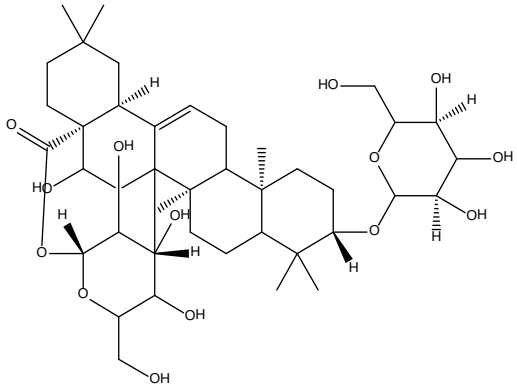
Eclipta alba contains the triterpenoid saponins eclalbasaponin I (3), eclalbasaponin II (4), eclalbasaponins III–VI, XI and XII, eclipta saponin C and D, eclalbatin (6), the flavonoids apigenin and luteolin7-glucoside (5), as well as the coumestans wedelolactone (1), demethylwedelolactone (2), isodemethylwedelolactone and strychnolactone (8). Alkaloids include 25-β-hydroxyverazine and ecliptalbine, as well as small amounts of nicotine (0.078%) in the aerial portions. Other constituents are α-formylterthienyl, α-terthienyl, polyacetylenicthiophenes, dithienylacetylineesters I, II, and III, β-sitosterol (7), stigmasterol, daucosterol, stigmasterol–3-*O*-glucoside, nonacosanol, stearic acid, lacceroic acid, 3,4-dihydroxy benzoic acid, α-amyrin, ursolic acid and oleanolic acid (Caldecott 2006).



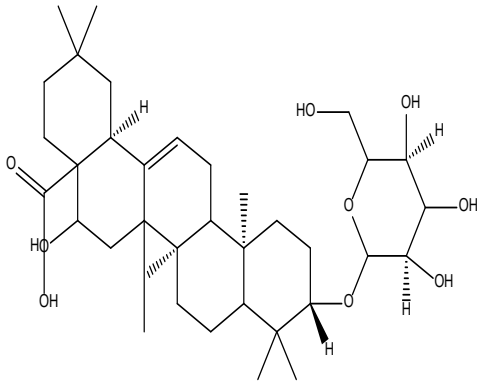
Demethylwedelolactone (1)



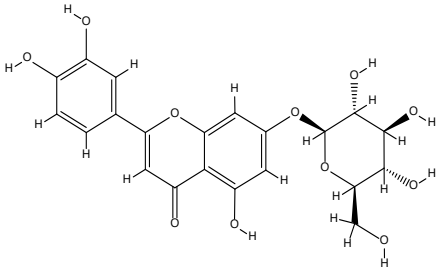
Wedelolactone (2)



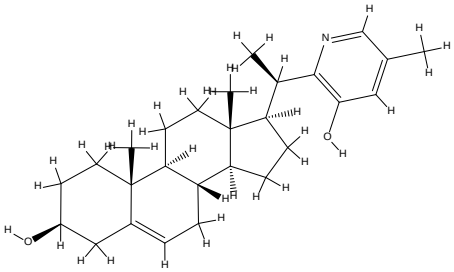
Eclalbosaponin I (3)



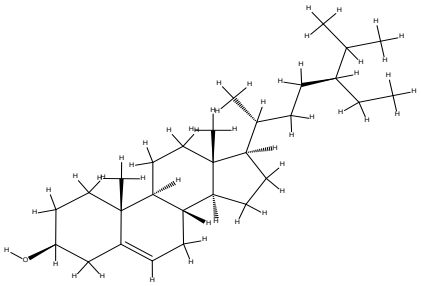
Eclalbasaponin II (4)



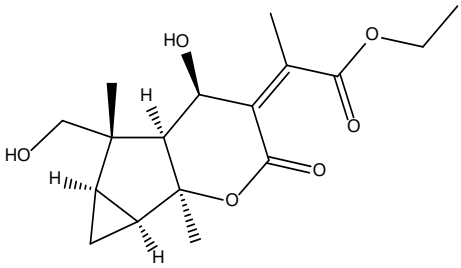
Luteolin 7-glucoside (5)



Ecliptalbine (6)



β-sitosterol (7)



Strychnolactone (8)

Figure 2.1C. Phytoconstituents of *Eclipta alba.*

2.1.4. *Medicinal uses*

Bhringaraj oil is a famous hair tonic for maintaining dark hair and reversing baldness. It is often translated as 'king of the hair'. It is decocted in coconut oil and as this is a 'cooling' oil it is used externally for 'hot' and inflammatory head problems such as headaches, sinusitis and ear infections. The herb also benefits heat problems (Caldecott 2006). *Eclipta alba* is also used as medicine alterative, anti-inflammatory, hemostatic, antipyretic, vulnerary, tonic, cholagogue, hepatoprotective. Ayurveda action of *Eclipta alba* given below in the table 2.1.

Table 2.1. *Ayurveda action of Eclipta alba*

Rasayana Rejuvenative	***Raktastambhana*** Stops bleeding
Medhya Promotes the intellect	***Amanasaka*** Destroys *ama*
Kesya Benefits hair	***Visaghna*** Destroys poisons in the body
Dantya Benefits the teeth	***Caksusya*** Benefits the eyes
Tvacya Benefits itching	***Krmighna*** Destroys worms
Kusthahaghna Destroys skin diseases	***Yakrdottejaka*** Benefits the liver
Raktasodhana Purifies the blood	***Pandughna*** Reduces anaemia

Eclipta alba use with different plants in combinations for treatment of various diseases

* Amalaki*,* sariva*, triphala* for hair problems from high *pitta*.
* Manjishtha, kutki, neem, pippali for hepatitis and liver conditions.
* Jatamamsi, brahmi and shankhapushpi for mental disorders from high *vata* and *pitta*.
* Black pepper for stimulating *rasa* and *rakta dhatu agni* and treating anaemia.
* Turmeric, neem, licorice for dermatological conditions due to high *kapha* and *vata.*
* Manjishtha, ashoka, lotus node for bleeding conditions from high *pitta* (Pole et al, 2006).

2.1.5. *Ethnopharmacological relevance*

The whole plant of *Eclipta alba* works as a best medicine for hair growth. The presence of β-sitosterol in *Eclipta alba* help to rebuild hair in androgenic alopecia (Roy et al., 2008).

2.2. *Hibiscus rosa-sinensis* – plant profile

2.2.1. *Botanical taxonomy*

Scientific classification			Vernacular names		
Kingdom	:	Plantae	English	:	Chinese hibiscus
Division	:	Magnoliophyta	Sanskrit	:	Japa
Class	:	Magnoliopsida	Hindi	:	Jasum, Gulhar
Order	:	Malvales	Bengali	:	Jaba
Family	:	Malvaceae	Tamil	:	Sembaruthi
Genus	:	Hibiscus	Telugu	:	Mandara
Species	:	*Rosa sinensis*	Malayalam	:	Bunga Raya

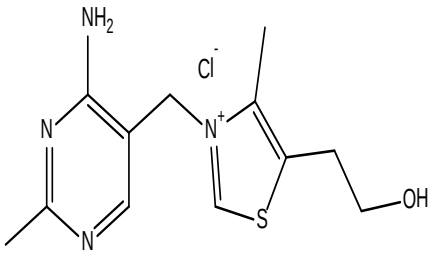
2.2.2. *Plant description*

The plant *Hibiscus rosa-sinensis* is a perennial shrub with tap root. The leaves are 3.5-12 cm in length and 2-5.5 cm wide. Leaves are simple ovate or ovate-lanceolate. Leaves are entire at the base and coarsely toothed at the apex. Taste is mucilaginous. Flowers are pedicillate, Actinomorphic, pentamerous and complete. Corolla consists of 5 petals, red in colour and about 3 inches in diameter, generally available in many areas within its hardiness range. *Hibiscus rosa-sinensis* are native to tropical Asia. A native of south eastern Asia (China), the plant is commonly found throughout the tropics and as a house plant throughout the world. Most ornamental varieties are hybrids. The present wide range of cultivars is considered to be a complex of inter specific hybrids, between 8 or more different species originating from the African east coast and islands in the Indian and pacific ocean (Rao et al., 2014).

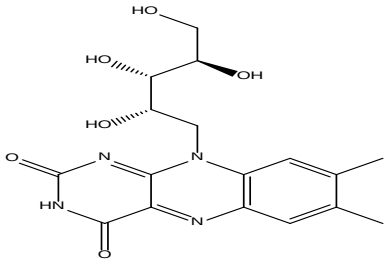
Figure 2.2A. *Hibiscus rosa-sinensis* plant. **Figure 2.2B.** *Hibiscus rosa-sinensis* flower.

2.2.3. Chemical constituents of *Hibiscus rosa-sinensis*

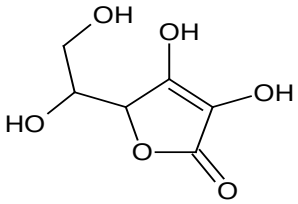
The edible portion of the flower (61.6 %) was reported to have the following nutrient composition (per 100 g): moisture 89.8 %, nitrogen 0.064 %, fat 0.36 %, crude fiber 1.56 %, calcium 4.04 mg, phosphorus 26.68 mg, iron 1.69 mg, thiamine 0.031 mg (9), riboflavin 0.048 mg (10), ascorbic acid 4.16 mg (11) and niacin0.61 mg (12). Petals of *Hibiscus rosa-sinensis* were reported to contain quercetin-3-di-*O*-β-D-glucoside; quercetin-3-7-di-*O*-β-D -glucoside; quercetin-3-*O*-β-D-sophorotrioside; and kaempferol and kaempferol-3-*O*-β-D-xylosylglucoside. The major anthocyanin contained in the red flowers of *H. rosa- sinensis* was cyanidin-3-sophoroside. red-petalled varieties of *H. rosa-sinensis* were found to have more number of anthocyaninbands compared with that observed in yellow-yellow orange varieties. Thevarieties in the different coloured groups differed in the quantitative distribution of anthocyanins, leucoanthocyanins, flavonol and carotenoids. Flavonoid aglycones found in the flowers (per g fresh tissues) included quercetin 7 mg (16) and cyanidin 36 mg. The flowers were also reported to contain the following flavones: quercetin-3-diglucoside (18), quercetin-3,7-diglucoside (14), cyanidin-3,5-diglucoside (15) and cyanidin-3-sophoroside-3-5 glucoside from deep yellow and white flowers and from ivory white flowers is kaempferol-3- xylosylglucoside (13) (Lim, 2014). Leaves and stems contain β-sitosterol, stigma sterol, taraxeryl acetate (19) and three cyclopropane compounds and their derivatives. Fatty acids, fatty alcohols and hydrocarbons were identified from *Hibiscus rosa-sinensis* leaves (Tiwari et al., 2015).



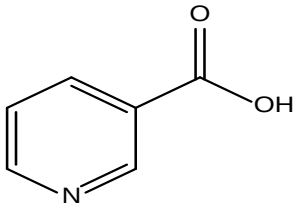
Thiamin (9)



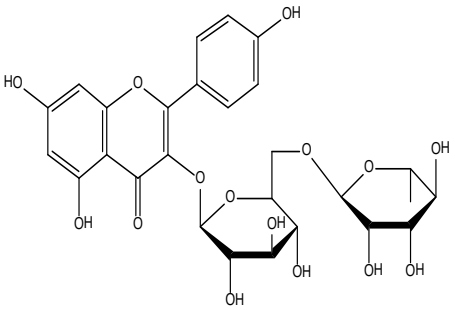
Riboflavin (10)



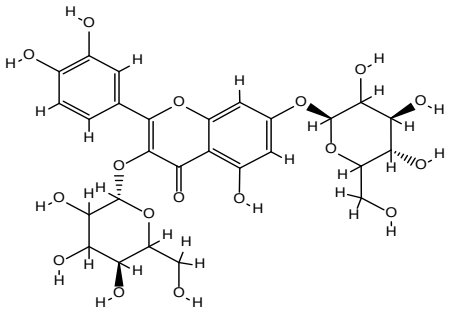
Ascorbic acid (11)



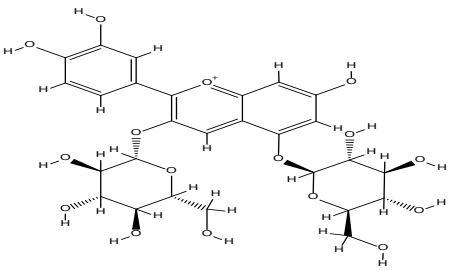
Niacin (12)



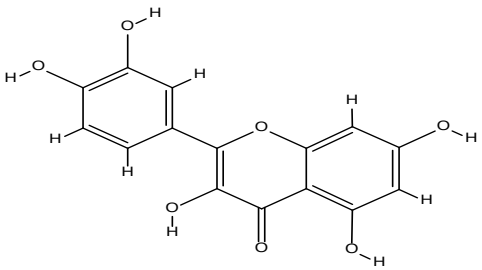
Kaempferol-3- xylosylglucoside (13)



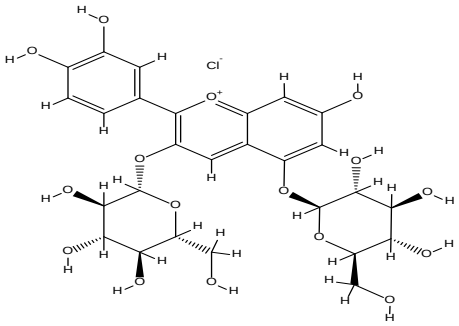
Quercetin-3,7-diglucoside (14)



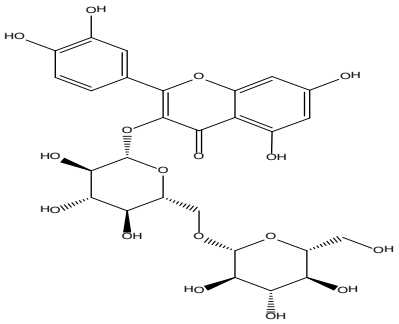
Cyanidin- 3,5-diglucoside (15)



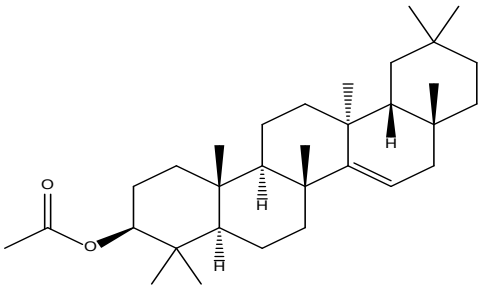
Quercetin (16)



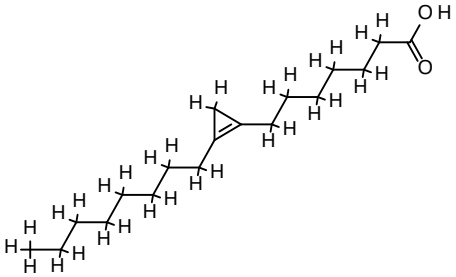
Cyanin Chloride (17)



Quercetin-3-diglucoside (18)



Taraxerol acetate (19)



Malvalic acid (20)

Figure 2.2C. Phytoconstituents of *Hibiscus rosa-sinensis.*

2.2.4. *Medicinal uses*

The aqueous-ethanolic extract of aerial parts of *Hibiscus rosa-sinensis* were reported for its use in constipation and diarrhea. The alcoholic extract of flowers of *Hibiscus rosa-sinensis* has been proved to possess anticonvulsant property. In traditional medicine, the leaves of the plant are used in fatigue and skin disease. Fresh root juice of the plant is given for gonorrhea and powder root for menorrhagia. Flowers of the plant are used in epilepsy, leprosy, bronchial catarrh and diabetes. An infusion of the petal is widely used in ayurvedic medicine in India as a demulcent refrigerant drink in fever and decoction is given in bronchial catarrh. It has been reported that the plant flower possesses anti-spermatogenic and androgenic, anti-tumour and anticonvulsant activities. The use of flower to treat heart disorders has also been described has demonstrated the anti-diabetic activity of *Hibiscus rosa-sinensis* in diabetic rural population. Infusion of the petals is given as refrigerant and demulcent. Leaves are used as laxative while root is used in cough (Upadhyay and Upadhyay., 2011).

2.2.5. *Ethnopharmacological relevance*

Traditionally, *Hibiscus rosa-sinensis* is used for the treatment of various diseases including alopecia (Upadhyay et al, 2011), (Upadhyay et al, 2013), (Adhirajan et al, 2003). Apart from this it is also used for anti-dandruff agent (Vyjayanthi et al., 2013).

2.3. *Solanum nigrum* - plant profile

2.3.1. Botanical taxonomy

Scientific classification			Vernacular names		
Kingdom	:	Plantae	Sanskrit	:	Dhvansamaci
Division	:	Tracheophyta	English	:	Garden night shade
Class	:	Magnoliopsida	Hindi	:	Makoya
Order	:	Solanales	Bengali	:	Gudakamai
Family	:	Solanaceae	Punjabi	:	Mako
Genus	:	Solanum	Telugu	:	Kamanchi
Species	:	Solanum Nigrum L.	Tamil	:	Manarthakkali

2.3.2. *Plant description*

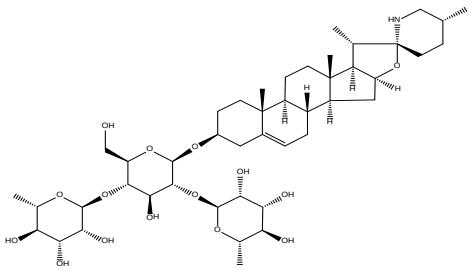
Solanum nigrum is 25-100 cm tall, erect annual herb, pubescent with simple hairs. Stems are often angular, sparsely-pubescent. The fruits are dull black, 8-10 mm in diameter. The leaves are ovate, the bases are cuneate, 4-10 and 3-7 cm wide, pubescent, coarsely dentate, the apex is obtuse. Inflorescences are extra axillary umbels, the calyx cup-shaped, the corolla is white, the lobes ovate-oblong, pubescent abaxially, ciliate spreading. Filaments are 1-1.5 mm long; anthers are 2.5- 3.5 mm long. Two varieties of Solanum nigrum found one is black colour fruit and second is reddish brown colour fruit. In both varieties black colour fruit are toxic (Chauhan et al., 2012).

Figure 2.3A. *Solanum nigrum* plant.

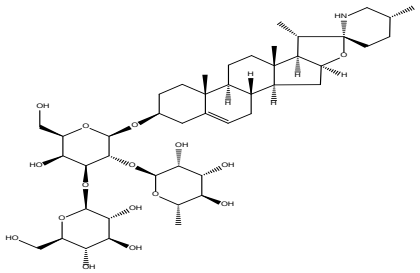
Figure 2.3B. *Solanum nigrum* berries.

2.3.3. Chemical constituents of *Solanum nigrum*

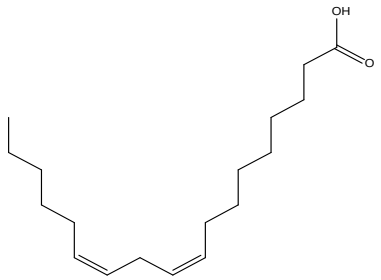
Phytochemical investigation of whole plant reported that which contain alkaloids, flavonoids, tannins, saponins, glycosides, proteins, carbohydrates, coumarins & phytosterols. It has been found that *Solanum nigrum* contains the substances, such as total alkaloid, steroid alkaloid, steroidal saponins and glycoprotein, exhibiting anti-tumor activity. Researchers studied the chemical characterization of osmotin - like protein from this plant. New glycoprotein (150 KDa) has been isolated from this plant which consist carbohydrate content (69.74%) and protein content (30.26%) which contain more than 50% hydrophobic amino acids such as glycine and proline. Small unripe fruits of *Solanum nigrum* had a high concentration of solasodine, but both the concentration and the absolute amount per fruit decreases with fruit maturation. The berries of *Solanum nigrum* from New Zealand have recently been studied and found to contain 4 steroidal alkaloid glycosides, solamargine (21), solasonine (22), α and b- solanigrine. The berries of Solanum nigrum have been found to contain a saturated steroidal genin, which has been identified as tigogenin by mixed melting point and IR spectroscopy. One spirosestanol glycoside and two furostanol glycosides have been isolated from a methanol extract of the stems and roots of *Solanum nigrum*. Some researchers found the presence of ascorbic acid (24) in the fruits of *Solanum nigrum* and the concentration of ascorbic acid is more in fruit than root. Six new steroidal saponins, solanigrosides C-H, and one known saponin, degalactotigonin, were isolated from the whole plant of *Solanum nigrum*. Some researchers isolated two new steroidal saponins, named nigrumnins I and II, together with two known saponins were obtained from the whole plant of *Solanum nigrum*. Recently phytochemical analysis of *Solanum nigrum* has resulted in the isolation of two novel disaccharides. Their structures were determined as ethyl b-D-thevetopyranosyl-(1-4)-b-D-oleandropyranoside and ethyl b-D-thevetopyranosyl-(1-4)-a-D-oleandropyranoside, respectively, by chemical and spectroscopic methods. *Solanum nigrum* seeds have high lipid content. Their protein content and minerals elements (Mg being prominent) are considerable and *Solanum nigrum oil is* an important source of linoleic acid (23). Chemical structures of some phytoconstituents from *Solanum nigrum* given below (Saleem et al., 2009).



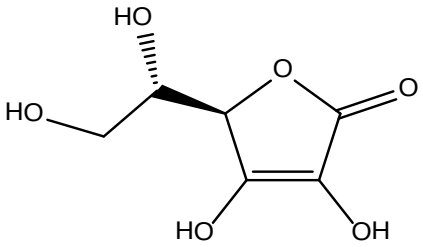
Solamargine (21)



Solasonine (22)



Linoleic acid (23)



Ascorbic acid (24)

Figure 2.3C. Phytoconstituents of *Solanum nigrum.*

2.3.4. *Medicinal uses*

The berries and leaves are mainly used for medicinal purposes, besides the other parts of the whole plant. The leaves are used as poultice for rheumatic and gouty joints (Disease causing the joints to swell and become painful), skin diseases, used in the treatment of antituberculosis and are said to produce diaphoresis. Leaves are also used in dropsy, nausea and nervous disorders. The decoction of the berries and flowers are useful in cough, erysipelas (specific, acute, cutaneous inflammatory disease caused by a haemolytic streptococcus and are characterized by red hot). These are remedy for pulmonary tuberculosis and Bronchitis, diuretic. The juice of the berries used as an antidiarrheal, opthalmopathy and hydrophobia. It is also used in anasarca and heart disease. Berries are used to possess tonic, diuretic and cathartic properties. Seeds are useful in giddiness and dipsia. They are also useful in inflammations and skin diseases. The roots are useful in otopathy, ophthalmopathy, rhinopathy and hepatitis. The whole plant used as antiseptic, anti-inflammatory, expectorant, cardiotonic, digestive, diuretic, laxative, diaphoretic, sedative, swelling, cough, asthma The plant is also effective in curing cardiopathy, leprosy, haemorrhoids, nephropathy, ophthalmopathy, dropsy and general debility. Decoction of the plant depresses the CNS and reflexes of the spinal cord (Saleem et al., 2009).

2.3.5. *Ethnopharmacological relevance*

The berries of *Solanum nigrum* used as medicine for alopecia (Patel et al., 2015), (Rao., 1985).

Chapter - 3

Scope, objective and plan of work

3.1. Scope and rationale of the present study

3.2. Objective of the work

3.3. Framework of the study

3.1. Scope and rationale of the present study

Natural products from plants are rich sources used for treating a number of diseases. Most of the herbal drugs are a mixture of a number of plant ingredients. Their synergistic effect increases the efficacy of the drug in curing the diseases. In this modern time, the knowledge and experience of usage of herbs are being blend with advanced technology to develop a safe and elegant herbal product, which has wider range of people acceptability. The present study is aim to screening the extract *Eclipta alba*, *Hibiscus rosa-sinensis*, *Solanum nigrum* to investigate the effect of 5α-reductase inhibitory activity. Alopecia (hair loss or baldness) is due to over expression of 5α- reductase enzyme which converts testosterone (T) to dihydrotestosterone (DHT). Considerably higher DHT concentration results in loss of hair. Inhibition of 5α-reductase activity is considered as a promising strategy for treatment of alopecia disorder. At present a number of synthetic remedies are there for treatment of this disorder, which include corticosteroids, dithranol, tretinoin, minoxidil, zinc, systematic cortisone, finasteride, azelaic acid etc but not a single or multiple drug therapy is giving satisfactory and permanent results to the alopecia patients. Besides, a number of side effects are associated with the use of these synthetic compounds, including erythema, scaling, pruritus, dermatitis, itching etc. Additionally, inhibition of 5α-reductase is also useful for improvement of benign prostatic hyperplasia (BPH). This unfavorable hair loss, BPH and side effects of synthetic remedies has been of great concern, and it is necessary to search for potent 5α- reductase inhibitors. Therefore, these plant materials can be use as raw material for treatment and controlling alopecia disorder as well as cosmetic formulation.

3.2. Objective of the study

I. To discover and characterize new 5α-reductase inhibitor from plants that can helps in preventing alopecia.

II. To understand 5α-reductase activators and inhibitors that regulates the hair growth cycle.

III. To evaluate the efficacy of plant extracts by *in-vitro* enzyme assay.

IV. The inhibitory strength is compared with standard inhibitor like finasteride (a potent 5α-reductase inhibitor) and their inhibitory mechanisms are evaluated.

3.3. Framework of the study

Extraction of following plants *Eclipta alba* (whole plant), *Hibiscus rosa-sinensis* (flower), *Solanum nigrum* (berries) by cold maceration in methanol to get the different polarity extracts.

- Selection of the plant on the basis of traditional uses of hair care plants.
- Collection and authentication of plants.
- Extraction of plant materials by process of cold maceration.
- Concentrated by rotary vacuum evaporator and store in proper condition.
- HPLC and HPTLC analysis of secondary metabolites of plant extracts.
- Heavy metals estimation of plant materials by atomic atomic absorption spectroscopy.
- *In-vitro* screening of 5α-reductase inhibition assay.
- Preparation and evaluation of different hair growth formulations based on above three plants (hair gel, lotion, cream).
- Preparation and evaluation of different β-sitosterol gel hair growth formulation.
- Standardization and quantification of herbal formulation by HPTLC or HPLC.
- Evaluation of 5α-reductase inhibitory potential of herbal hair growth formulation compare to the marketed hair growth formulation.
- Analysis of obtained results and apply suitable statistical parameter for better understanding of performed activity.

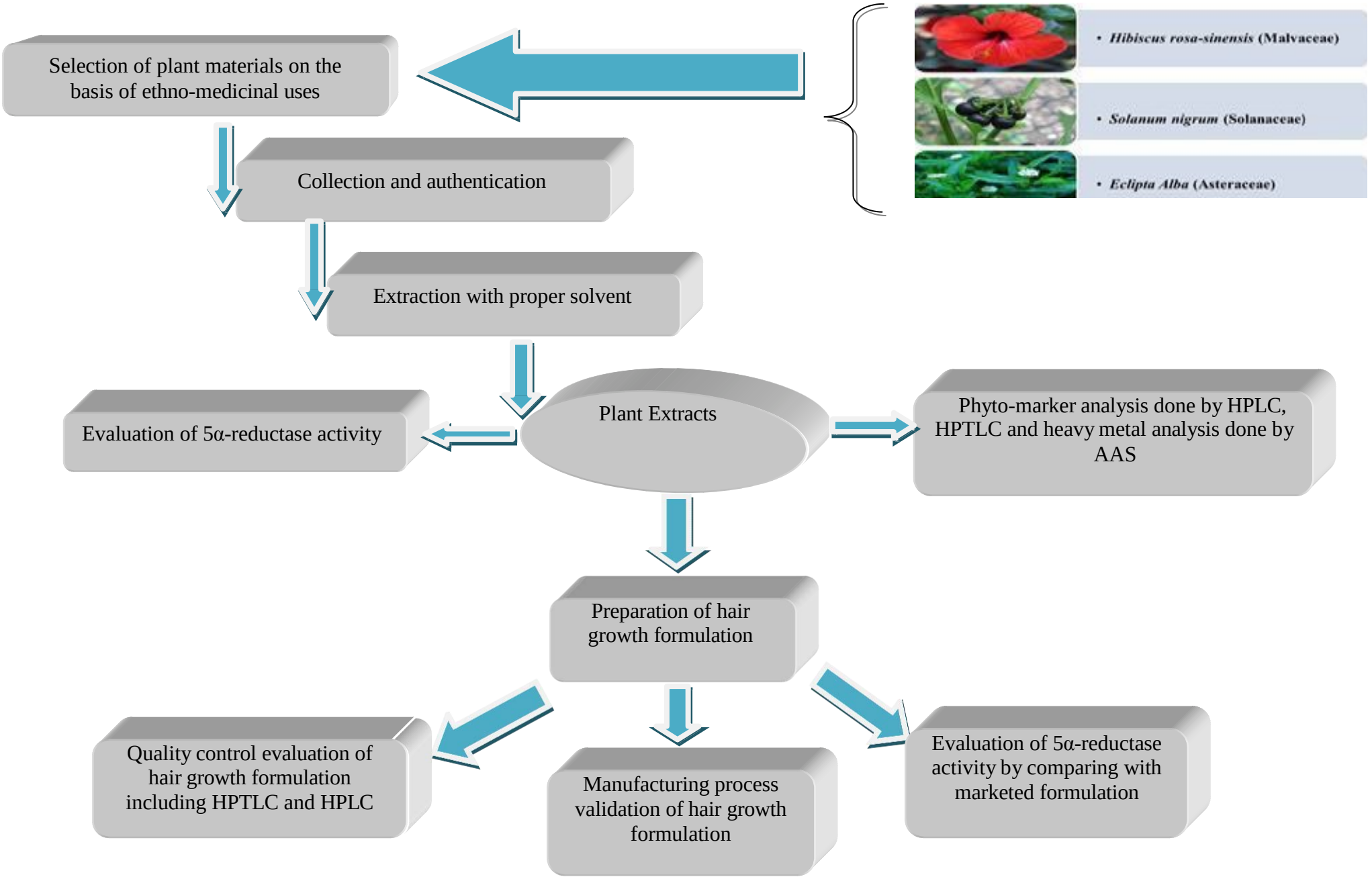


Figure 3.1. Framework of plan of the study

Chapter - 4

Collection, authentication, extraction and phytochemical screening of plant material

4.1. Collection and authentication of *Eclipta alba*
4.2. Extraction procedure of *Eclipta alba*
4.3. Phytochemical screening of *Eclipta alba*
4.4. Results

4.5. Collection and authentication of *Hibiscus rosa-sinensis*
4.6. Extraction procedure of *Hibiscus rosa-sinensis*
4.7. Phytochemical screening of *Hibiscus rosa-sinensis*
4.8. Results

4.9. Collection and authentication of *Solanum nigrum*
4.10. Extraction procedure of *Solanum nigrum*
4.11. Phytochemical screening of *Solanum nigrum*
4.12. Results

4.1. Collection and authentication of *Eclipta alba*

Eclipta alba whole plant was purchased from local vender. It was authenticated by Dr. S. Rajan, Field Botanist, Survey of Medicinal Plants and Collection Unit, Emerald, Tamilnadu, India. A voucher specimen (specimen no. SNPS/JU/2015/1089) was deposited to School of Natural Product Studies (Figure 4.1), Jadavpur University, Kolkata, India for future reference.

Figure 4.1. Voucher Specimen *Eclipta alba* whole plant.

4.2. Extraction procedure of *Eclipta alba*

Eclipta alba whole plant was purchased from local vender. It was cut into small pieces by knife. 250 g of dried small pieces *Eclipta alba* (whole plant) was taken in two separate 2000 mL conical flask and added 1000 mL of methanol and 1000 mL of petroleum ether. It was kept for 72 hrs in air tight condition at 25 to 30 °C temperature. After that, it was filtrated by normal filter paper. Filtrate was kept in a 1000 mL beaker. After filtration, the filtrate was concentrated by rotary evaporator at 40 to 45°C temperature and other ambient condition. The percentage yield of extraction was 1.16 % w/w. The extract was stored in glass vials in air tight condition at room temperature with proper label.

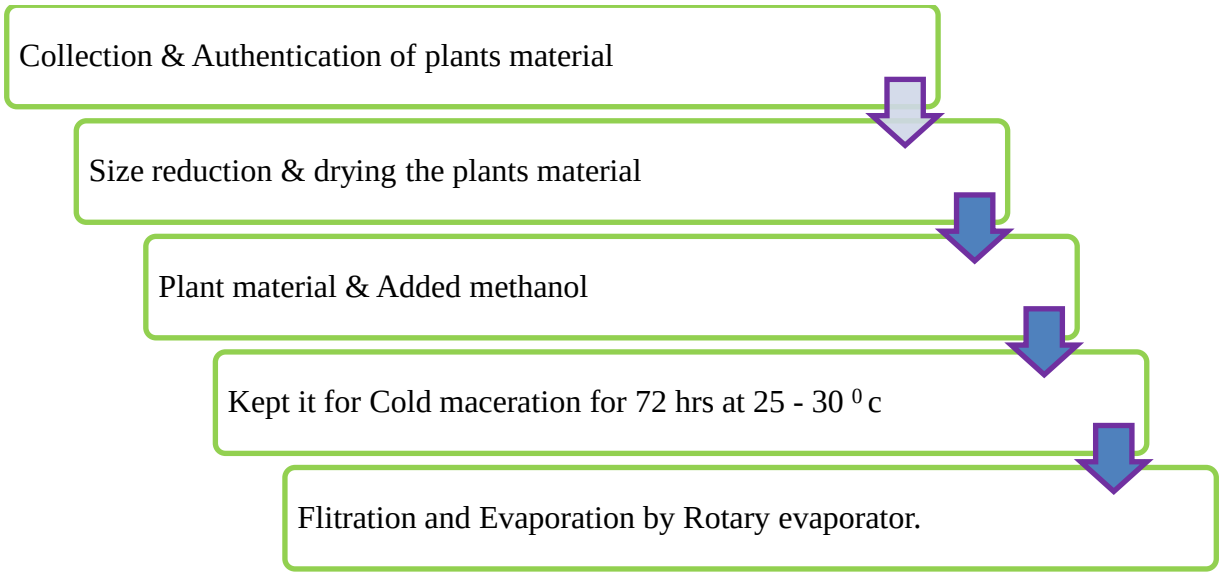


Figure 4.2. Extraction procedure of cold maceration process.

4.3. Phytochemical screening of *Eclipta alba* extract

Detection of phytosterols (Mukherjee, 2002)

Libermann-Burchard Test	10 mg of extract was dissolved in 1mL of chloroform. 1 mL of acetic anhydride was added following the addition of 2 mL of concentrated sulphuric acid, a reddish violet color developed, indicating the presence of steroids.
Salkowski Test	1 mL of concentrated sulphuric acid was added to 10 mg of extract dissolved in 1 mL of chloroform. A reddish-blue color exhibited by chloroform layer and green fluorescence by the acid layer suggested the presence of steroids.

Detection of triterpenoids (Harborne, 1998)

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

Detection of flavonoids (Mukherjee, 2002; Trease and Evans, 2002)

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

Detection of alkaloids (Trease and Evans, 2002; Mukherjee, 2002)

Mayer's test	1.2 mL of extract was taken in a test tube. 0.2 mL of dilute hydrochloric acid and 0.1 mL of Mayer's reagent were added. Formation of yellowish buff colored precipitate gives positive test for alkaloid.
Dragendroff's test	0.1 mL of dilute hydrochloric acid and 0.1 mL of Dragendroff's reagent were added in 2 mL solution of extract in a test tube. Development of orange brown colored precipitate suggested the presence of alkaloid.
Wagner's test	2 mL of extract solution was treated with dilute hydrochloric acid and 0.1 mL of Wagner's reagent. Formation of reddish brown precipitate indicated the positive response for alkaloid.

Detection of alkaloids (Trease and Evans, 2002; Mukherjee, 2002)

Biuret test	1 mL of 40% NaOH mixed with 2 drops of 1% copper sulphate was added to the extract, a violet color indicated the presence of proteins.

Detection of protein and amino Acid (Harborne, 1998)

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

Detection of deoxy sugars (Harborne, 1998)

Keller Kiliani test	To 1 g of the sample, 10 mL of 70% ethanol were added and boiled for 2-3 min. it was filtered and to the 5 mL of the filtrate, 5 mL of distilled water and 0.5 mL strong lead acetate solution were added. It was filtered and 5 mL of chloroform were added to the filtrate. Excess chloroform was pipetted off and gentle evaporation of chloroform was done on a porcelain dish. It was cooled and to the residue, 3 mL of glacial acetic acid and 2 drops of 5% ferric chloride were added. The solution was transferred to the surface of 2 mL concentrated sulphuric acid. Reddish brown color (which changed to bluish green to dark on standing) at the junction confirmed the presence of deoxy sugars in the sample.

Detection of reducing sugars (Mukherjee, 2002)

Fehling's test	5 mL of the extract solution, mixed with 5 mL of Fehling's solution was boiled for 5 minutes. Formation of brick red colored precipitate demonstrated the positive test for reducing sugars.
	To 5 mL of the extract solution, 5 mL of Benedict's solution was added in a test tube and boiled for few min. Development of brick red precipitate confirmed the presence of reducing sugars.

Detection of glycosides (Harborne, 1998)

Legal test	Extract was dissolved in pyridine, sodium nitroprusside solution was added to it and made alkaline. Pink red color was produced.
Baljet test	To a drug extract, sodium picrate solution was added. Yellow to orange color was produced.
Borntrager's test	Few mL of dil. sulphuric acid added to the test solution. Boiled, filtered and extracted the filtrate with ether or chloroform. Then organic layer was separated to which ammonia was added, pink red color was produced in organic layer.
Keller Killiani test	Sample was dissolved in acetic acid containing trace of ferric chloride and transferred to the surface of conc. sulphuric acid. At the junction of liquid reddish brown color was produced which gradually becomes blue.

Detection of phenolic compounds and tannins (Tyler et al., 1988)

Ferric chloride test	5 mL of extract solution was allowed to react with 1 mL of 5% ferric chloride solution. Greenish black coloration indicated the presence of tannins.
Potassium dichromate test	5 mL of the extract was treated with 1 mL of 10% aqueous potassium dichromate solution. Formation of yellowish brown precipitate suggested the presence of tannins.

Detection of saponins (Harborne, 1998)

Foam test	1 mL solution of the extract was diluted with distilled water to 20 mL and shaken in a graduated cylinder for 15 min.

	Development of stable foam suggested the presence of saponins.
Potassium dichromate test	1 mL extract was treated with 1% lead acetate solution. Formation of white precipitate indicated the presence of saponins.

Preparation of the reagents were carried out by the following methods (Mukherjee, 2002):

Mayer's reagent	:	1.36 g of mercuric iodide in 60 mL of water mixed with a solution which contains 5 g of potassium iodide in 20 mL of water.
Libermann-Burchard reagent	:	5 g of acetic anhydride was carefully mixed under cooling with 5 mL concentrated sulphuric acid; this mixture was added continuously to 50 mL of absolute ethanol with cooling.
Dragendorff's reagent	:	1.7 g basic bismuth nitrate and 20 g tartaric acid are dissolved in 80 mL of water. This solution was mixed with a solution containing 16 g potassium iodide and 40 mL of water.
Fehling's solution A	:	34.64 g copper sulphate was dissolved in a mixture of 0.5 mL of sulphuric acid and sufficient water to produce 500 mL.
Fehling's solution B	:	176 g of sodium potassium tartarate and 77 g of NaOH are dissolved in sufficient water to produce 500mL. Equal volumes of solution A & B are mixed at the time of use.
Benedict's reagent	:	1.73 g of cupric sulphate, 1.73 g of sodium citrate and 10 g anhydrous sodium carbonate are dissolved in water and the volume was made up to 100 mL with water.
Molish's reagent	:	2.5 g of pure α-naphthol was dissolved in 25 mL of ethanol.

4.4. Results

Phytochemical screening of the methanolic extract of *Eclipta alba* showed presence of different type of phytoconstituents as depicted in table 4.1.

Table 4.1. Phytochemical screening of the methanolic extract of *Eclipta alba*

Test	Methanolic extract of *Eclipta alba*
Phytosterols	+
Triterpenoids	-
Flavonoids	+
Alkaloids	+
Protein and amino acids	+
Carbohydrates	+
Glycosides	+
Phenolic compounds and Tannins	+
Saponins	+

("+" Indicates positive; "-" indicates negative)

4.5. Collection and authentication of *Hibiscus rosa-sinensis*

Hibiscus rosa-sinensis flower was purchased from local vender. It was authenticated by Dr. S. Rajan, Field Botanist, Survey of Medicinal Plants and Collection Unit, Emerald, Tamilnadu, India. A voucher specimen (specimen no. SNPS/ JU/2015/1088) (Figure 4.2) was deposited to School of Natural Product Studies., Jadavpur University, Kolkata, India for future reference.

Figure 4.2. Voucher Specimen *Hibiscus rosa-sinensis*.

4.6. Extraction procedure of *Hibiscus* rosa-sinensis

Hibiscus rosa-sinensis flower was purchased from local vender. It was crushed into coarse powder by machine. 500 g of coarse dry power of *Hibiscus rosa-sinensis* (flower) was taken in a 2000 mL conical flask and added 1000 mL of methanol. It was kept for 72 hrs in air tight condition at 25 to 30 °C temperature. After that, it was filtrated by normal filter paper. Filtrate was kept in a 1000 mL beaker. After filtration, the filtrate was concentrated by rotary evaporator at 40 to 45 °C temperature and other ambient condition. The percentage yield of extraction was 1.85 % w/w. The extract was stored in glass vials in air tight condition at room temperature with proper label.

4.7. Phytochemical screening of *Hibiscus rosa-sinensis* extract

Phytochemical tests of the methanolic extract of *Hibiscus rosa-sinensis* flower were performed following the methods as described in Chapter-4, Section-4.3. Pages: 36-39.

4.8. Results

Phytochemical screening of the methanolic extract of *Hibiscus rosa-sinensis* flower showed presence of different type of phytoconstituents as depicted in table 4.2.

Table 4.2. Phytochemical screening of the methanolic extract of *Hibiscus* rosa-sinensis flower

Test	Methanolic extract of *Hibiscus* rosa-sinensis flower
Phytosterols	+
Triterpenoids	+
Flavonoids	+
Alkaloids	+
Protein and amino acids	+
Deoxy sugars	-
Reducing sugars	+
Glycosides	-
Phenolic compounds and Tannins	-
Saponins	-

("+" Indicates positive; "-" indicates negative)

4.9. Collection and authentication of *Solanum nigrum*

Solanum nigrum berries were purchased from local vender. It was authenticated by Dr. S. Rajan, Field Botanist, Survey of Medicinal Plants and Collection Unit, Emerald, Tamilnadu, India. A voucher specimen (specimen no. SNPS/JU/2015/1090) (Figure 4.2) was deposited to School of Natural Product Studies, Jadavpur University, Kolkata, India for future reference.

Figure 4.3. Voucher Specimen *Solanum nigrum* barriers.

4.10. Extraction procedure of *Solanum nigrum*

Solanum nigrum berries were purchased from local vender. It was crushed into coarse powder by machine. 500 g of coarse dry power of *Solanum nigrum* (berries) was taken in 2000 mL conical flask and added 1000 mL of methanol. It was kept for 72 hrs in air tight condition at 25 to 30 °C temperature. After that, it was filtrated by normal filter paper. Filtrate was kept in a 1000 mL beaker. After filtration, the filtrate was concentrated by rotary evaporator at 40 to 45 °C temperature and other ambient condition. The percentage yield of extraction was 2.45 % w/w. The extract was stored in glass vials in air tight condition at room temperature with proper label.

4.11. Phytochemical screening of *Solanum nigrum* berries extract

Phytochemical tests of the methanolic extract of *Solanum nigrum* berries were performed following the methods as described in Chapter-4, Section-4.3. Pages: 36-39.

4.12. Results

Phytochemical screening of the methanolic extract of *Solanum nigrum* berries showed presence of different type of phytoconstituents as depicted in table 4.3.

Table 4.3. Phytochemical screening of the methanolic extract of *Solanum nigrum* berries

Test	Methanolic extract of *Solanum nigrum* berries extract
Steroids	-
Triterpenoids	+
Flavonoids	+
Alkaloids	+
Protein and amino acids	+
Glycosides	+
Phenolic compounds and Tannins	+
Saponins	+

("+" Indicates positive; "-" indicates negative)

Chapter - 5

Standardization of plant material by HPTLC, HPLC and AAS

5.1. Standardization of β-sitosterol *in Eclipta alba* whole plant by HPTLC

5.2. Standardization of linoleic acid *in Solanum nigrum* berries by HPTLC

5.3. Standardization of quercetin *in Hibiscus rosa-sinensis* flower by HPTLC

5.4. Standardization of β-sitosterol *in Eclipta alba* whole plant by HPLC

5.5. Standardization of quercetin *in Hibiscus rosa-sinensis* flower by HPLC

5.6. Atomic absorption spectroscopy study of heavy metal analysis of plant extracts

5.0. Standardization of plants material by HPTLC

HPTLC is a method to standardize and identify the chemical component which is expected to be present in a medicinal plant. This is done from regulatory perspective to ensure the quality, efficacy and safety of the herbal drugs present in a plant. Thus it provides a very reliable way of determining the purity and percentage content of the active biomarker in the plant extracts. In this chapter the standardization and quantification of the three plant extracts have been described namely *Eclipta alba*, *Hibiscus rosa-sinensis*, *Solanum nigrum*.

5.1. Standardization of β-sitosterol *in Eclipta alba* whole plant by HPTLC

5.1.1. *Equipments and reagents*

The CAMAG HPTLC system consisting of WINCATS software, LINOMAT V automatic sample applicator, and automatic development chamber, scanning densitometer CAMAG scanner 3 and photo documentation apparatus CAMAG reprostar 3 were used. Stationary phase used was aluminum based silica gel plate 60 F_{254} (Merck, Mumbai) with 10 cm x 10 cm in a particle size of 5-10 µm. All the solvents were used of analytical grade.100 µl syringe (HAMILTON, Switzerland) was used for sample application on HPTLC plates. β-sitosterol was purchased from sigma Aldrich. Methanol and toluene of analytical grade were purchased from Merck (Mumbai, India). Whatman's syringe filter (NYL 0.45 µm) was used for the filtration of samples and standard.

5.1.2. *Preparation of standard solution*

About 1 mg of β-sitosterol standard was weighed and put in to 1 mL eppendorf tube. 1.0 mL of methanol was added with it and mixed in vortex mixture till the material completely dissolved. It was then filtered through 0.45 µ syringe filter and kept for further study.

5.1.3. *Preparation of sample solution*

About 10.0 mg of lyophilize methanolic and 5 mg of petroleum ether extract of *Eclipta alba*(whole plant) was taken in separate 1 mL of eppendorf tube. 1.0 mL of methanol and petroleum ether was added in methanolic extract and petroleum ether extract of *Eclipta alba* respectively and mixed in vortex mixture and put to ultrasonication bath till the material completely dissolved. It was then filtered through 0.45 µ syringe filter and kept for further study.

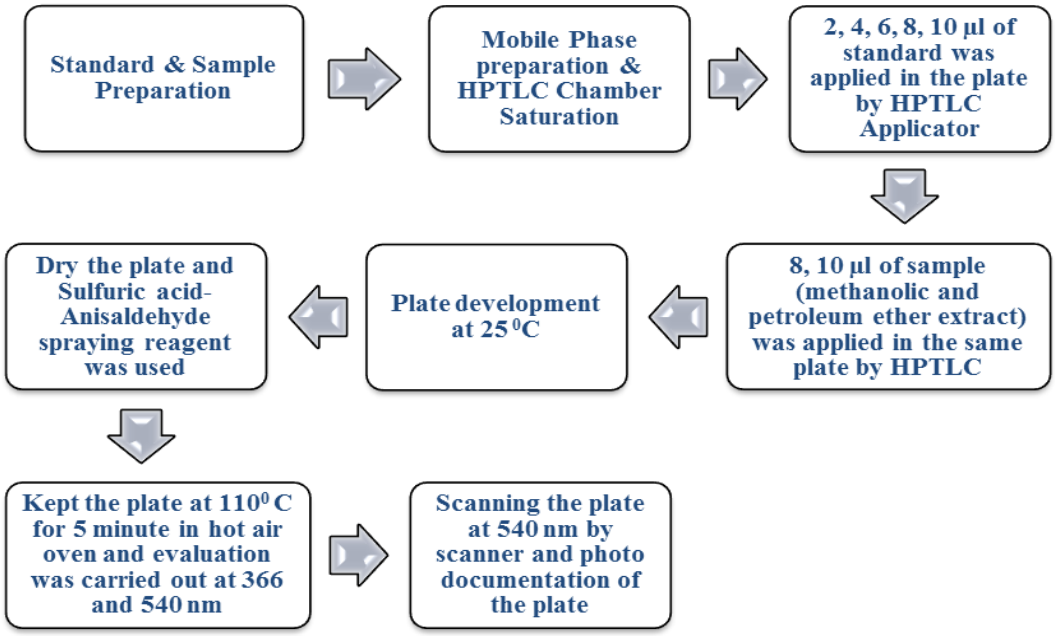


Figure 5.1. Standardization of β-sitosterol (standard) *in Eclipta alba* whole plant by HPTLC.

5.1.4. *Chromatographic conditions*

HPTLC analysis was performed using isocratic technique. Mobile phase was optimized with toluene and methanol in a ratio of 90:10 v/v. The temperature was kept at 25 ^{0}c and mobile phase was developed in a twin trough glass chamber. Standard stock solution was applied consequently in the range of 2-10 µl with 2 µl gradual increment. Methanolic and petroleum ether extract of *Eclipta alba* (whole plant) was applied 8 and 10 µl each at the concentration 10 mg/mL and 5 mg/mL respectively. All total 9 tracks in HPTLC plate were used for standardization including standard and sample solution respectively in a band wise fashion. After development, plates were dried by hand dryer. The dry plate was treated with sulfuric acid-anisaldehyde spraying reagent. The plate was kept at 110 °C for 5 min in hot air oven and evaluation was carried out at 366 and 540 nm. Colored bands were observed at 540 nm.

5.1.5. *Results and discussion*

The percentage content of β-sitosterol in *Eclipta alba* methanolic and petroleum ether extract was found to be 0.10% and 4.65% respectively. This was determined by a calibration curve with the equation of Y = 3447.759*X - 1121.100 (correlation coefficient = 0.9686 and standard deviation = ± 5.20%) as shown in Fig 5.2 where X represents amount of β-sitosterol and Y represents area under the curve.

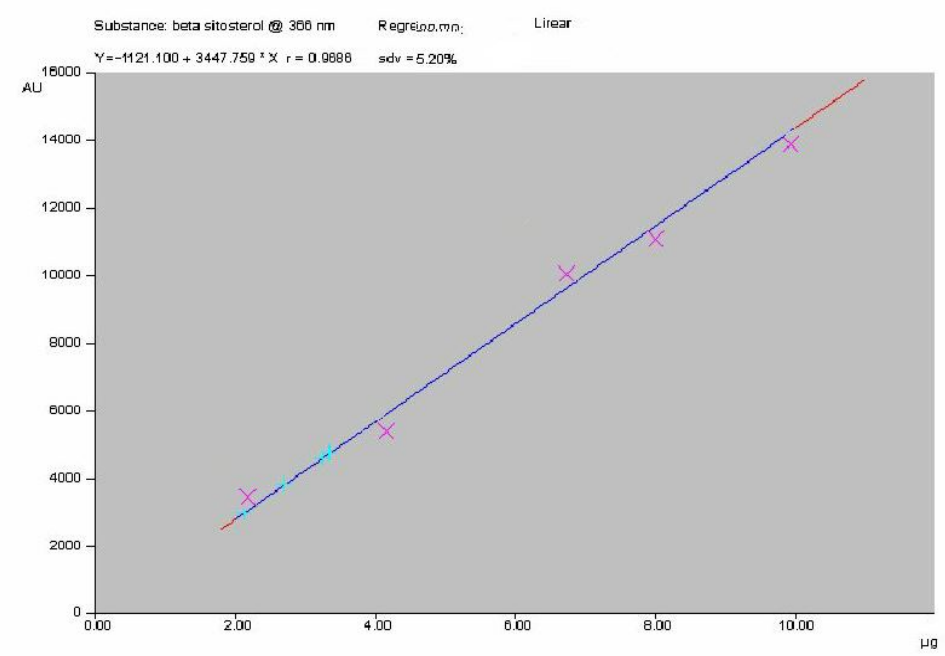


Figure 5.2. Calibration curve of β-sitosterol.

R_f value of standard β-sitosterol was found to be 0.66. Specificity was confirmed by comparing the R_f of standard and sample (Figure 5.3 - 5.5).

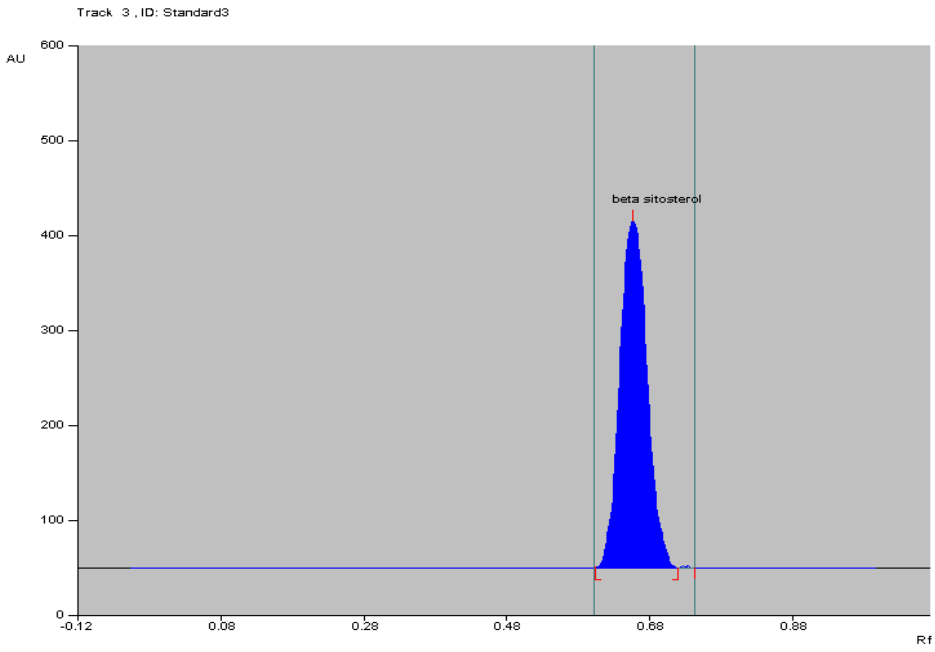


Figure 5.3. HPTLC chromatogram of standard β-sitosterol.

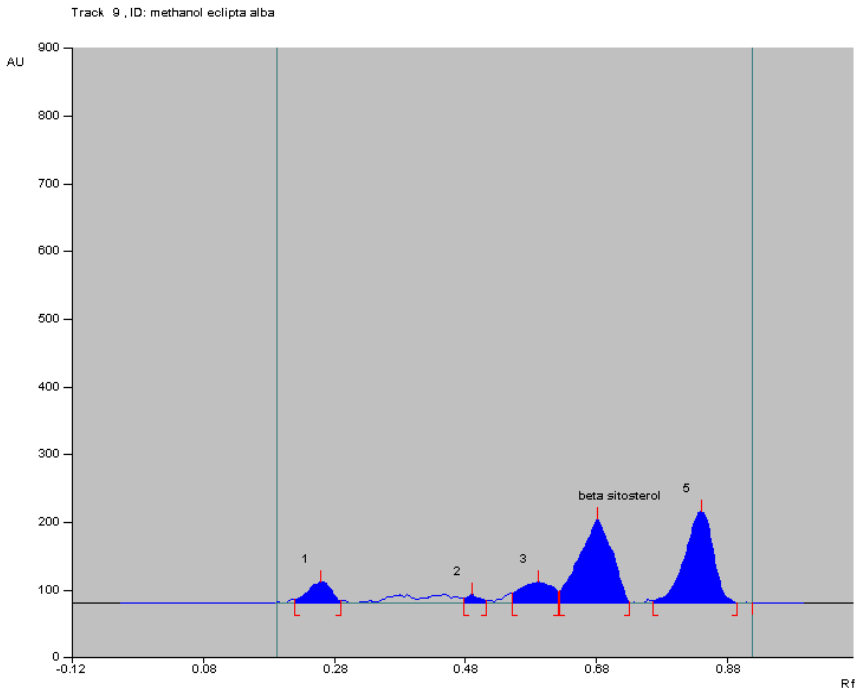


Figure 5.4. HPTLC chromatogram of methanolic extract of *Eclipta alba.*

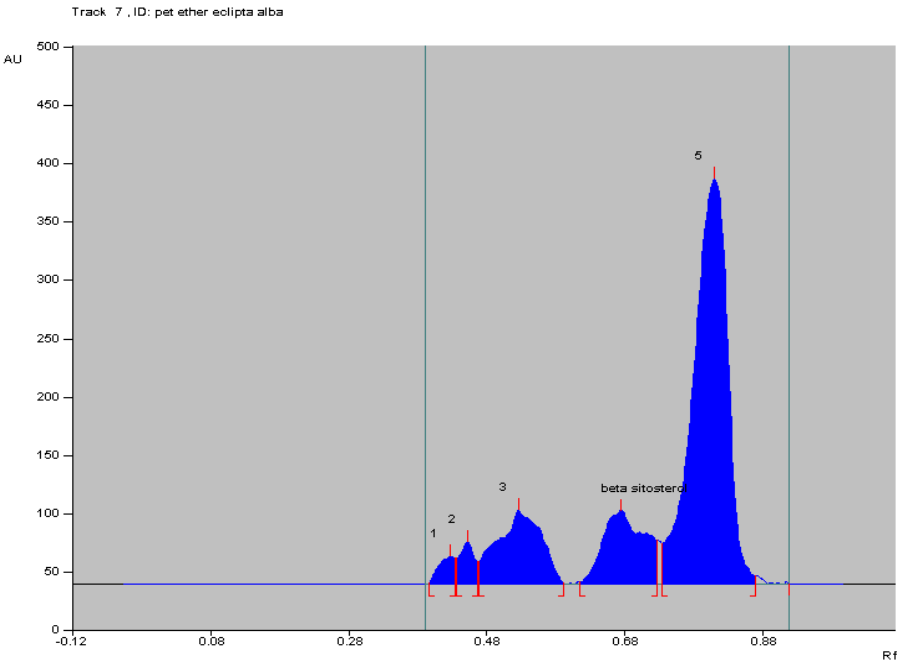


Figure 5.5. HPTLC chromatogram of petroleum ether extract of *Eclipta alba.*

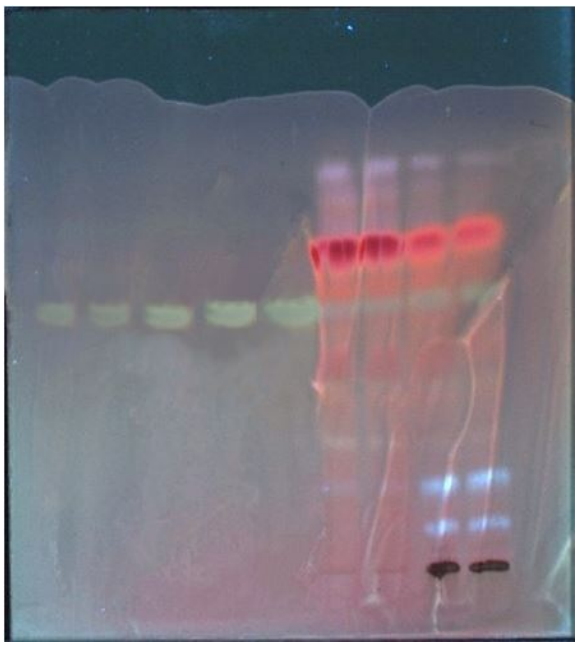


Figure 5.6. Photodocumentaton of *Eclipta alba* whole plant extract at 366 nm.

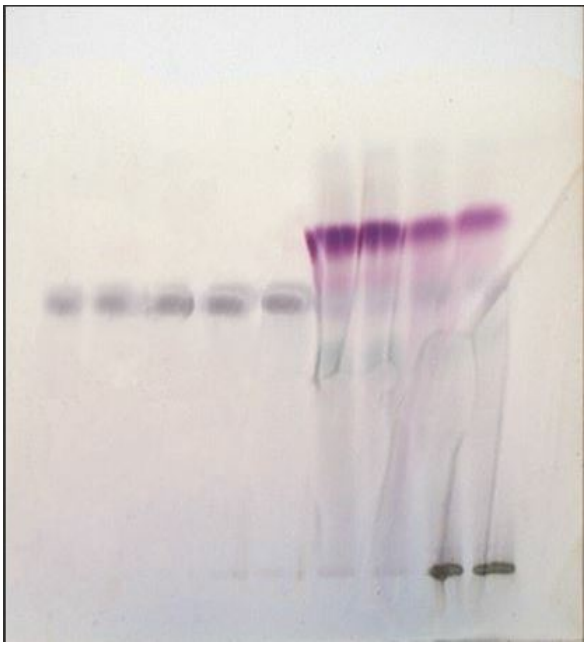


Figure 5.7. Photodocumentaton of *Eclipta alba* whole plant extract at 540 nm.

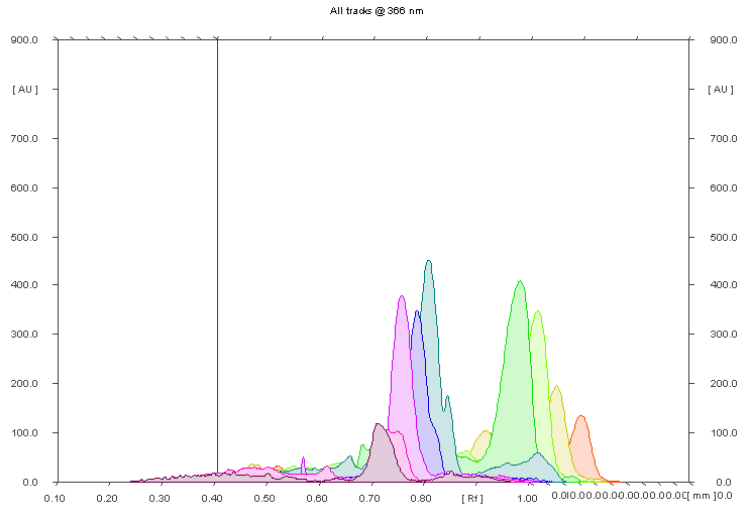


Figure 5.8. 3D Chromatogram of *Eclipta alba* and standard β-sitosterol at 366 nm.

5.2. Standardization of linoleic acid *in Solanum nigrum* berries by HPTLC

5.2.1. *Equipments and reagents*

The CAMAG HPTLC system consisting of WINCATS software, LINOMAT V automatic sample applicator, and automatic development chamber, scanning densitometer CAMAG scanner 3 and photo documentation apparatus CAMAG reprostar 3 were used. Stationary phase was used as aluminum based silica gel plate 60 F_{254} (Merck, Mumbai)

with 10 cm x 10 cm in a particle size of 5-10 µm. All the solvents were used of analytical grade. 100 µl syringe (HAMILTON, Switzerland) was used for sample application on HPTLC plates. Linoleic acid was purchased from Sisco research laboratories (SRL). Methanol, n-hexane and ethyl acetate (analytical grade) were procured from Merck (Mumbai, India). All the samples were filtered through Whatman's syringe filter (NYL 0.45 µ).

5.2.2. *Preparation of standard solution*

About 1 mg of linoleic acid standard was weighed and put in to 1 mL eppendorf tube. 1.0 mL of methanol was added with it and mixed in vortex mixture till the material completely dissolved. It was then filtered through 0.45 µ syringe filter and kept for further study.

5.2.3. *Preparation of sample solution*

About 5 mg of *Solanum nigrum* berries methanolic extract was dissolved in 1 mL methanol in an eppendorf tubes. It was then mixed in vortex mixture and put to ultrasonication bath till the material completely dissolved. Then it was filtered through 0.45 µ syringe filter and kept for further study.

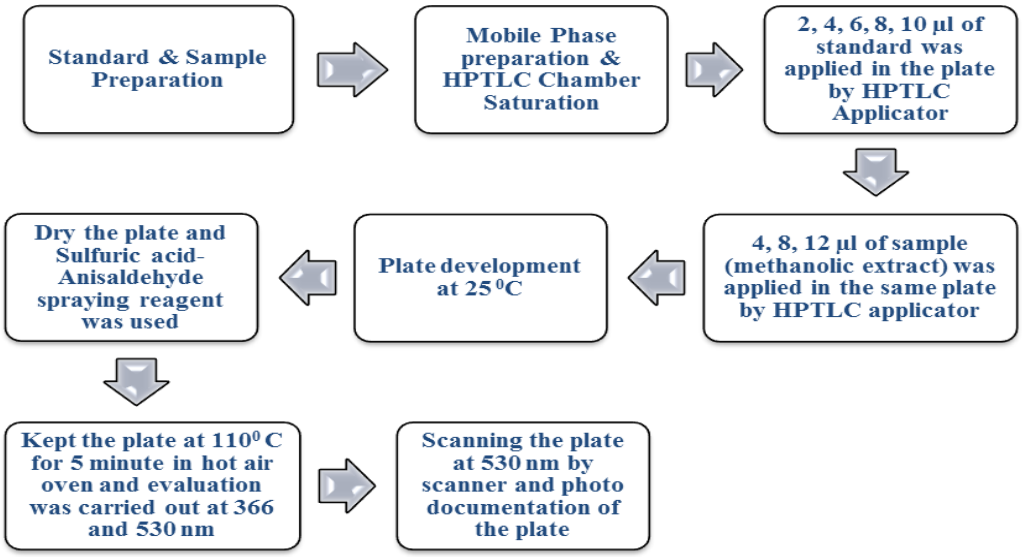


Figure 5.9. Standardization of linoleic acid (standard) *in Solanum nigrum* berries by HPTLC.

5.2.4. *Chromatographic conditions*

HPTLC analysis was performed using isocratic technique. The mobile phase was optimized with n-hexane and ethyl acetate in a ratio of 5:4 v/v. The temperature was kept at 25 °C and mobile phase was developed in a twin trough glass chamber. The standard solution was applied 2, 4, 6, 8, 10 µL and sample solution was applied consequently in the range of 4, 8 and 12 µL. The total 8 tracks in HPTLC plate were

used for the development of 5 standard and 3 sample solution respectively in a band wise fashion. After development the plate it was dried. Then the dry plate was treated with sulfuric acid-anisaldehyde spraying reagent. The plate was kept at 110 °C for 5 min in hot air oven and evaluation was carried out at 366 and 540 nm.

5.2.5. *Results and discussion*

The percentage content of linoleic acid in *Solanum nigrum* berries methanolic extract was found to be 9.21 %. This was determined by a calibration curve with the equation of Y = 3141.508*X + 1366.840 (correlation coefficient = 0.9954 and standard deviation = ± 5.46%) as shown in Fig 5.10 where X represents amount of linoleic acid and Y represents area under the curve.

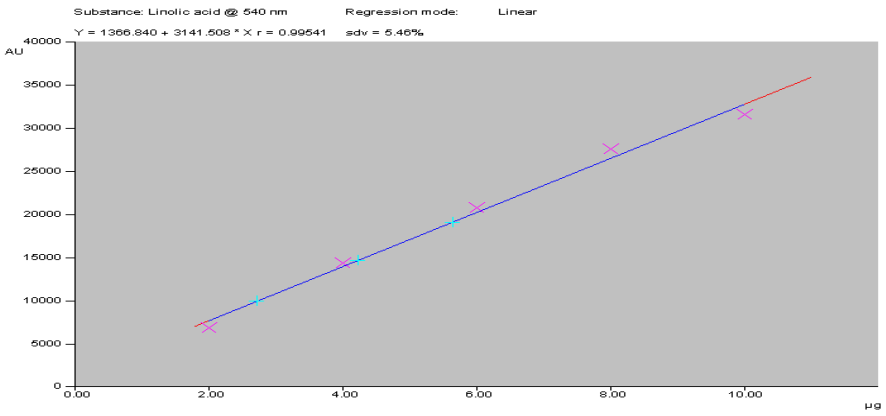


Figure 5.10. Calibration curve of linoleic acid.

R_f value of standard linoleic acid was found to be 0.56. Specificity was confirmed by comparing the R_f of standard and sample (Figure 5.11- 5.12).

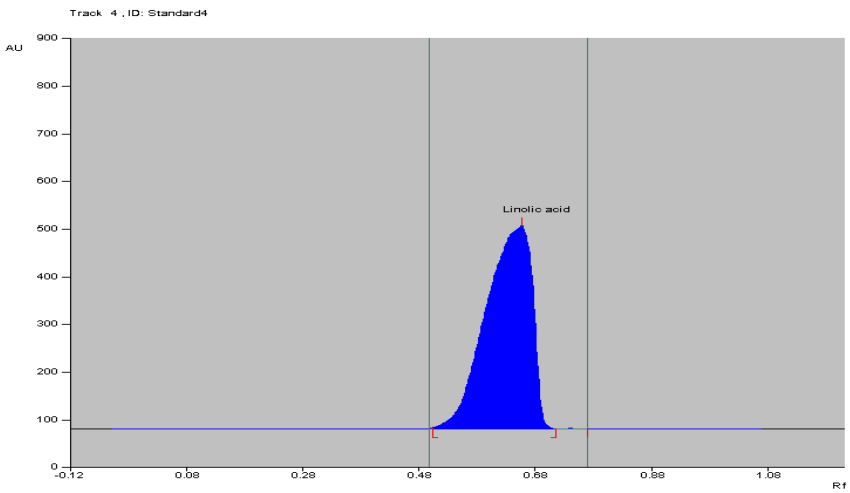


Figure 5.11. HPTLC chromatogram of standard linoleic acid.

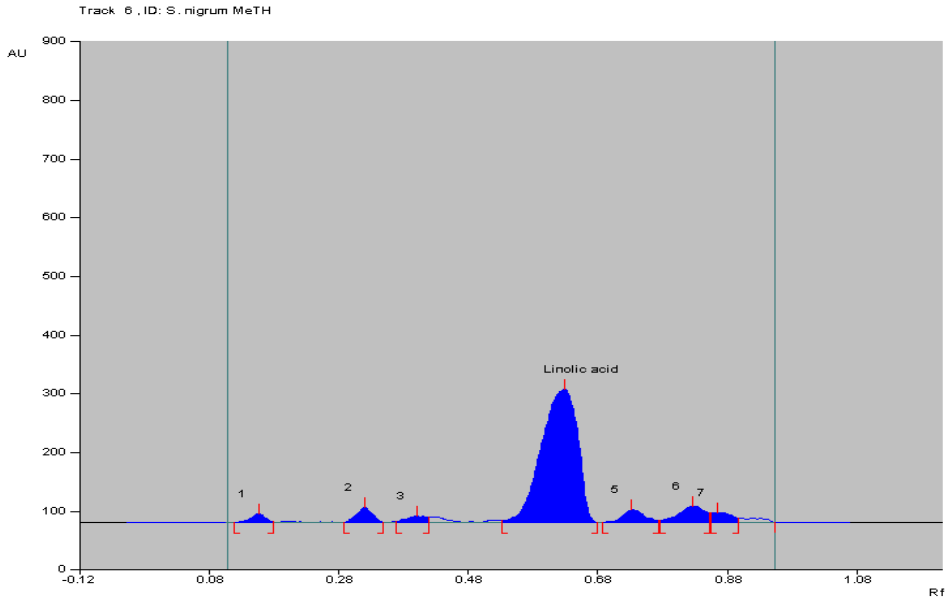


Figure 5.12. HPTLC chromatogram of *Solanum nigrum* berries methanolic extract.

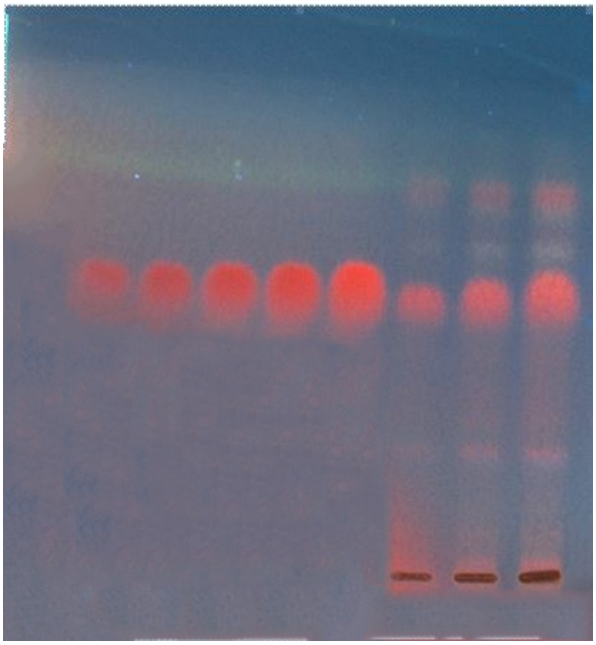


Figure 5.13. Photodocumentaton of *Solanum nigrum* berries methanolic extract at 366 nm.

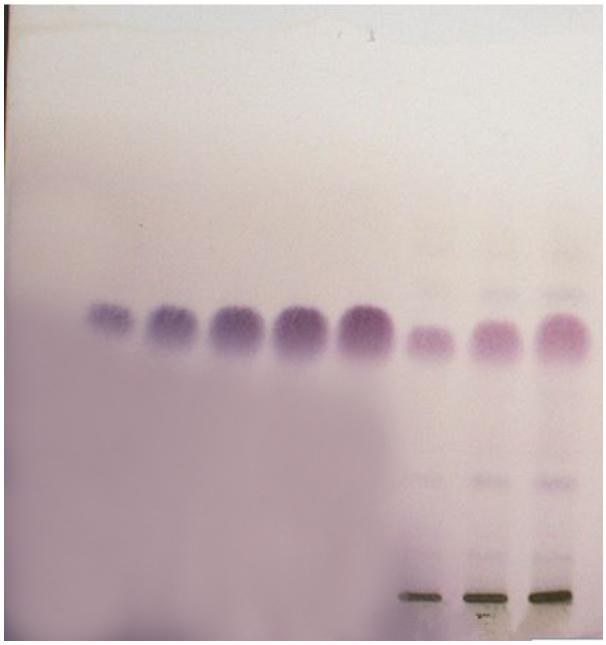


Figure 5.14. Photodocumentaton of *Solanum nigrum* berries methanolic extract at 540 nm.

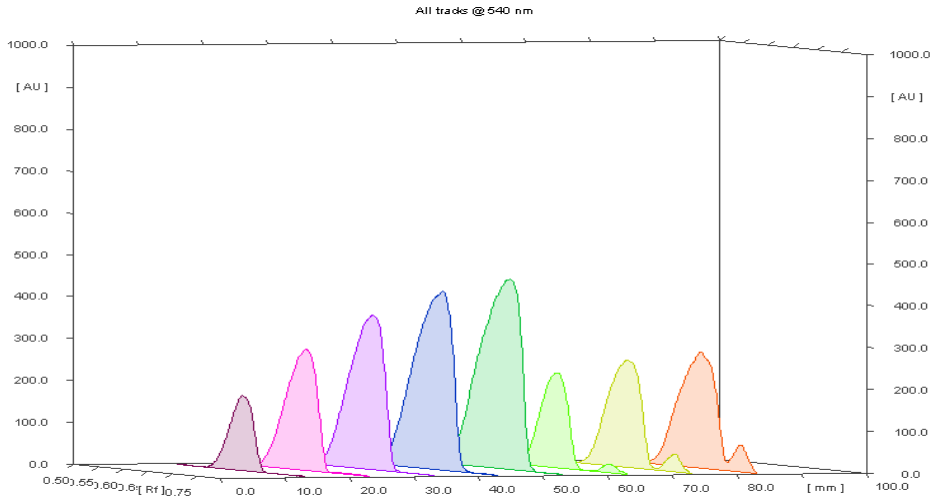


Figure 5.15. 3D Chromatogram of *Solanum nigrum and* standard linoleic acid at 540 nm.

5.3. Standardization of quercetin *in Hibiscus rosa-sinensis* flower by HPTLC

5.3.1. *Equipments and reagents*

The CAMAG HPTLC system consisting of WINCATS software, LINOMAT V automatic sample applicator, and automatic development chamber, scanning densitometer CAMAG scanner 3 and photo documentation apparatus CAMAG reprostar-3 were used. Stationary phase was used as aluminum based silica gel plate 60 F_{254} (Merck, Mumbai) with 10 cm x 10 cm in a particle size of 5-10 µm. All the solvents were used of analytical grade. 100 µl syringe (HAMILTON, Switzerland) was used for sample application on HPTLC plates. Quercetin was present in lab**.** Methanol, toluene and ethyl acetate (analytical grade) were procured from Merck (Mumbai, India). All the samples were filtered through Whitman's syringe filter (NYL 0.45 µ).

5.3.2. *Preparation of standard solution*

About 1 mg of quercetin standard was weighed and put in to 1 mL eppendorf tube. 1.0 mL of methanol was added with it and mixed in vortex mixture till the material completely dissolved. It was then filtered through 0.45 µ syringe filter and kept for further study.

5.3.3. *Preparation of sample solution*

About 10 mg of *Hibiscus rosa-sinensis* flower methanolic extract was dissolved in 1 mL methanol in eppendorf tube. It was then mixed in vortex mixture and put to ultrasonication bath till the material completely dissolved. Then it was filtered through 0.45 µ syringe filter and kept for further study.

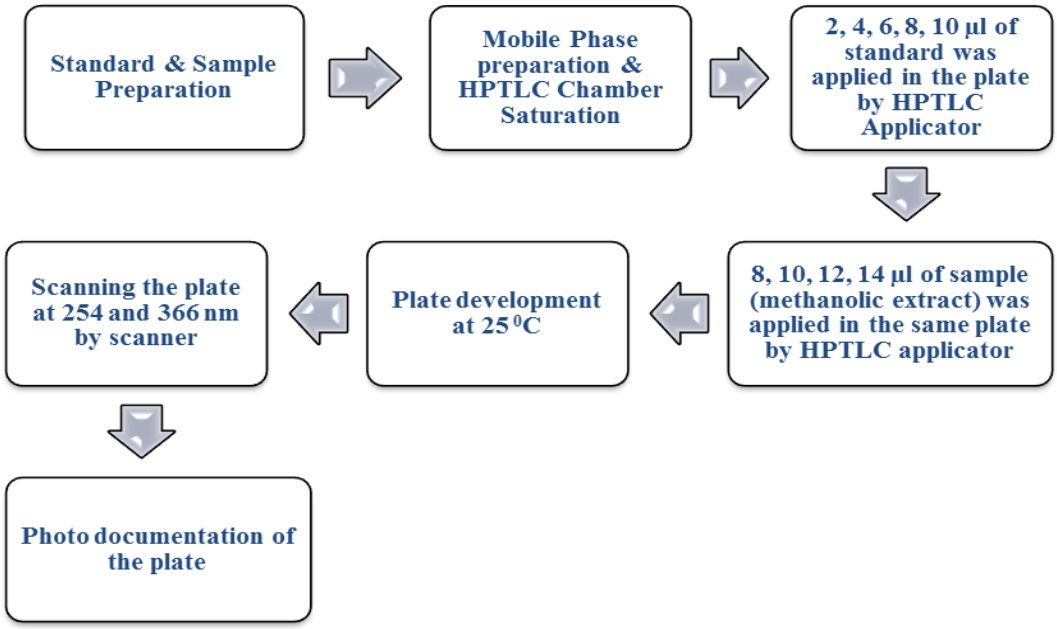


Figure 5.16. Standardization of quercetin *in Hibiscus rosa-sinensis* flower by HPTLC.

5.3.4. *Chromatographic conditions*

HPTLC analysis was performed using isocratic technique by external methods. The mobile phase was optimized with toluene: ethyl acetate: methanol in a ratio of 5: 3: 2 v/v. The temperature was kept at 25 °C and mobile phase was developed in a twin trough glass chamber. The standard solution was applied 2, 4, 6, 8, 10 µL and sample solution was applied consequently in the range of 8, 10, 12 and 14 µL. Total 9 tracks in HPTLC plate were used for the development of 5 standard and 4 sample solution respectively in a band wise fashion. After drying the colored bands were observed at 254 to 360 nm.

5.3.5. *Results and discussion*

The percentage content of quercetin in methanolic extract of *Hibiscus rosa-sinensis* flower was found to be 0.30 % w/w. This was determined by a calibration curve with the equation of Y = 3303.952*X + 2991.844 (correlation coefficient = 0.9960 and standard deviation = ± 2.22%) as shown in Fig 5.17 where X represents amount of quercetin and Y represents area under the curve.

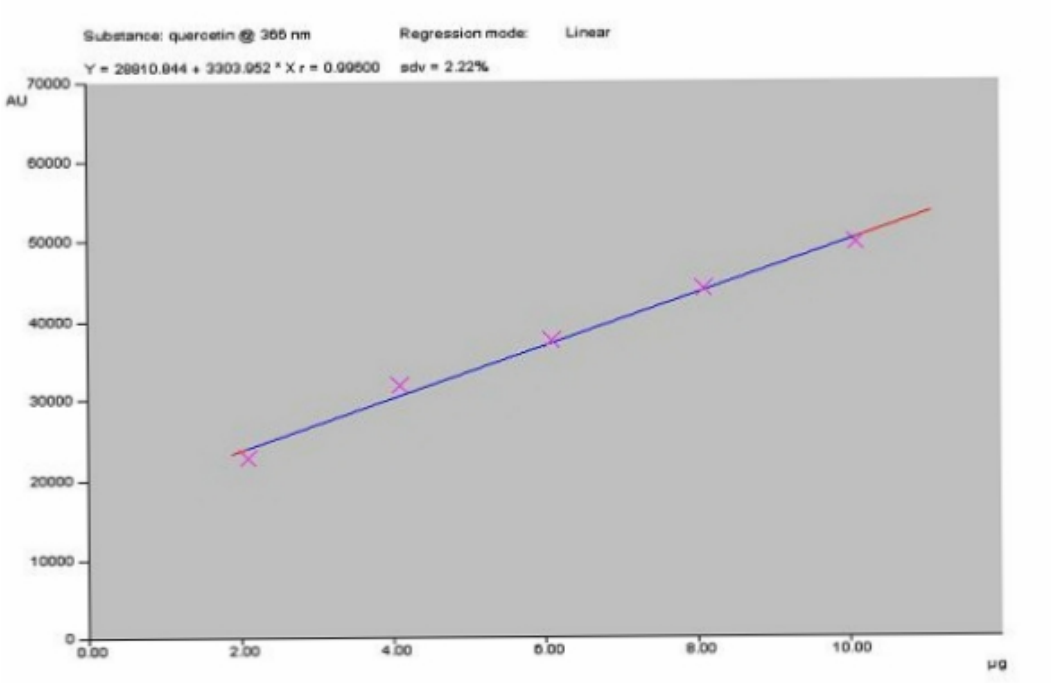


Figure 5.17. Calibration curve of standard quercetin.

R_f value of standard quercetin was found to be 0.52. Specificity was confirmed by comparing the R_f of standard and sample (Figure 5.18 - 5.19).

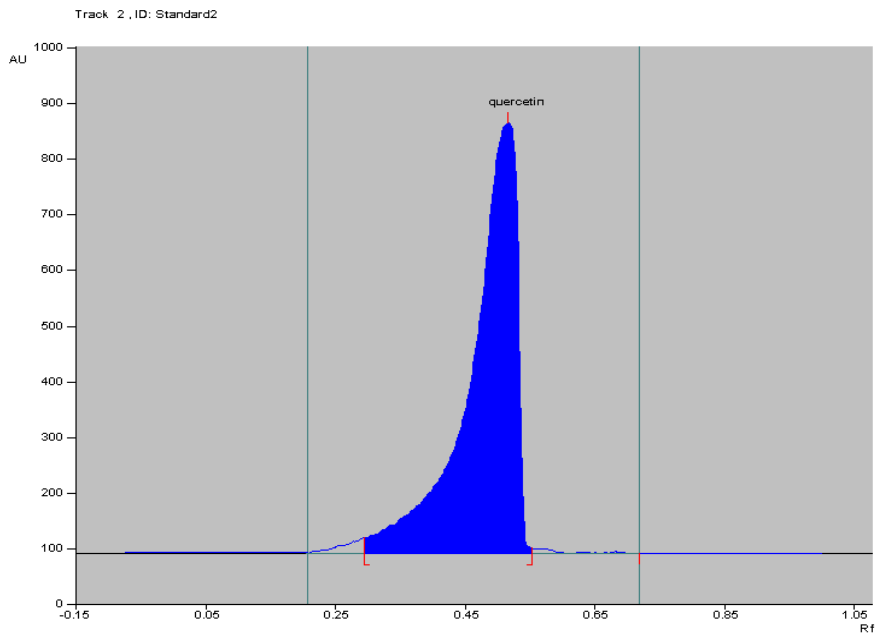


Figure 5.18. HPTLC chromatogram of standard quercetin.

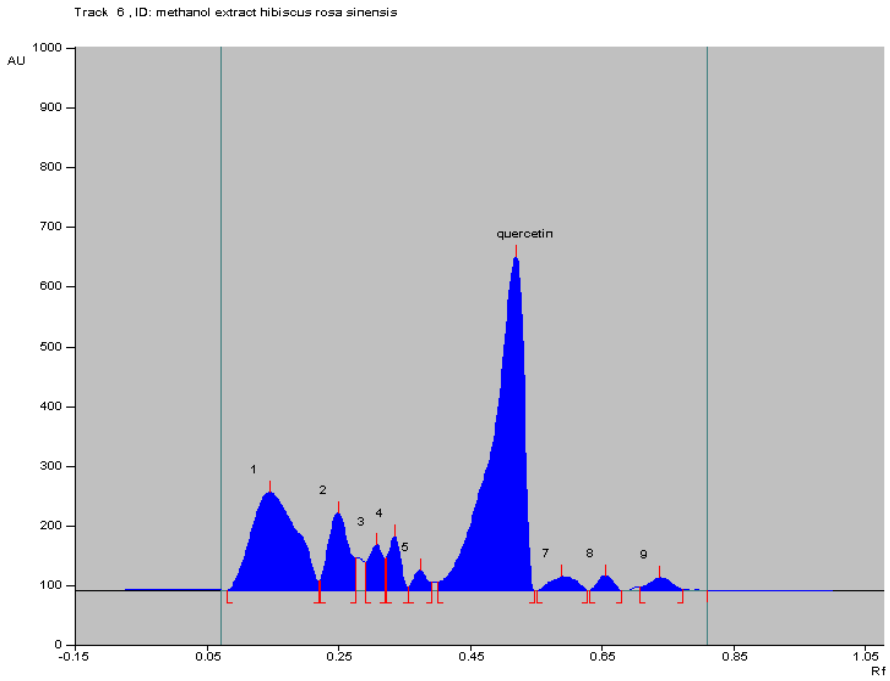


Figure 5.19. HPTLC chromatogram of methanolic extract of *Hibiscus rosa-sinensis* flower.

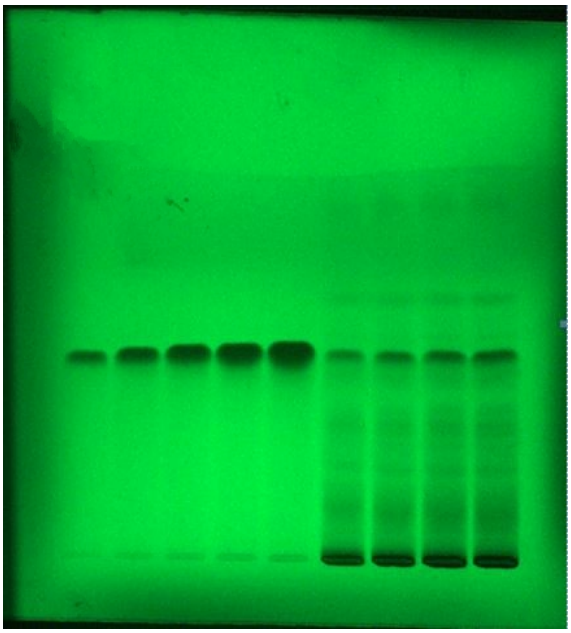


Figure 5.20. Photodocumentaton of methanolic extract of *Hibiscus rosa-sinensis* flower at 254 nm.

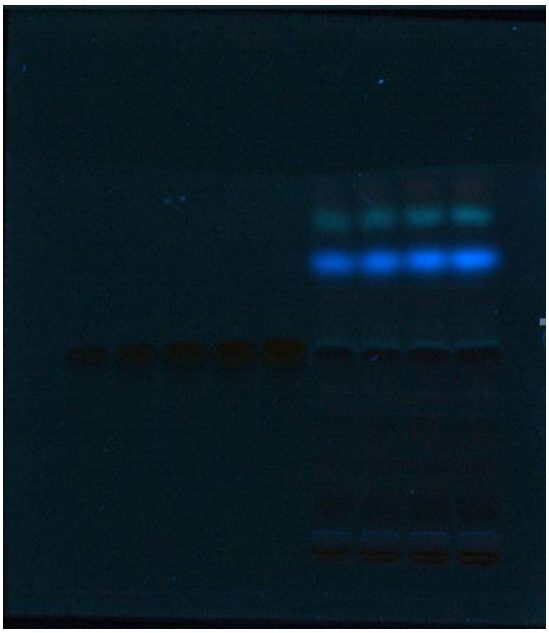


Figure 5.21. Photodocumentaton of methanolic extract of *Hibiscus rosa-sinensis* flower at 366 nm.

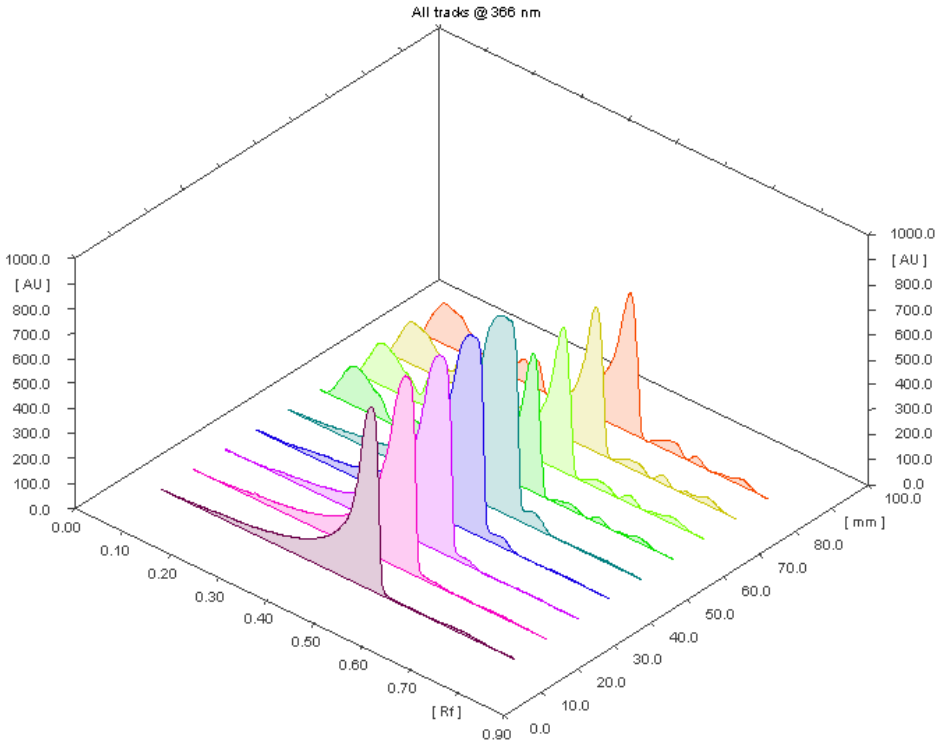


Figure 5.22. 3D Chromatogram of *Hibiscus rosa-sinensis* and standard quercetin at 540 nm.

5.4. Standardization and quantification of β-sitosterol in *Eclipta alba* by HPLC

5.4.1. *Equipments and reagents*

RP-HPLC system (Shimadzu Prominence, Kyoto, Japan) equipped with two Shimadzu LC-20 AD UFLC reciprocating pumps, a variable Shimadzu SPD-M20A Prominence PDA detector and a Rheodyne manual injector with a loop size of 20 µl was used. The peak area was calculated with LC solution software. The analysis was carried out in isocratic condition using a C_{18} reverse phase column having dimension of 250 mm (length) ×4.6 mm (width) with a particle size of 5 µm (Phenomenex-Luna C_{18}, Torrance, CA, USA).100 µl syringe (HAMILTON, Switzerland) was used for sample injection on HPLC. β-sitosterol was purchased from sigma Aldrich. Methanol, acetonitrile analytical grade were purchased from Merck (Mumbai, India). All other solvents were used of analytical grade. Whatman's syringe filter (NYL 0.45 µm) was used for the filtration of samples and standard.

5.4.2. *Preparation of standard solution*

About 1 mg of β-sitosterol standard was weighed and put in to separate 1 mL eppendorf tube. 1.0 mL of methanol in added in 1 mL eppendorf tube and mixed in vortex mixture

till the material completely dissolved. Volume was made with methanol to obtain standard stock solution of concentration 1000 µg/mL. It was then filtered through 0.45 µ syringe filter and kept for further study. Further dilutions were made as required with methanol for calibration curve.

5.4.3. *Preparation of sample solution*

About 1 mg of petroleum ether extract of *Eclipta alba* was weighed and taken in a eppendorf tube. 1 mL petroleum ether added to the centrifuge tube. Mixed in vortex mixture for 15 min and put to ultrasonication bath for 30 min. It was then filtered through 0.45 µ syringe filter and kept it in 1 mL eppendorf tube for further study. Sample solution obtained concentration was 1000 µg/mL.

5.4.4. *Chromatographic conditions*

HPLC analysis was performed using isocratic technique. Mobile phase was optimized with of Acetonitrile and Methanol in the ratio of 70:30 v/v respectively. The temperature was kept at 25 °c and mobile phase was inject 20 µl. Standard stock solution of biomarker was applied consequently in the range of 2.5 to 15 µl with 2.5 µl gradual increment for calibration curve. Sample solution was applied 20 µl. Stationary phase used RP C18 (250 x 4.6, 5 µm). Detection was carried out at 210 nm wavelength. Flow rate was adjusted to 1 mg/mL.

Table 5.1. HPLC Method Parameters

HPLC System	**:**	Shimadzu HPLC with rheodyne injector.
Pump	**:**	Binary pump.
Detector	**:**	PDA Multi-wavelength detector.
Stationary phase	**:**	RP-C18 (250 mm × 4.6 mm i.d., particle size 5 µm) column.
Mobile phase	**:**	Acetonitrile: Methanol (70:30 v/v).
Detection wavelength	**:**	UV detector, 210 nm.
Flow rate	**:**	1 mL/min.
Sample size	**:**	20 µL.

5.4.5. *Results and discussion*

The percentage content of β-sitosterol in *Eclipta alba* petroleum ether extract was found to be 4.67 % w/w. This was determined by a calibration curve with the equation of Y = 2649.8*X + 228397 (correlation coefficient = 0.9923 as shown in Fig 5.23 where X represents amount of β-sitosterol and Y represents area under the curve.

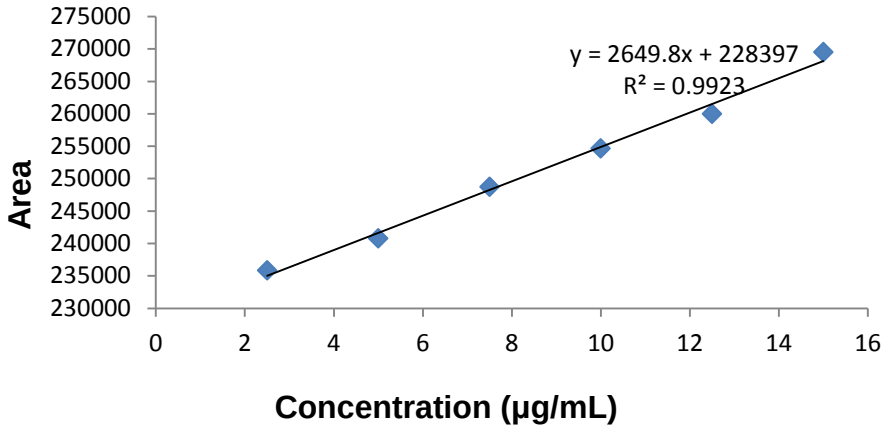


Figure 5.23. Calibration curve of β-sitosterol.

Retention time (R_t) value of standard β-sitosterol was found to be 2.325 min. Specificity was confirmed by comparing the R_t of standard and sample (Figure 5.24 -5.25).

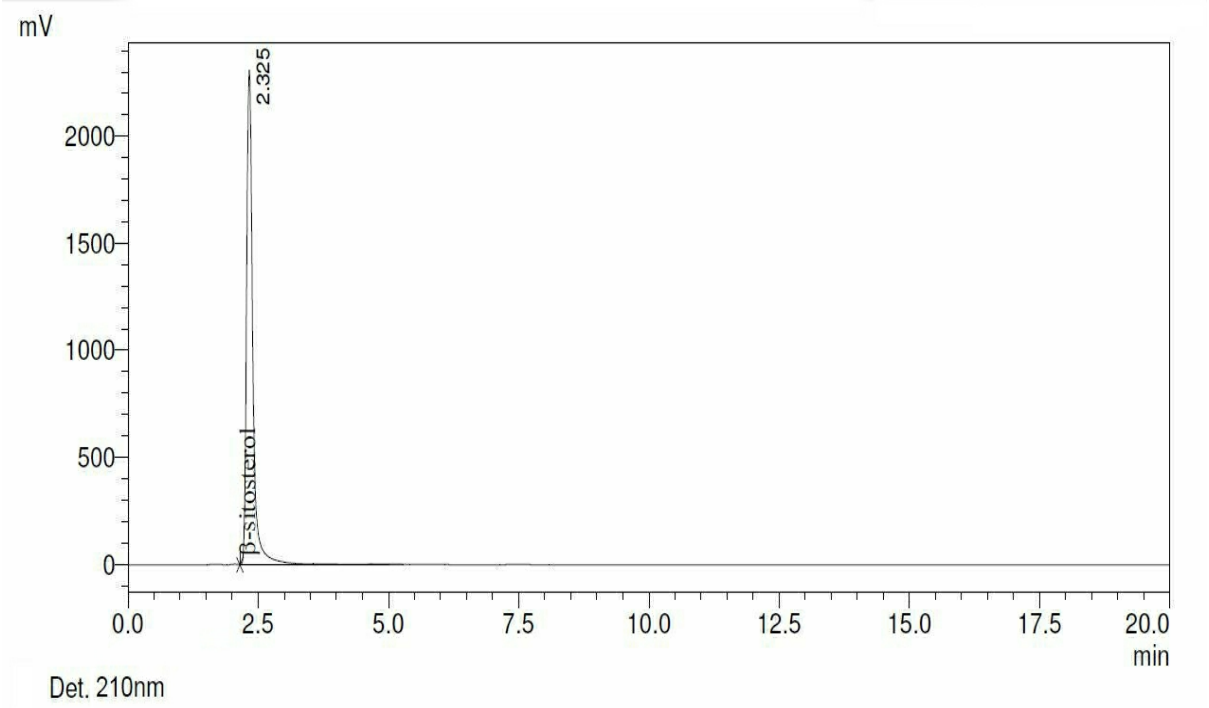


Figure 5.24. Chromatogram of β-sitosterol**.**

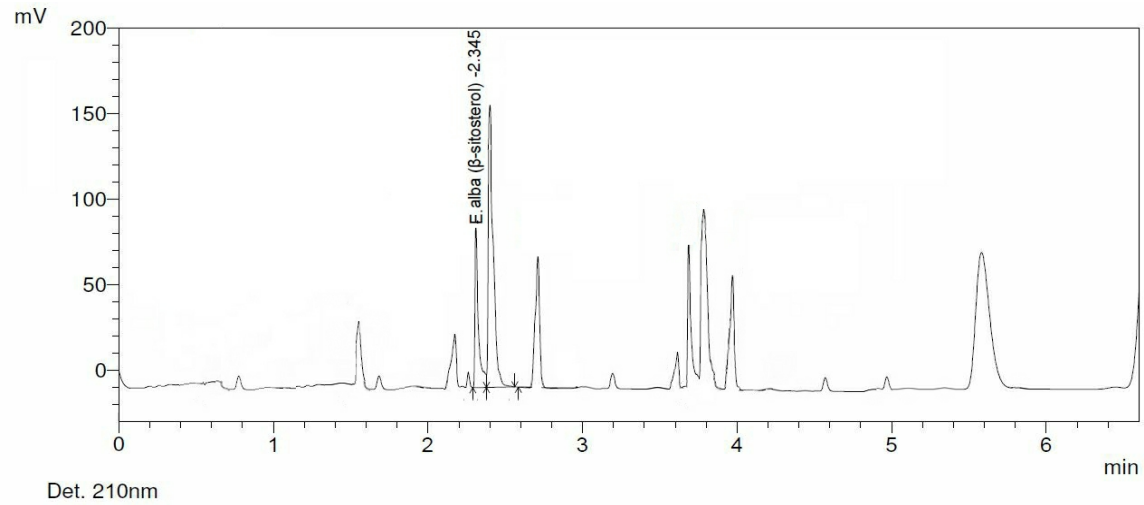


Figure 5.25. Chromatogram of *Eclipta alba* petroleum ether extract.

5.5. Standardization of quercetin *in Hibiscus rosa-sinensis* flower by HPLC

5.5.1. *Equipments and reagents*

RP-HPLC system (Shimadzu Prominence, Kyoto, Japan) equipped with two Shimadzu LC-20 AD UFLC reciprocating pumps, a variable Shimadzu SPD-M20A Prominence PDA detector and a Rheodyne manual injector with a loop size of 20 µl was used. The peak area was calculated with LC solution software. The analysis was carried out in isocratic condition using a C_{18} reverse phase column having dimension of 250 mm (length) ×4.6 mm (width) with a particle size of 5 µm (Phenomenex-Luna C_{18}, Torrance, CA, USA). Quercetin was taken from lab. Methanol, acetonitrile analytical grade were purchased from Merck (Mumbai, India). All other solvents were used of analytical grade. Whatman's syringe filter (NYL 0.45 µm) was used for the filtration of samples and standard.

5.5.2. *Preparation of standard solution*

About 1 mg of quercetin standard was weighed and put in to separate 1 mL eppendorf tube. 1.0 mL of methanol in added in 1 mL eppendorf tube and mixed in vortex mixture till the material completely dissolved. It was then filtered through 0.45 µ syringe filter and kept for further study. Volume was made with methanol to obtain standard stock solution of concentration 1000 µg/mL. It was then filtered through 0.45 µ syringe filter and kept for further study. Further dilutions were made as required with methanol for calibration curve.

5.5.3. *Preparation of sample solution*

About 1 mg of *Hibiscus rosa-sinensis* flower methanolic extract was weighed and taken in a centrifuge tube. 1 mL methanol added to the eppendorf tube. Mixed in vortex

mixture for 15 min and put to ultrasonication bath for 30 min. It was then filtered through 0.45 µ syringe filter and kept it in 1 mL eppendorf tube for further study. Sample solution obtained concentration was 1000 µg/mL.

5.5.4. *Chromatographic conditions*

HPLC analysis was performed using isocratic technique by external methods. Mobile phase was optimized with of Methanol: 0.1% ortho-phosphoric acid (65:35%). respectively. The temperature was kept at 25 °c and mobile phase was inject 20 µl. Standard stock solution of biomarker was applied consequently in the range of 2.5 to 15 µl with 2.5 µl gradual increment for calibration curve. All the parameters are given in table 5.2.

Table 5.2. HPLC Method Parameters

HPLC System	:	Shimadzu HPLC with rheodyne injector.
Pump	:	Binary pump.
Detector	:	PDA Multi-wavelength detector.
Stationary phase	:	RP-C18 (250 mm × 4.6 mm i.d., particle size 5 µm) column.
Mobile phase	:	Methanol: 0.1% ortho phosphoric acid (65:35%).
Detection wavelength	:	UV detector, 369 nm.
Flow rate	:	1 mL/min.
Sample size	:	20 µl.

5.5.5. *Results and discussion*

The percentage content of quercetin in methanolic extract of *Hibiscus rosa-sinensis* flower was found to be 0.37 % w/w. This was determined by a calibration curve with the equation of Y = 95635*X - 66568 (correlation coefficient = 0.9931) as shown in Fig 7.4 where X represents amount of quercetin and Y represents area under the curve.

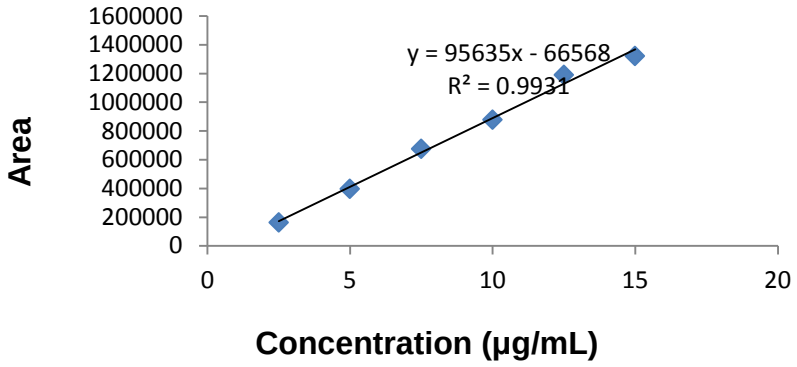


Figure 5.26. Calibration curve of quercetin.

R_t value of standard quercetin was found to be 5.034 min. Specificity was confirmed by comparing the R_t of standard and sample (Figure 5.27 – 5.28).

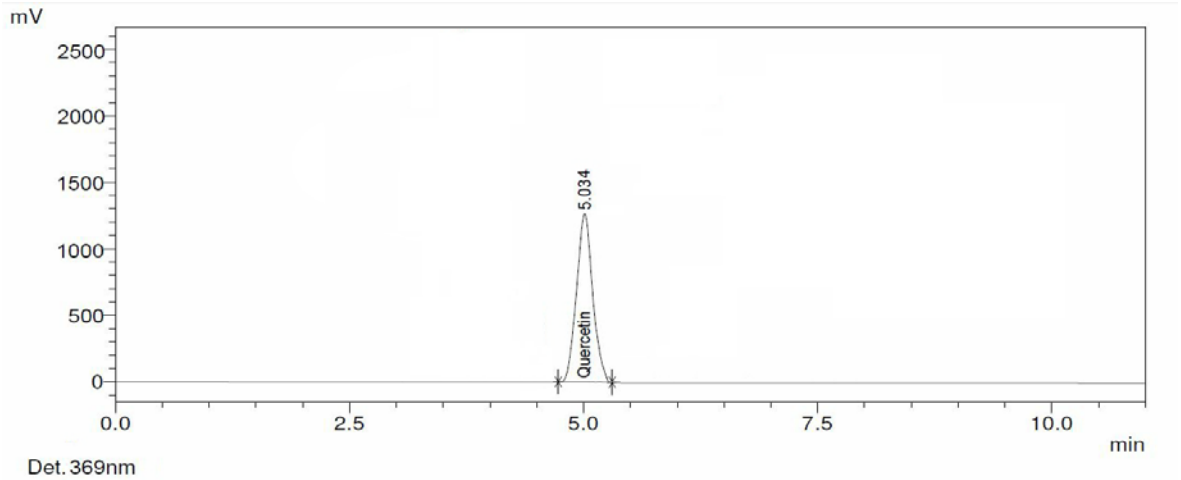


Figure 5.27. Chromatogram of standard quercetin.

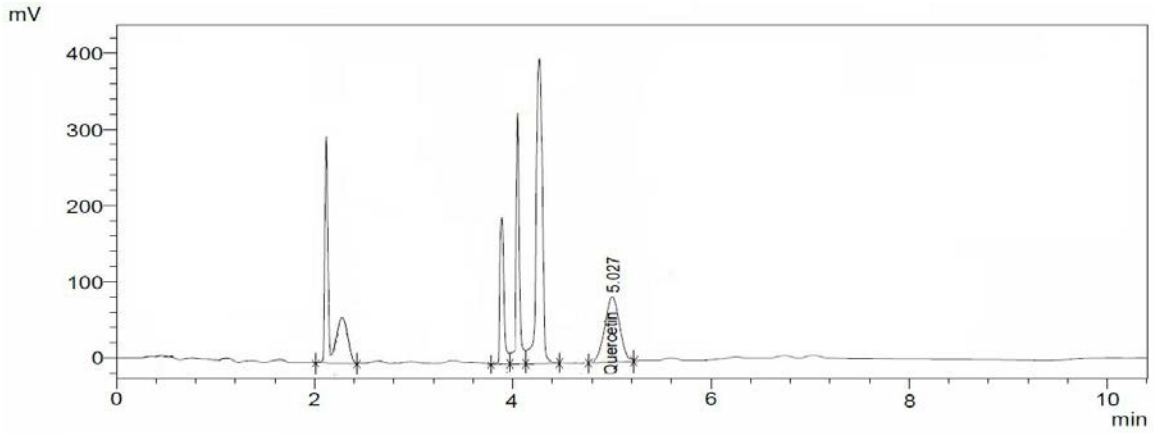


Figure 5.28. Chromatogram of *Hibiscus rosa-sinensis* flower methanolic extract.

5.6. Atomic absorption spectroscopy (AAS) study of heavy metal analysis of plant extracts

Medicinal plants have been used to treat many ailments since the ayurvedic age. Trace elements present in plant play an important role in healing and prevention of many diseases. Elements such as iron (Fe), copper (Cu), cobalt (Co), nickel (Ni), zinc (Zn), magnesium, manganese (Mn), molybdenum, chromium (Cr), vanadium, lithium, selenium, fluorine (F) and iodine (I). But less quantities of heavy metals like lead (Pb), cadmium (Cd), arsenic (As) and mercury (Hg) are toxic for human health when exposed for a long time (Lobet et al., 2003). According to World Health organization (WHO, 1989) the maximum permissible levels in food and drug materials of heavy metals are given only for arsenic (As),cadmium (Cd) and lead (Pb) which are 1.0, 0.3 and 10 mg/kg,

respectively. These heavy metals are toxic to human. For example, Lead (Pb) can cause renal tumors; cadmium (Cd) can cause osteomalacia and pyelonephritis. Chromium is responsible for causing respiratory diseases as well as nephrotoxic ailments. Occupational exposure to hexavalent chromium (Cr) compounds causes different diseases. Skin contact of chromium compounds can result in skin allergies, dermatitis, dermal necrosis and dermal corrosion (Kota's and Stasicka., 2000). Thus, especially medicinal plants, is part of quality control, which has been established by their purity, safety and efficacy (Mukherjee et al., 2003).

5.6.1. *Instrumentation of AAS*

As early as 1860, Kirchhoff described the basic principle of atomic absorption spectra. It was not until 1955, the analytical back ground for its analytical applications were demonstrated by Walsh, Alkemade and Milatz. The simplicity of this technique marks it an attractive tool for the analysis of many elements. In atomic absorption spectrometry, the elements are transformed into the atomic vapour form by drawing an aerosol of the sample solution into an open flame. Most of the freed atoms are then excited by exposure to a suitable source of radiation. The radiation absorbed by the unexcited atoms is related to the sample concentration. In this sense atomic absorption spectrometry then could be envisaged as the inverse of emission spectrometry where the radiation emitted by the thermally excited atoms is related to concentration. It should be emphasized that usually the fraction of atoms excited by heat (via flame or an electric arc) is relatively small for most elements.

An atomic absorption spectrometer consists of the following elements.

Source: Single-element or multi-element hollow cathode tubes generally are employed as sources in atomic absorption. Less frequently, the bright continuum of a xenon arc has been used as a source. Collision of these atoms with an inert gas such as argon induces excitation of the metal atoms and subsequent emission of characteristic radiation. **Burner:** The quality of the burner, the type of fuel and the ratio of fuel to oxidise are the important factors which affect the result of analysis by an atomic absorption instrument. The burner can be compared to a sampling cell in a spectrometer. **Monochromator**: The monochromator should be able to pass the resonance line and filter out other. **Phototube** and **Amplifier**: Following factors are affecting the atomic absorption spectrometer. In general, an organic solvent enhances the absorption signal and therefore, it may alter the absorption intensity. These can bond strongly with metals and tend to reduce the signal intensity. EDTA could eliminate such effect.

5.6.2. *Chemicals and reagents*

Nitric acid (HNO_3), perchloric acid ($HClO_4$), hydrochloric acid (HCl) and sulfuric acid (H_2SO_4) were used and all were of analytical grade. The concentration of stock solution was 1000 ppm. All the metals were procured from Merck (Darmstadt, Germany).

5.6.3. *Sample preparation*

For the analysis, samples were ground to a fine powder and dried at 55 to70 ^{0}C for 6–8 hrs in a controlled environment, to remove moisture. Immediately after drying, accurately weighed 1.0 g was placed in a flask and treated with 12 mL of concentrated HNO_3 for 24 hrs. A mixture of H_2SO_4 and HNO_3 (3:1) was added 5 mL in each of the conical flask. The mixture was heated at 120–130 ^{0}C for 5–6 hrs, until fumes stop and until the resulting solution was become clear. It was cooled at room temperature and filtered using Whatman filter paper no. 42. The entire filtrate was mixed and made the volume up to 50 mL with Milli-Q water. A blank was also prepared for every sample in the same way. Each sample was aspirated twice and the experiment was repeated for five times.

Table 5.3. Instrumental condition for trace and heavy metal analysis by atomic absorption spectrometry

Elements / AAS specification	Copper	Chromium	Nickel	Arsenic	Lead	Mercury
Wavelength	324.8	357.9	232.0	193.7	217.0	253.7
Current (mA)	5.0	5.0	9.0	12.0	9.0	3.0
Flame	AA	AA	AA	AA	AA	-
Fuel (L/min)	3.05	2.90	2.94	2.40	2.90	7.66
Slit width(nm)	0.5	0.5	0.5	0.5	0.5	0.5
Working range (ppm)	1-5	2-8	3-10	0.04-0.1	2-10	1-0.2
Read time (sec)	3	3	3	3	3	3
Wash time (sec)	10	10	10	10	10	10

AA - Air acetylene flame, AAS - Atomic absorption spectrophotometer

5.6.4. *Statistical analysis*

The data were represented as the mean ± SEM using the Graph-Pad Prism Version 6.0 on the basis of the number of samples analyzed.

5.6.5. *Result*

Table 5.4. Result of heavy metal analysis by atomic absorption spectrometry

Plants used	Copper (ppm ±SEM)	Chromium (ppm ±SEM)	Cadmium (ppm ±SEM)	Lead (ppm ±SEM)	Arsenic (ppm ±SEM)	Mercury (ppm ±SEM)
Eclipta alba	1.151 ± 0.031	0.308 ±0.012	0.021 ±0.035	0.860 ±0.009	0.081 ±0.007	0.036 ±0.010
Hibiscus rosa-sinensis	2.605 ±0.045	0.450 ±0.025	0.018 ±0.050	0.630 ±0.025	0.041 ±0.014	0.023 ±0.006
Solanum nigrum	1.549 ±0.087	0.304 ±0.055	0.012 ±0.150	0.945 ±0.005	0.034 ±0.011	0.040 ±0.015

5.6.6. *Conclusion*

The concentration of trace metals and heavy metals of three plants were determined by AAS. The quantitative determinations were carried out using standard calibration curve obtained by the standard solution of metals having optimal detectable concentration ranges. The concentration of the metals obtained in plant material was expressed in terms of parts per million. The levels of heavy metals quantified in all the plant samples were found within prescribed limits.

Chapter - 6

Evaluation of 5α-reductase inhibitory potential

6.1. 5α-reductase inhibitory potential *Eclipta alba*

6.2. 5α-reductase inhibition of *Hibiscus rosa-sinensis* flower (methanolic extract)

6.3. 5α-reductase inhibition of *Solanum nigrum* plant berries (methanolic extract)

6.4. 5α-reductase inhibition of β-sitosterol, quercetin, linoleic acid biomarker

6.5. Comparison study of 5α-reductase inhibitory potential of plants

6.6. Conclusion

6.1. 5α-reductase inhibitory potential *Eclipta alba*

6.1.1. *Reagent used*

Testosterone was obtained from marketed formulation Testoviron Depot injection (100 mg/mL), finasteride was obtained from marketed formulation Fincar tablet (contains 5 mg finasteride in each tablet), NADPH was purchased from Sisco research laboratories (SRL) and Tris buffer were purchased from Sigma-Aldrich. Ethylenediamine tetraacetic acid (EDTA), sodium Phosphate and sucrose were purchased from Merck, Mumbai. Methanol, ethanol (95%), ethyl acetate and petroleum ether (60–80°C) (analytical grade) were purchased from Merck, Mumbai. All other chemicals used in the study were of analytical grade.

6.1.2. *Reagent and chemical preparation*

6.1.2.1. *Tissue homogenization medium*

0.0372 g EDTA, 10.95 g sucrose and 0.760 g tri-sodium phosphate dodecahydrate dissolved in 100 mL water, pH was adjusted to 6.5.

6.1.2.2. *Preparation of NADPH solution*

Stock NADPH solution was prepared by dissolving of 0.0083 g NADPH in 10 mL methanol (1000 µM). 1.54 mL NADPH stock solution was diluted with 68.46 mL methanol for preparation of 22 µM working solution (70 mL).

6.1.2.3. *Preparation of testosterone solution*

Stock solution: Testosterone stock was prepared by dissolving 28 µL of testosterone solution (injection) in 9972 µL methanol. 28 µL of testosterone solution contains 0.0028 g of testosterone in 10 mL methanol (1000 µM).

Working solution: 3.52 mL stock was diluted with 43.48 mL methanol for preparation of 75 µM working solutions (46 mL).

6.1.2.4. *Preparation of finasteride solution*

Stock solution: 0.1151 g finasteride cursed power (tablet) was dissolve in 10 mL methanol. 0.1151 g finasteride cursed power contains approximate 0.0037 g of pure finasteride. Sonication was carried out for 1 hour and vortexed for 15 min. Then filtration was carried out by 0.45 µ syringe filter, and filtrate was collected in two separate eppendorf tube.

Working solution: 2 µL of stock finasteride solution diluted with 1998 µL methanol to obtained 1 µM working solutions. Further dilution was done 0.1, 0.2, 0.4, 0.6, 0.8 µM respectively for determine the IC_{50} value.

6.1.2.5. *Preparation of Tris HCl*

7.88 g tris HCl dissolved in 100 mL water.

6.1.3. *Methods of preparation 5α-reductase solution*

6.1.3.1. *Isolation of 5α-reductase enzyme solution*

Rats were anesthetized with diethyl ether. Testes were quickly removed. Isolated testis was wash with 0.9% normal saline. Testes were minced in small piece and homogenized with medium (20 mM sodium phosphate, pH 6.5, containing 0.32 M sucrose and 1 mM EDTA). The homogenate was centrifuged at 4000 rpm (716 g) for 15 min. the Supernatant was used as source of enzyme. Protein concentration in the supernatant was determined by Bradford method of protein estimation (Nahata & Dixit, 2013).

6.1.3.2. *Estimation of protein concentration*

6.1.3.2.1. *BSA standard curve*

1 mg/mL stock BSA solution was prepared in deionized water. Two fold serial dilutions were prepared from stock with the concentrations of 0.5, 0.25, 0.125 mg/mL. Different concentrations of prepared BSA solution (5 µL) were added to 96 well micro plate. 200 µL of Bradford reagents were added to the BSA solutions. Absorbance was measured at 592 nm. Standard curve was plotted with the concentrations of standards against the absorbance at 592 nm.

6.1.3.2.2. *Protein concentration of testis solution*

5 µL of rat testis solutions were added to micowell plate. 200 µL of Bradford reagent was added into the test solution. Absorbance was measured at 592 nm. Concentration of the protein was determined from BSA standard curve.

6.1.3.2.3. *Optimum protein concentration for the assay*

Protein concentration of isolated testis was found 16.631 mg/mL. Further the solution was diluted to 100 µg/mL by tissue homogenization medium for enzyme assay.

6.1.4. *Preparation of NADPH stranded curve*

Standard curve of NADPH was prepared in methanol at 340 nm using concentration of 1, 3, 5, 8, 10, 12, 15 and 20 µg/mL.

Standard curve of NADPH was prepared in methanol at 340 nm using concentration of 1, 3, 5, 8, 10, 12, 15 and 20 µg/mL. Equation of straight line was y = 0.0244x + 0.00056, and the correlation coefficient (r^2) was = 0.9978 indicating good linearity between absorbance and concentration.

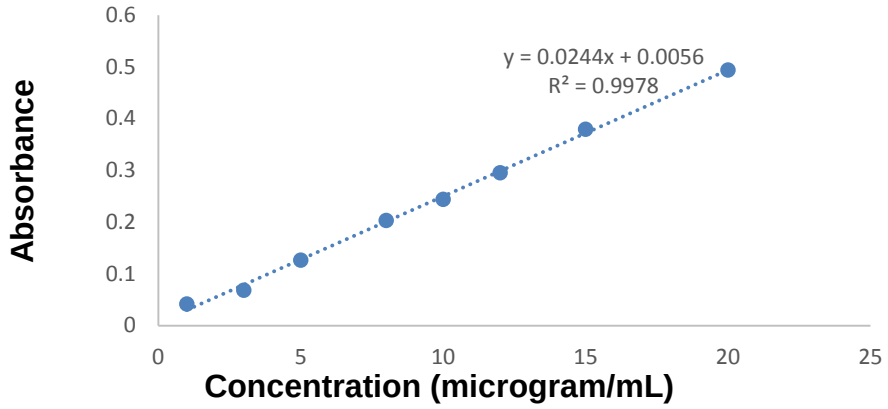


Figure 6.1. Calibration curve of NADPH.

6.1.5. *Preparation of plants extract*

About 10 mg of *Eclipta alba* methanolic and petroleum ether extract was taken in separate 2 mL of eppendorf tube and mixed with 2 mL methanol and petroleum ether respectively. It was mixed in vortex mixture and put to ultrasonication bath till the material completely dissolved. It was then filtered through 0.45 µ syringe filter and kept for further study. Further dilution was done 25, 50, 75, 100, 150, 200, 300 µg/mL respectively.

6.1.6. *Assay procedure of 5α-reductase inhibition of Eclipta alba*

5α-reductase inhibition assays were performed based on the method described by Nahata & Dixit, 2013. In brief, rat testis homogenate containing enzyme, T, NADPH and test samples were mix together. The detail reaction mixtures have been described in Table 6.1. All the reaction mixtures were incubated at 37^{o}C for 30 min. Absorbance was measured spectrophotometrically at 340 nm. Corresponding concentration of NADPH of the test samples were calculated from the NADPH standard curve. Residual amount of NADPH in the reaction mixture were determined. % scavenging of NADPH was determined from the NADPH concentration. Blank absorbance was deducted from the test samples to get net absorbance of NADPH. The 5α-reductase inhibition was calculated for individual test substances which reflect the original efficacy of the test substances against the enzyme. % inhibition of 5α-reductase was determined by % NADPH scavenging potential.

Table 6.1. Enzyme, substrate and coenzyme mixture

Sample ID	Methanol (mL)	Tris HCL (mL)	NADPH (mL)	Enzyme (mL)	Finasteride / (mL)	Test sample (mL)	Vortex and incubate at 37°C for 10 min	Testosterone (mL)	Vortex and incubate at 37°C for 30 min	Total volume (mL)
Blank Control	4	4	3	1						12
Negative control	2	4	3	1				2		12
Finasteride		4	3	1	2			2		12
Test samples		4	3	1		2		2		12

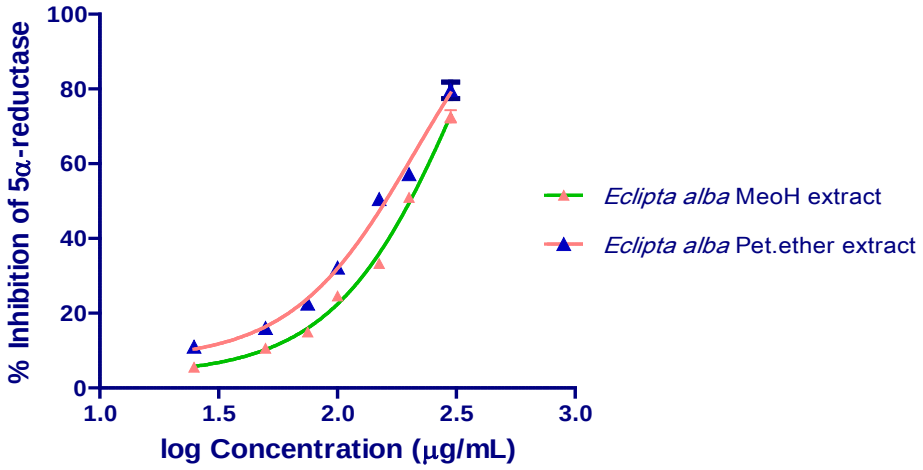
Absorbance at 340 nm, Blank Absorbance = 0.236
Net absorbance of test = (Test Absorbance – Blank Absorbance)
Calculate the NADPH concentration in each tube from NADPH standard curve prepared previously.
Percentage inhibition = (100 – [(54.78-net absorbance of test)/54.78) × 100]
Percentage inhibition of different concentrations of test samples have to determine in order to calculate IC_{50} value of the test extracts.

6.1.7. *Statistical analysis*

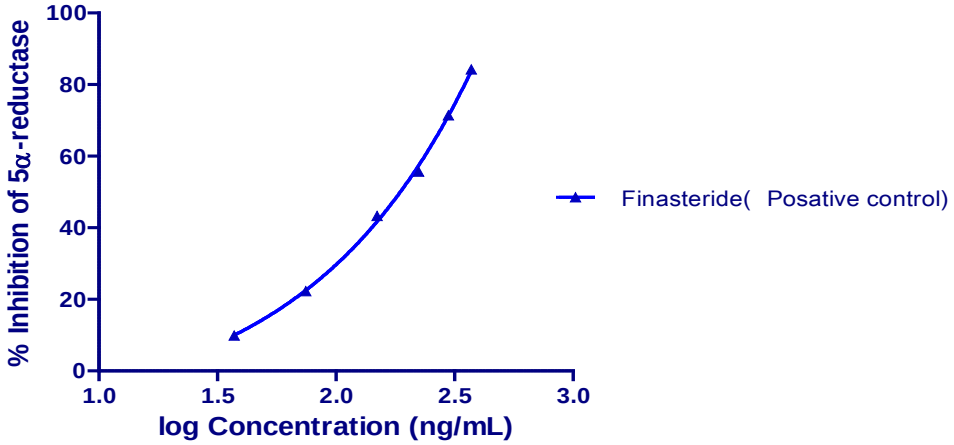
The IC_{50} values were expressed as mean ± standard error mean by plotting the curve with percentage of inhibition versus concentrations of the individual experiments measured. Statistical analysis was performed by one way analysis of variance (One way ANOVA) followed by Bonferroni post hoc test using Graph Pad prism version 6.0. The P was considered as less than 0.05 significance difference as compared to reference standard.

6.1.8. **Results and discussion**

IC_{50} value of *Eclipta alba* methanolic extract and petroleum ether extract (whole plant) is 206.31 ± 9.21 and 173.42 ± 11.35 (µg/mL). Whereas 5α-reductase inhibition of finasteride shown to be 202.59 ± 2.26 (ng/mL). 5α-reductase inhibition of *Eclipta alba* and finasteride shown in table 6.2.



(A)



(B)

Figure 6.2. 5α-reductase inhibition of *Eclipta alba* (A) and finasteride (B).

6.2. 5α-reductase inhibition of *Hibiscus rosa-sinensis* flower extract

6.3.1. *Preparation of sample of plants extract*

About 10 mg of *Hibiscus rosa-sinensis* flower methanolic extract was taken in 2 mL of eppendorf tube. It was mixed with methanol in vortex mixture and put to ultrasonication bath till the material completely dissolved. It was then filtered through 0.45 µ syringe filter and kept for further study. Further dilution was done 25, 50, 100, 150, 200 µg/mL respectively.

6.3.2. *Assay procedure 5α-reductase inhibition of Hibiscus rosa-sinensis*

5α-reductase inhibition assays were performed based on the method described by Nahata & Dixit, 2013. In brief, rat testis homogenate containing enzyme, T, NADPH and test samples were mix together. The detail reaction mixtures have been described in Table 6.1. All the reaction mixtures were incubated at 37 °C for 30 min. Absorbance was measured spectrophotometrically at 340 nm. Corresponding concentration of NADPH of the test samples were calculated from the NADPH standard curve. Residual amount of NADPH in the reaction mixture were determined. % scavenging of NADPH was determined from the NADPH concentration. Blank absorbance was deducted from the test samples to get net absorbance of NADPH. The 5α-reductase inhibition was calculated for individual test substances which reflect the original efficacy of the test substances against the enzyme. % inhibition of 5α-reductase was determined by % NADPH scavenging potential.

Absorbance taken at 340 nm, Blank Absorbance = 0.236

Net absorbance of test = (Test Absorbance – Blank Absorbance)

Calculate the NADPH concentration in each tube from NADPH standard curve prepared previously.

Percentage inhibition: = (100 – [(54.78-net absorbance of test)/54.78) × 100]

Percentage inhibition of different concentrations of test samples have to determine in order to calculate IC_{50} value of the test extracts.

6.3.3. **Results and discussion**

IC_{50} value of *Hibiscus rosa-sinensis* flower methanolic extract was 157.57 ± 11.12 (µg/mL). Whereas 5α-reductase inhibition of finasteride shown to be 202.59 ± 2.26 (ng/mL).

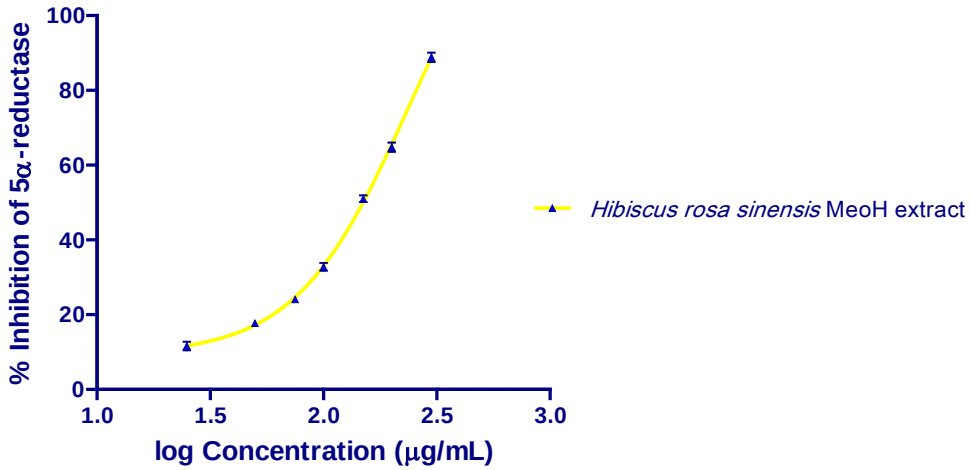


Figure 6.3. 5α-reductase inhibition of *Hibiscus rosa-sinensis* flower extract.

6.3. 5α-reductase inhibition of *Solanum nigrum* plant berries extract

6.3.1. Sample preparation of plants extract

About 10 mg of *Solanum nigrum* plant berries methanolic extract was taken in 2 mL of eppendorf tube and added 2 mL methanol. It was mixed in vortex mixture and put into ultrasonication bath till the material completely dissolved. It was then filtered through 0.45 µ syringe filter and kept for further study. Further dilution was done 25, 50, 100, 150, 200, 300, 350 µg/mL respectively.

6.3.2. Assay procedure of 5α-reductase inhibition of Solanum nigrum plant berries extract

5α-reductase inhibition assays were performed based on the method described by Nahata & Dixit, 2013. In brief, rat testis homogenate containing enzyme, T, NADPH and test samples were mix together. The detail reaction mixtures have been described in Table 6.1. All the reaction mixtures were incubated at 37^{o}C for 30 min. Absorbance was measured spectrophotometrically at 340 nm. Corresponding concentration of NADPH of the test samples were calculated from the NADPH standard curve. Residual amount of NADPH in the reaction mixture were determined. % scavenging of NADPH was determined from the NADPH concentration. Blank absorbance was deducted from the test samples to get net absorbance of NADPH. The 5α-reductase inhibition was calculated for individual test substances which reflect the original efficacy of the test substances against the enzyme. % inhibition of 5α-reductase was determined by % NADPH scavenging potential.

Absorbance taken at 340 nm, Blank Absorbance = 0.236

Net absorbance of test = (Test Absorbance – Blank Absorbance)

Percentage inhibition of different concentrations of test samples have to determine in order to calculate IC_{50} value of the test extracts.

6.3.3. **Results and discussion**

IC_{50} value of *Solanum nigrum* plant berries methanolic extract is 216.09 ± 14.32 (µg/mL). Whereas 5α-reductase inhibition of finasteride shown to be 202.59 ± 2.26 (ng/mL). IC_{50} value of plant extract shown in figure 6.4.

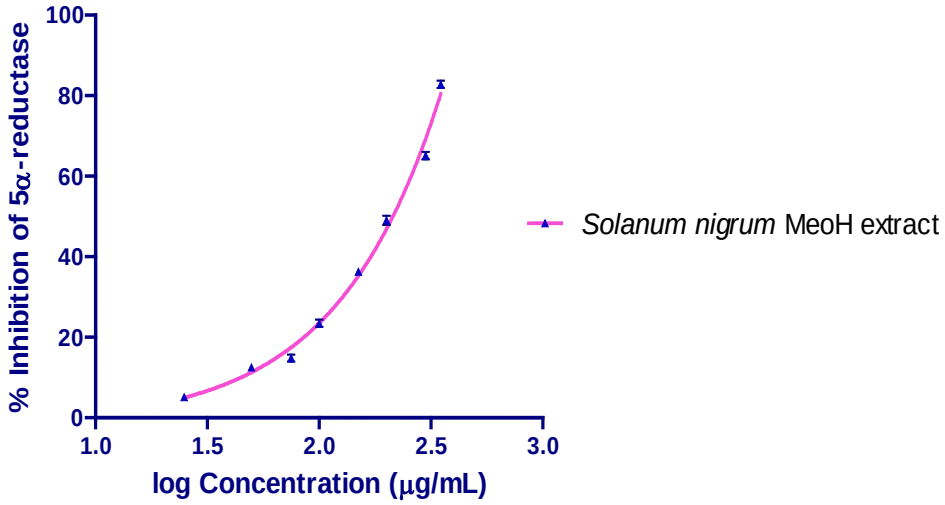


Figure 6.4. 5α-reductase inhibition of *Solanum nigrum.*

6.4. 5α-reductase inhibition of β-sitosterol, quercetin, linoleic acid biomarkers

6.4.1. Sample preparation of plant biomarkers

About 2 mg of β-sitosterol, quercetin, linoleic acid plant biomarker was taken in separate 2 mL of eppendorf tubes and added 2 mL methanol in it. It was mixed in vortex mixture and put into ultrasonication bath till the material completely dissolved. It was then filtered through 0.45 µ syringe filter and kept for further study. Further dilution was done 25, 50, 75, 100, 150, 200 µg/mL respectively. β-sitosterol, quercetin, linoleic acid was used for standardized *Eclipta alba*, *Hibiscus rosa sinensis, Solanum nigrum* plant extracts.

6.4.2. Assay procedure of 5α-reductase inhibition of biomarkers

5α-reductase inhibition assays were performed based on the method described by Nahata & Dixit, 2013. In brief, rat testis homogenate containing enzyme, T, NADPH and test samples were mix together. The detail reaction mixtures have been described in Table 6.1. All the reaction mixtures were incubated at 37 °C for 30 min. Absorbance was measured spectrophotometrically at 340 nm. Corresponding concentration of NADPH of the test samples were calculated from the NADPH standard curve. Residual amount of NADPH in the reaction mixture were determined. % scavenging of NADPH was determined from the NADPH concentration. Blank absorbance was deducted from the test samples to get net absorbance of NADPH. The 5α-reductase inhibition was calculated for individual test substances which reflect the original efficacy of the test substances against the enzyme. % inhibition of 5α-reductase was determined by % NADPH scavenging potential.

Net absorbance of test = (Test Absorbance – Blank Absorbance)
Calculate the NADPH concentration in each tube from NADPH standard curve prepared previously.
Percentage inhibition: = (100 – [(54.78-net absorbance of test)/54.78) × 100]
Percentage inhibition of different concentrations of test samples have to determine in order to calculate IC_{50} value of the test extracts.

6.4.3. **Results and discussion**

IC_{50} value of β-sitosterol, quercetin, linoleic acid was 44.12 ± 2.09, 103.72 ± 3.46, 119.17 ± 5.45. Whereas 5α-reductase inhibition of finasteride shown to be 202.59 ± 2.26 (ng/mL). IC_{50} values of β-sitosterol, quercetin, linoleic acid markers shown in figure 6.5.

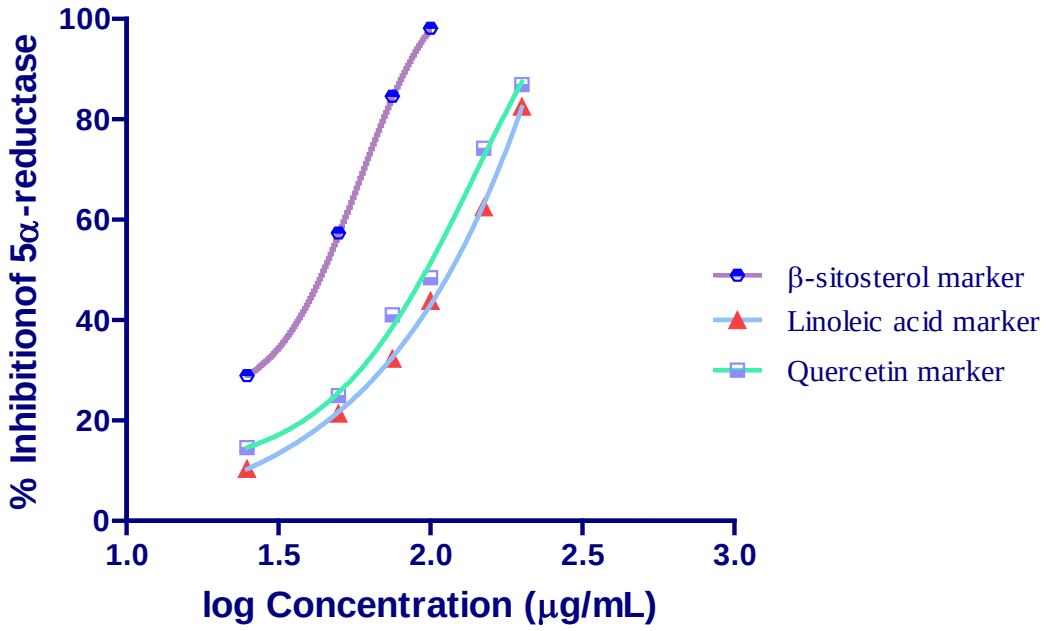


Figure 6.5. 5α-reductase inhibition of β-sitosterol, quercetin, linoleic acid.

6.5. Comparison study of 5α-reductase inhibitory potential of plants.

Table 6.2. Comparison study of 5α-reductase inhibitory potential of plants

Name of plants	IC_{50} (1)	IC_{50} (2)	IC_{50} (3)	Mean	SEM
Solanum nigrum	216.09	235.70	205.85	219.21	8.75
Eclipta alba methanolic extract	206.54	221.50	173.59	200.54	14.51
Eclipta alba petroleum ether extract	173.42	189.54	148.32	168.42	11.99
Hibiscus rosa-sinensis	157.57	131.25	161.15	149.99	9.42
β-sitosterol	44.12	41.45	51.72	45.76	3.07
Quercetin	103.72	107.56	109.46	119.00	1.68
Linoleic acid	119.17	115..41	126.84	123.01	3.36
Finasteride (Std)	0.2025	0.3106	0.4542	0.322	0.07

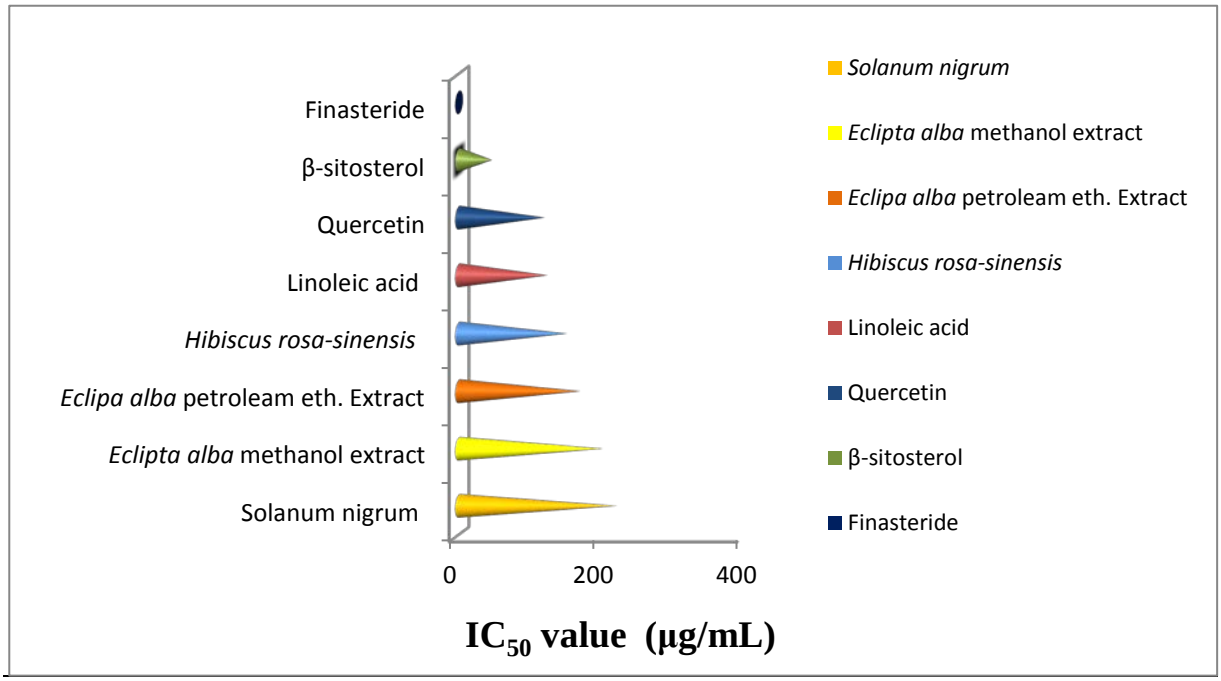


Figure 6.6. Comparison study of 5α-reductase inhibitory potential of plants.

6.6. Conclusion

The results showed significant IC_{50} values of 200.54 ± 14.51, 168.42 ± 11.99, 149.99 ± 19.42, 219.21 ± 8.75, 45.76 ± 3.07, 119.00 ± 1.68, 123.01 ± 3.36 (µg/mL) for *Eclipta alba* methanolic extract and petroleum ether extract (whole plant), *Hibiscus rosa-sinensis* flower methanolic extract, *Solanum nigrum* plant berries methanolic extract, β-sitosterol, quercetin, linoleic acid biomarkers respectively. Among them, *Hibiscus rosa-sinensis* and *Eclipta alba* petroleum ether extract have found potential 5α-reductase inhibition activity in respect to standard drug finasteride (IC_{50} value: 202.59 ± 2.26) (ng/mL).

Chapter- 7

Preparation and evaluation of different hair growth formulations

7.1. Preparation and evaluation of hair growth gel formulation

7.2. Preparation and evaluation of hair growth cream formulation

7.3. Preparation and evaluation of hair growth lotion formulation

7.4. Preparation and evaluation of β-sitosterol hair growth gel formulation

7.0. Preparation and evaluation of different hair growth formulations

Herbal medicine has become an item of global importance both medicinal and economical. Although usage of these herbal medicines has increased, their quality, safety and efficiency are serious concerns in industrialized and developing countries. Herbal remedies are getting increasing patient compliance as they are devoid of typical side effects of allopathic medicines. The present work has been undertaken with the aim to formulate different hair growth formulation like gel, cream, lotion based on *Hibiscus rosa sinensis*, *Eclipta alba*, *Solanum nigrum* plant extracts.

7.1. Preparation and evaluation of hair growth gel formulation

7.1.1. *Materials and method*

Hibiscus rosa sinensis flower methanolic extract, *Eclipta alba* whole plant methanolic and petroleum ether extract, *Solanum nigrum* berries methanolic extract, carbopol 940, propylene glycol, polyvinyl pyrrolidine, DMDM hydration, potassium sorbet, sodium benzoate, triethanolamine, EDTA disodium, igsurf 1540, water.

7.1.2. *Preparation method of hair gel formulation*

The gel was prepared using dried *Hibiscus rosa sinensis* flower methanolic extract, *Eclipta alba* whole plant methanolic and petroleum ether extract, *Solanum nigrum* berries methanolic extract. The gel was prepared using carbopol 940 (1%), propylene glycol, DMDM hydration, potassium sorbet, sodium benzoate, EDTA disodium, triethanolamine and purified water in quantity to prepare 100 g gel. Water required for the formulations was divided into two parts. In one parts the exact amount of extract was dissolved and to this calculated quantity of igsurf 1540, DMDM hydration, potassium sorbet, sodium benzoate, and EDTA disodium and propylene glycol was added and in other part, carbopol 940 was dissolved and to this solution EDTA disodium, polyvinyl pyrrolidine was added. Both of these solutions were mixed in beaker and triethanolamine was added mixture drop wise to obtain gel consistency. Composition of poly herbal hair growth gel is given below Table 7.1.

7.1.3. *Active ingredient (label claim)*

Hibiscus rosa sinensis flower 1 *%, Eclipta alba* whole plant 1 %, *Solanum nigrum* plant berries 0.5 % extract*,* riboflavin 5 phosphate 0.02 %, pyridoxine hydrochloride 0.01 %.

Table 7.1. Composition of poly herbal hair growth gel

Sl. No		Ingredients	Specification	Quantity used on 100 g basis
1.	I.	*Hibiscus rosa sinensis* flower methanolic *extract*		1 g
2.		*Eclipta alba* methanolic extract		0.5 g
3.		*Eclipta alba* pet. Ether extract		0.5 g
4.		*Solanum nigrum* berries methanolic extract		0.5 g
5.		Riboflavin 5 phosphate	I.P	0.02 g
6.		Pyridoxine hydrochloride	I.P	0.01 g
7.		DMDM Hydration	I.P	0.3 g
8.		Potassium sorbet	I.P	0.1 g
9.		Sodium benzoate	I.P	0.3 g
10.		Propylene glycol	I.P	15 g
11.		Igsurf 1540	I.P	0.5 g
12.		Kollidon 30	I.P	0.2 g
13.		Purified water	I.P	38 g
14.	II.	Carbopol 940	I.P	1.0 g
15.		EDTA Disodium	I.P	0.5 g
16.		Purified water	I.P	40.0 g
17.	III.	Triethanolamine		Q.S.

7.1.4. *Manufacturing method*

Solution I: EDTA disodium mixed in 40 mL water until it gets dissolved. Then measured amount of carbopol 940 added and mixed for 30 minutes and let swell during one hour.

Solution II: Take 15 g of propylene glycol in a 50 mL beaker and heated 40-50°C in heating mantel for 4-5 minutes. Then igsurf 1540 was taken and added to it with constant stirring. Measured amount of *Hibiscus rosa-sinensis* flower methanolic extract, *Eclipta alba* whole plant methanolic and petroleum ether extract, *Solanum nigrum* berries methanolic extract is added to the above solution with constant stirring.

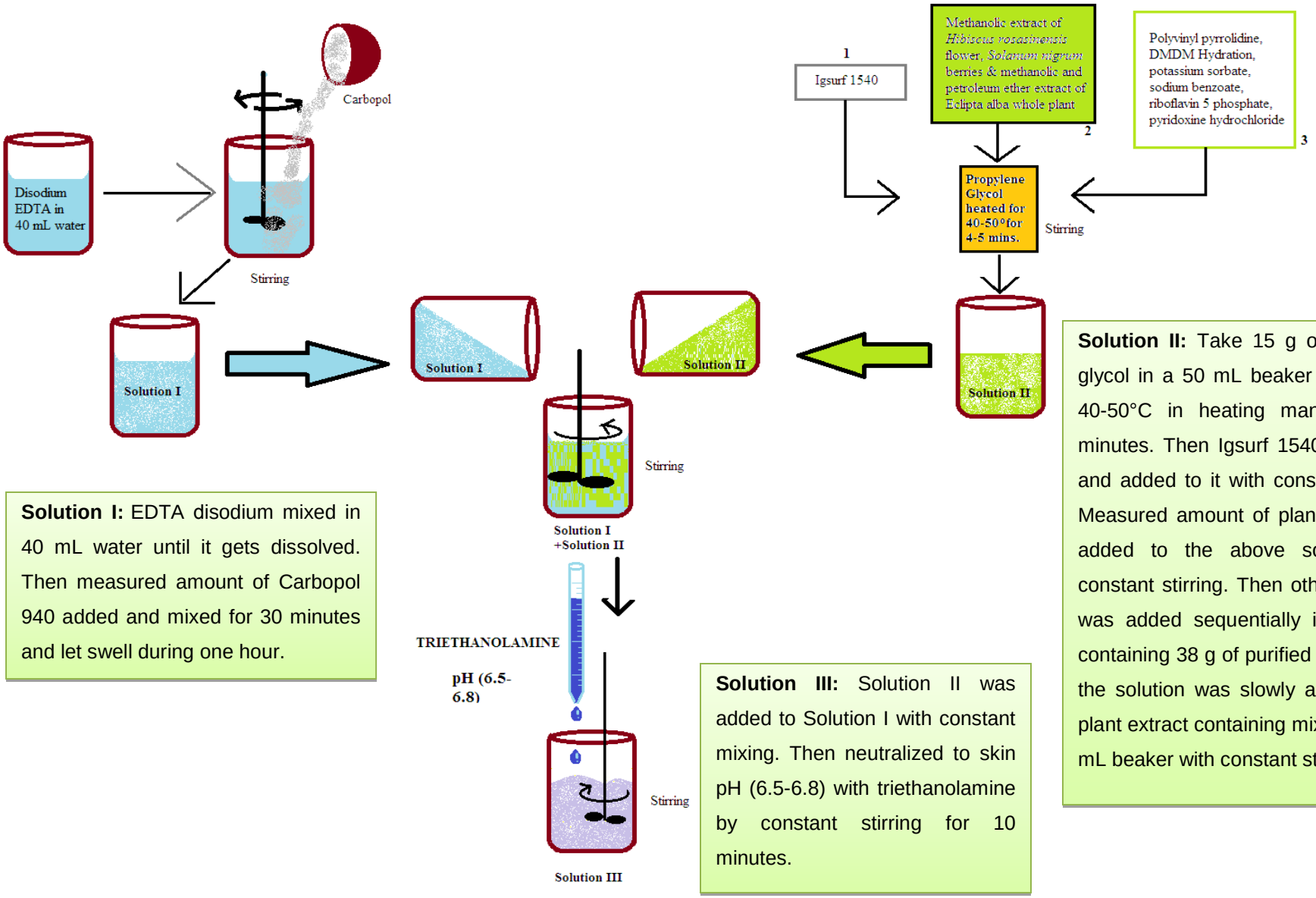
Polyvinyl pyrrolidine, DMDM hydration, potassium sorbet, sodium benzoate, riboflavin 5 phosphate and pyridoxine hydrochloride was added sequentially in a beaker containing 38 g of purified water. Then the solution was slowly added to the plant extract containing mixture into 50 mL beaker with constant stirring.

Solution III: Solution II was added to Solution I with constant mixing. Then neutralization was done to skin pH (6.5-6.8) with triethanolamine by constant stirring for 10 minutes.

Properties of the gel

Yellow color transparent gel.

Figure 7.1. Poly herbal hair growth gel.



Solution I: EDTA disodium mixed in 40 mL water until it gets dissolved. Then measured amount of Carbopol 940 added and mixed for 30 minutes and let swell during one hour.

Solution II: Take 15 g of Propylene glycol in a 50 mL beaker and heated 40-50°C in heating mantel for 4-5 minutes. Then Igsurf 1540 was taken and added to it with constant stirring. Measured amount of plant extracts is added to the above solution with constant stirring. Then other excipient was added sequentially in a beaker containing 38 g of purified water. Then the solution was slowly added to the plant extract containing mixture into 50 mL beaker with constant stirring.

Solution III: Solution II was added to Solution I with constant mixing. Then neutralized to skin pH (6.5-6.8) with triethanolamine by constant stirring for 10 minutes.

Figure 7.2. Schematic diagram of poly herbal hair gel preparation

7.1.5. *Evaluation of hair gel formulation*

7.1.5.1. *pH*

The pH of various gel formulations was determined by using digital pH meter. The measurement of pH of each formulation was done in triplicate and average values were calculated.

7.1.5.2. *Viscosity*

Brookfield viscometer is used for the measurement of viscosity of the prepared gel. The Brookfield viscometer is rotated at 100 rpm by spindle no.6. Each reading was taken after equilibrium of the sample at the end of two minutes. The samples were repeated three times.

7.1.5.3. *Spredibility*

It was determined by wooden block and glass slide apparatus. Weights about 20g were added to the pan and the time noted for upper slide (movable) to separate completely from the fixed slides. Spreadability was then calculated by using the formula,

$$S = M.L / T$$

Where, S = Spredibility, M = Weight tide to upper slide, L = Length of glass slide, T = Time taken to separate the slide completely from each other. The therapeutic efficacy of a formulation also depends upon its value.

7.1.5.4. *Physical appearance*

The gel formulations were evaluated in terms of physical character like phase separation & change in colour, odour & rheological parameters.

7.1.5.5. *Homogeneity*

All developed gels were tested for homogeneity by visual inspection after the gels have been set in the container. They were tested for their appearance and presence of any aggregates.

7.1.5.6. *Skin irritation test*

Test for irritation was performed on human volunteers. For each gel, five volunteers were selected and 1.0 g of formulated gel was applied on an area of 2 square inch to the back of hand. The volunteers were observed for lesions or irritation.

7.1.5.7. Drug content

In this method 1g of prepared gel is mixed with 10mL of drug soluble or extractable suitable solvent. Then HPLC or HPTLC was carried out. Drug content will be calculated the equation obtained by linear or calibration curve.

7.1.6. *Standardization of Herbal hair growth formulation by HPTLC*

HPTLC is a method to standardize and identify the chemical component which is expected to be present in a medicinal plant. This is done from regulatory perspective to ensure the quality, efficacy and safety of the herbal drugs present in a plant. Thus it provides a very reliable way of determining the purity and percentage content of the active biomarker in the plant extracts. In this chapter the standardization and quantification of the herbal hair growth formulation have been described. The hair growth formulation is prepared based on *Eclipta alba*, *Hibiscus rosa-sinensis* and *Solanum nigrum extract*.

7.1.6.1. *Standardization and quantification of β-sitosterol, quercetin and linoleic acid in Herbal hair growth Gel by HPTLC*

7.1.6.2. *Equipments and Reagents*

The CAMAG HPTLC system consisting of WINCATS software, LINOMAT V automatic sample applicator, and automatic development chamber, scanning densitometer CAMAG scanner 3 and photo documentation apparatus CAMAG reprostar 3 were used. Stationary phase used was aluminum based silica gel plate 60 F_{254} (Merck, Mumbai) with 20 cm x 10 cm in a particle size of 5-10 µm. All the solvents were used of analytical grade.100 µL syringe (HAMILTON, Switzerland) was used for sample application on HPTLC plates. β-sitosterol was purchased from Sigma Aldrich. Linoleic acid was purchased from Sisco Research Laboratories (SRL). Quercetin was present in Lab. Methanol, toluene, ethyl acetate of analytical grade were purchased from Merck (Mumbai, India). Whatman's syringe filter (NYL 0.45 µm) was used for the filtration of samples and standard.

7.1.6.3. *Preparation of standard solution*

About 1 mg of β-sitosterol, linoleic acid, quercetin standard was weighed and put in to separate 1 mL eppendorf tubes. 1.0 mL of methanol in added in each 1 mL eppendorf tube and mixed in vortex mixture till the material completely dissolved. It was then filtered through 0.45 µ syringe filter and kept for further study.

7.1.6.4. *Preparation of sample solution*

About 1 g of gel formulation was weighed and taken in a centrifuge tube. 10 mL methanol added to the centrifuge tube. Mixed in vortex mixture for 15 minutes and put to ultrasonication bath for 30 minutes. It was then filtered through 0.45 µ syringe filter and kept it in 1 mL eppendorf tube for further study.

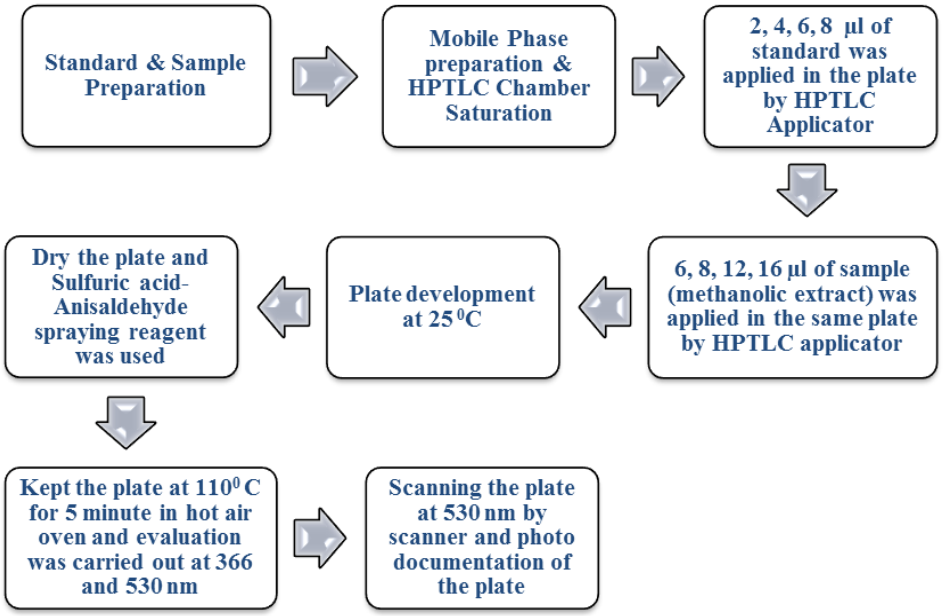


Figure 7.3. Standardization and quantification of β-sitosterol, quercetin and linoleic acid (standards) in Herbal hair growth gel by HPTLC.

7.1.6.5. *Chromatographic conditions*

HPTLC analysis was performed using isocratic technique. Mobile phase was optimized with toluene: ethyl acetate: methanol in a ratio of 5: 3: 2 v/v. The temperature was kept at 25 ^{0}C and mobile phase was developed in a twin trough glass chamber. Standard stock solution of three biomarkers β-sitosterol, quercetin and linoleic acid was applied consequently in the range of 2-8 µL with 2 µL gradual increments. Sample solution was applied 6, 8, 12, 16 µL respectively. All total 18 tracks in HPTLC plate were used for standardization including standard and sample solution respectively in a band wise fashion. After development, plates were dried by hand dryer. Colored bands were observed at 366 and 530 nm.

7.1.6.6. *Results and discussion*

The percentage content of β-sitosterol, quercetin and linoleic acid in herbal hair gel methanolic extract was found to be 0.1377, 0.120 and 0.379 % w/w respectively.

R_f value of standard β-sitosterol, quercetin and linoleic acid was found to be 0.72, 0.49, 0.61 respectively. Specificity was confirmed by comparing the R_f of standard and sample (Figure 7.4 -7.5, 7.6 - 7.7, 7.8 – 7.9).

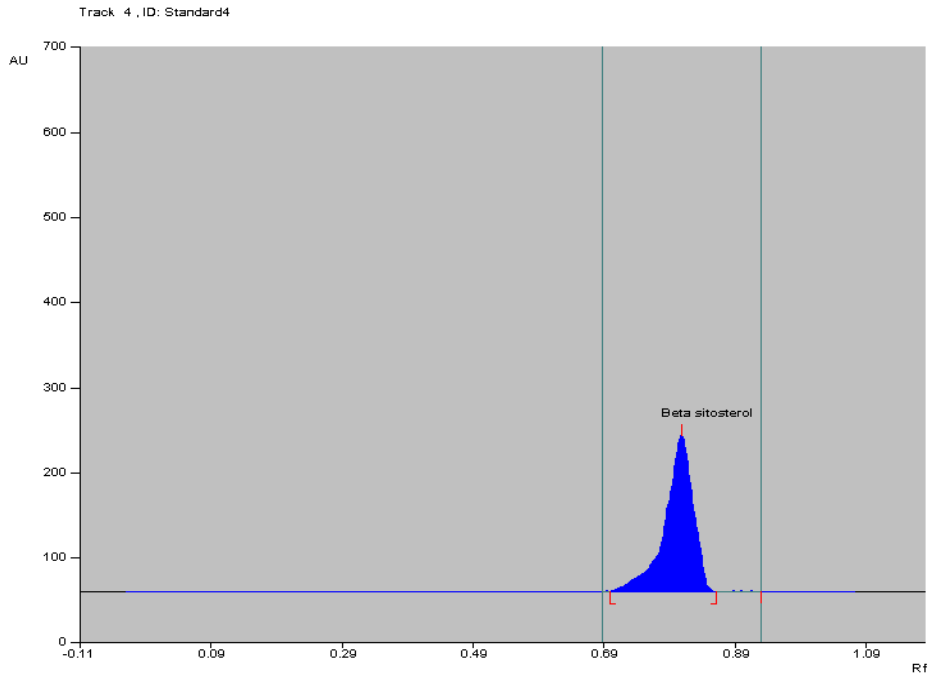


Figure 7.4. HPTLC chromatogram of standard β-sitosterol.

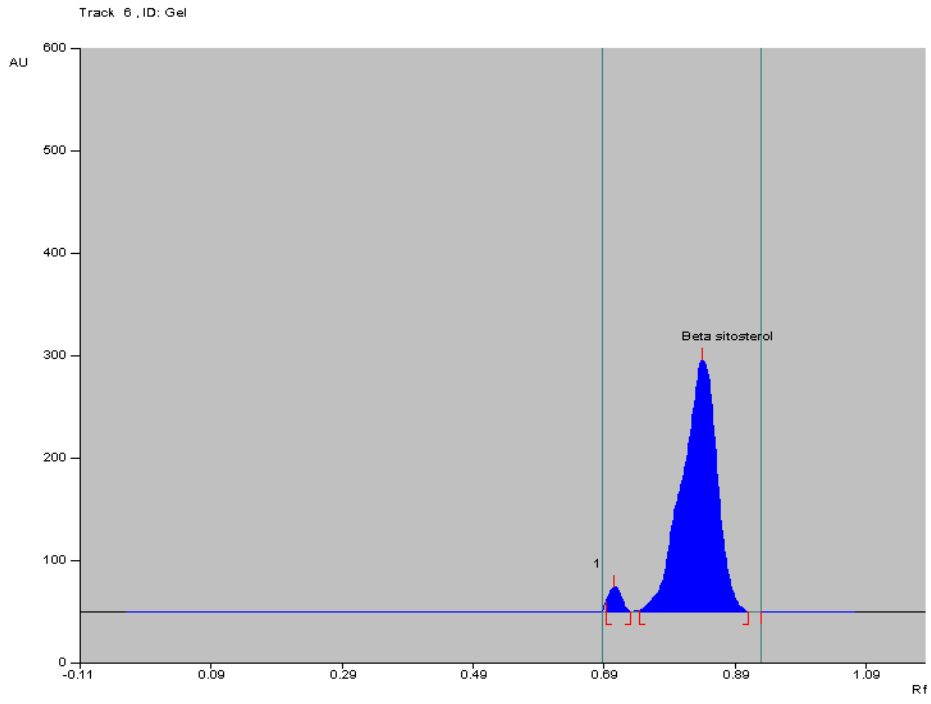


Figure 7.5. HPTLC chromatogram of β-sitosterol present in hair gel formulation.

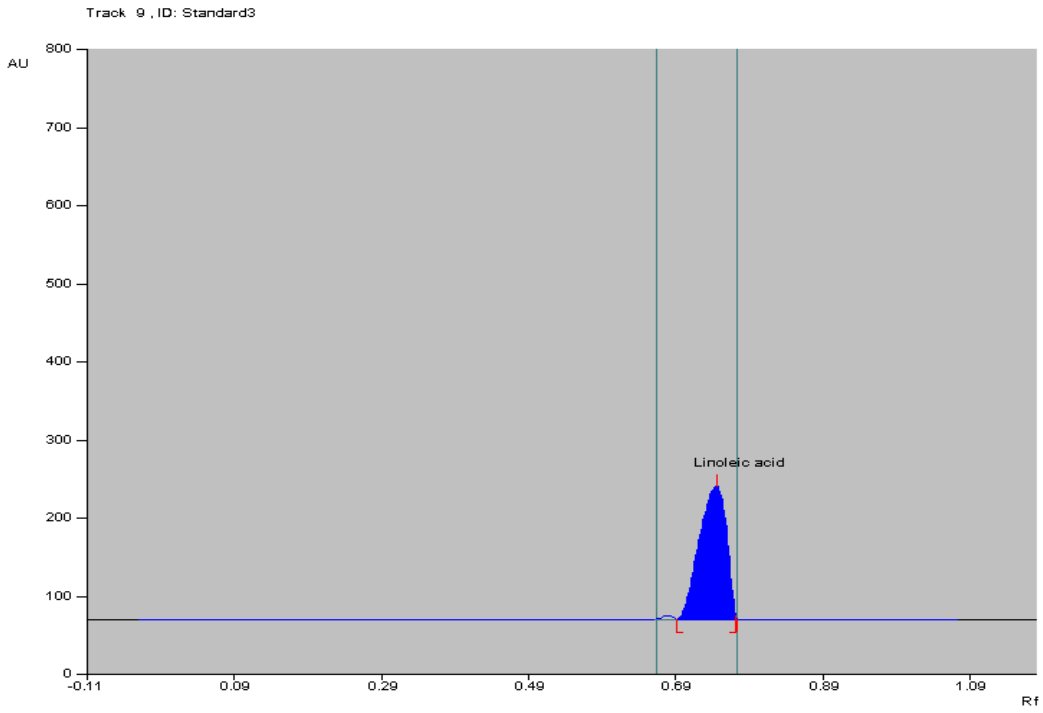


Figure 7.6. HPTLC chromatogram of standard linoleic acid.

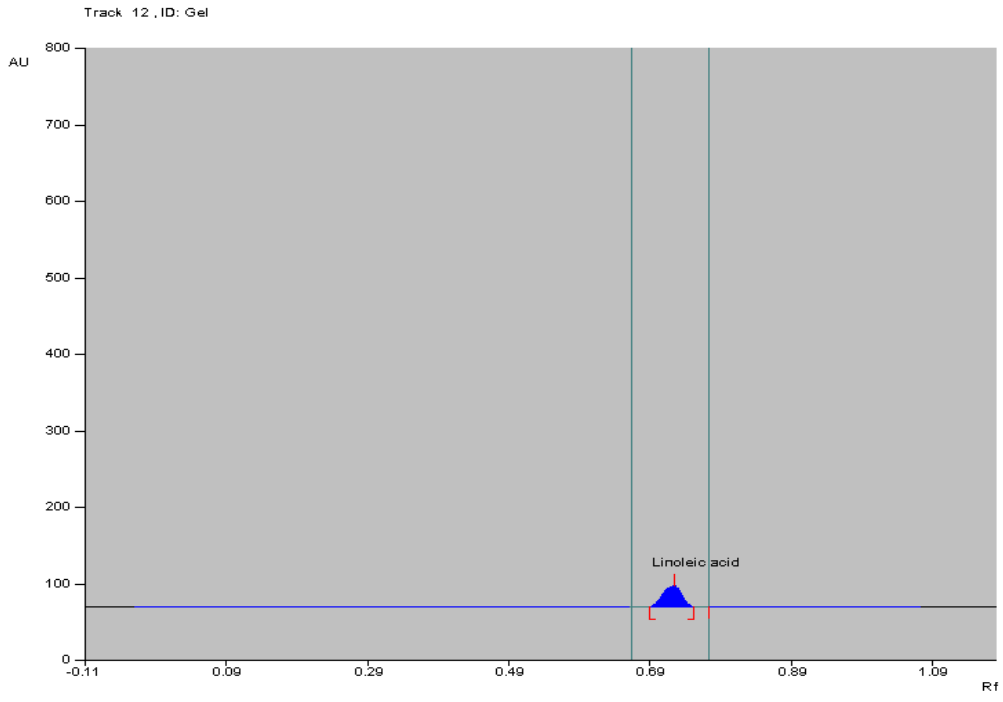


Figure 7.7. HPTLC chromatogram of linoleic acid present in hair gel formulation.

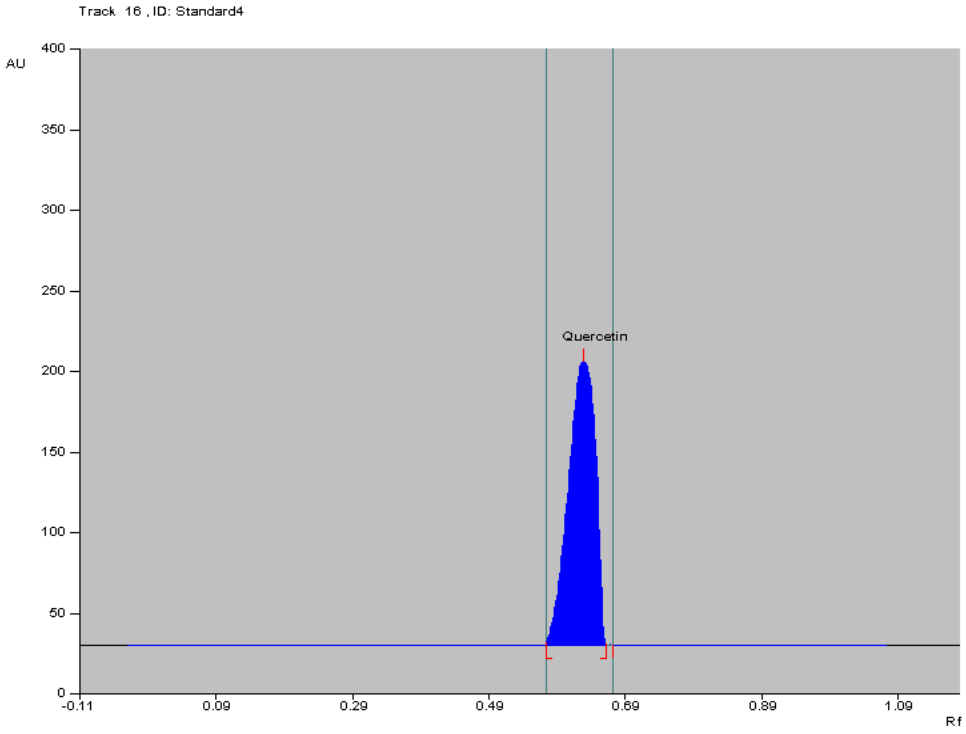


Figure 7.8. HPTLC chromatogram of standard quercetin.

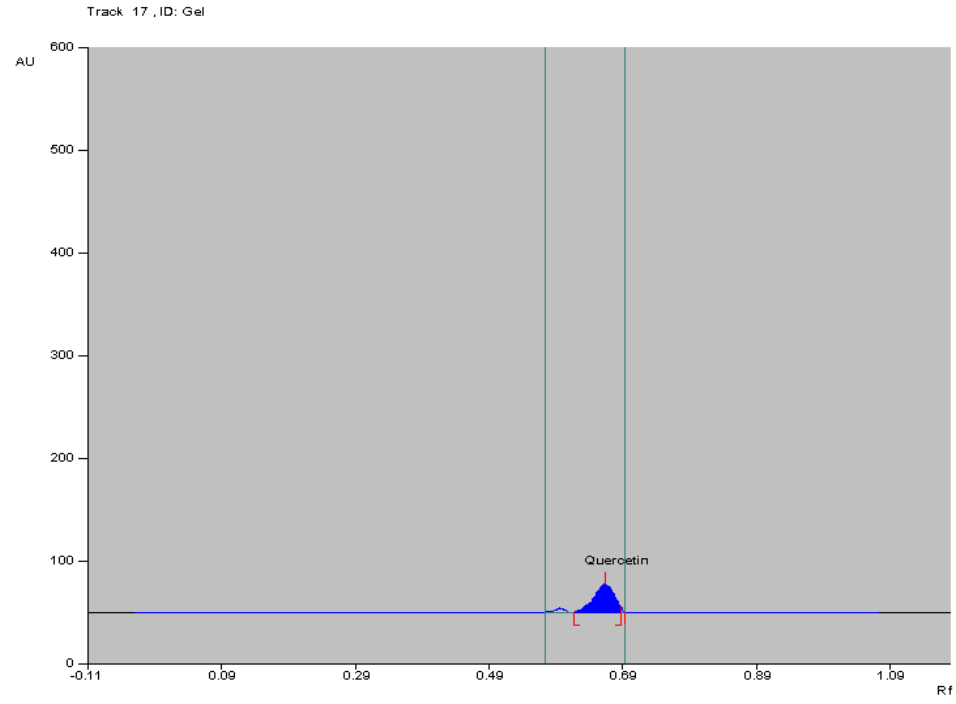


Figure 7.9. HPTLC chromatogram of quercetin present in hair gel formulation.

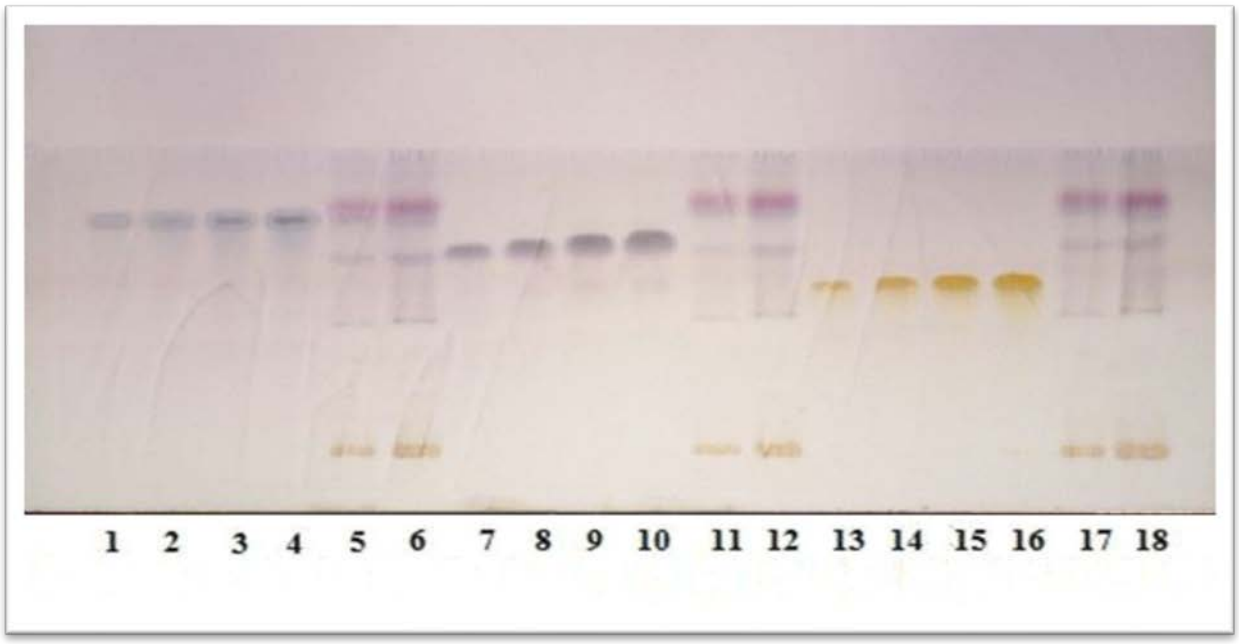


Figure 7.10. Photodocumentaton of hair gel formulation at 530 nm.

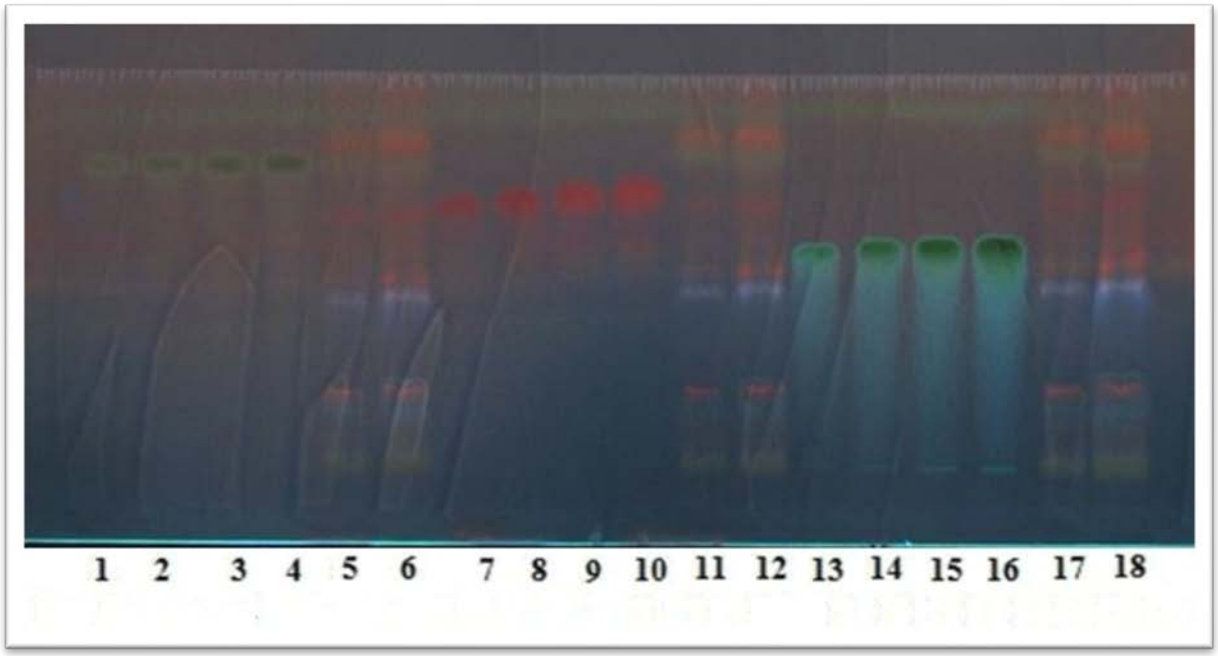


Figure 7.11. Photodocumentaton of hair gel formulation at 366 nm.

Track no: 1, 2, 3, 4 - standard β-sitosterol, track no: 7, 8, 9, 10 - linoleic acid, track no: 13, 14, 15, 16 - quercetin, track no: 5, 6, 11, 12, 17, 18 - gel formulation.

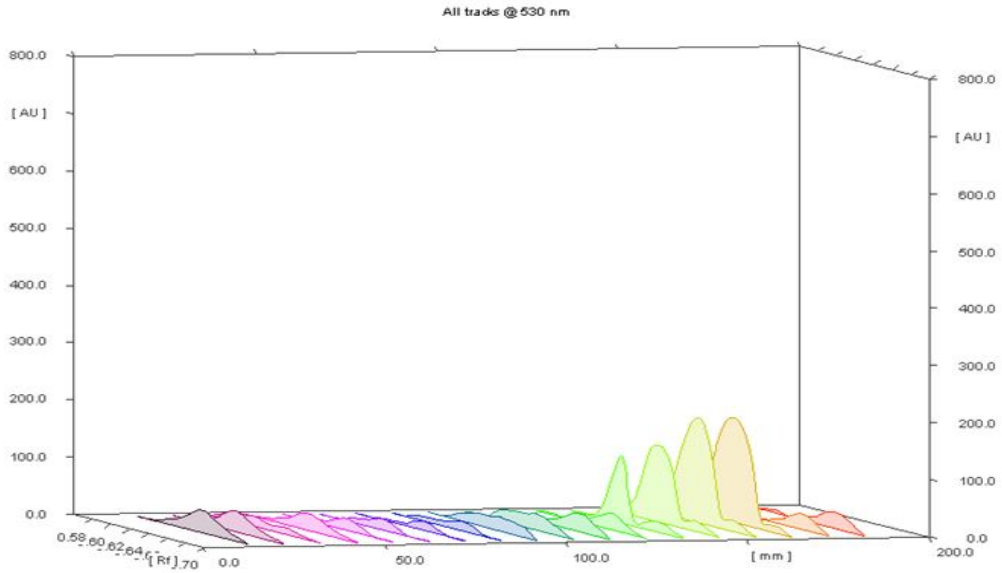


Figure 7.12. 3D chromatogram of herbal hair gel with β-sitosterol, quercetin and linoleic acid marker at 530 nm.

7.1.7. **Results and discussion**

The result of the gel formulation is good in appearance, homogeneity and easily spreadable and showed significant inhibition of 5α-reductase enzyme in in-vitro model. Further HPTLC method was done for quantification of plant biomarkers used in formulation. The results also showed that 5α-reductase enzyme inhibition effect of the formulation was better than the effect of marketed hair gel formulation.

Table 7.2. Evaluation parameters of hair gel formulations

Parameters	Results
pH	6.68
Viscosity	4731 cps
Drug content by HPTLC	β-sitosterol, quercetin and linoleic acid was found to be 0.1377, 0.120 and 0.379 % w/w respectively.
Physical appearance	Brownish yellow colour, odourless gel found.
Homogeneity	Homogeneous, smooth and consistent. No aggregates found.
Skin irritation test	Skin compatible, no irritation found.
Spredibility	11.05 (g-cm/sec)

7.2. Preparation and evaluation of hair growth cream formulation

7.2.1. *Materials and method*

Hibiscus rosa sinensis flower methanolic extract, *Eclipta alba* whole plant methanolic and petroleum ether extract, *Solanum nigrum* berries methanolic extract, stearic acid, potassium hydroxide, sodium carbonate, BHT, glycerin, propylene glycol, sodium methayl paraben, sodium propyl paraben, EDTA disodium, igsurf 1540, purified water.

7.2.2. *Preparation method of hair cream formulation*

The cream was prepared using dried *Hibiscus rosa sinensis* flower methanolic extract, *Eclipta alba* whole plant methanolic and petroleum ether extract, *Solanum nigrum* berries methanolic extract. The cream was prepared using stearic acid (17%), propylene glycol, glycerin, sodium methyl paraben, sodium propyl paraben, BHT, EDTA disodium, potassium hydroxide, sodium carbonate, igsurf 1540 and purified water in quantity to prepare 100 g cream. The formulation was divided into two parts that is aqueous phase and oil phase. In aqueous phase the exact amount of extract was dissolved in glycerin and to this calculated quantity of Igsurf 1540, sodium methayl paraben, sodium propyl paraben, EDTA disodium, purified water and in oil part, stearic acid in was dissolved in propylene glycol and potassium hydroxide, sodium carbonate, BHT, was added sequentially. Both of these solutions were mixed in beaker with constant stirring in heating condition to obtain desire cream. Composition of poly herbal hair growth cream given below in table 7.3.

Table 7.3. Composition of poly herbal hair growth cream

S.No		Ingredients	Specification	Quantity Used On 100g basis
1.	I.	Stearic acid	I.P	17 g
2.		Potassium hydroxide	I.P	0.5 g
3.		Sodium carbonate	I.P	0.5 g
4.		Propylene glycol	I.P	5 g
5.		BHT	I.P	0.01 g
6.	II.	Glycerin	I.P	6 g
7.		Igsurf 1540	-	0.5 g
8.		*Hibiscus rosa sinensis* methanolic extract	-	1 g
9.		*Eclipta alba* methanolic extract	-	0.5 g
10.		*Eclipta alba* pet. ether extract	-	0.5 g
11.		*Solanum nigrum* methanolic extract	-	0.5 g
12.	III.	Riboflavin 5 phosphate	I.P	0.02 g
13.		Pyridoxine hydrochloride	I.P	0.01 g
14.		EDTA Disodium	I.P	0.1 g
15.		Sodium methyl paraben	I.P	0.3 g
16.		Sodium propyl paraben	I.P	0.1 g
17.		Purified water	I.P	67 g

7.2.3. *Active ingredient (label claim)*

Hibiscus rosa sinensis flower 1 *%, Eclipta alba* whole plant 1 %, *Solanum nigrum* plant berries 0.5 % extract*,* riboflavin 5 phosphate 0.02 %, pyridoxine hydrochloride 0.01 %.

7.2.4. *Manufacturing method*

Solution I: Propylene glycol is heated in heating mantel to 60-70 °C. Then stearic acid mixed in Propylene glycol until it gets dissolved. Then measured amount of BHT, potassium hydroxide and sodium carbonate was added and mixed consecutively until it gets dissolved**.**

Solution II: Take 6 g of glycerin in a 50 mL beaker and heated 40-50 °C in heating mantel for 2-3 minutes. Then igsurf 1540 was taken and added to it with constant stirring. Measured amount of *Hibiscus rosa sinensis* flower methanolic extract, *Eclipta alba* whole plant methanolic and petroleum ether extract, *Solanum nigrum* berries methanolic extract is added to the above solution with constant stirring.

Solution III: Sodium methyl paraben, Sodium propyl paraben, EDTA disodium, riboflavin 5 phosphate and pyridoxine hydrochloride was added sequentially in a beaker containing 67 g of purified water. Then the solution was slowly added to the solution II and mixed constantly with a glass rod until it gets dissolved.

Finally, solution III was added to Solution I with constant mixing at 40-50^0 C.

Properties of the cream

Slightly greenish yellow color cream found.

Figure 7.13. Poly herbal hair growth cream.

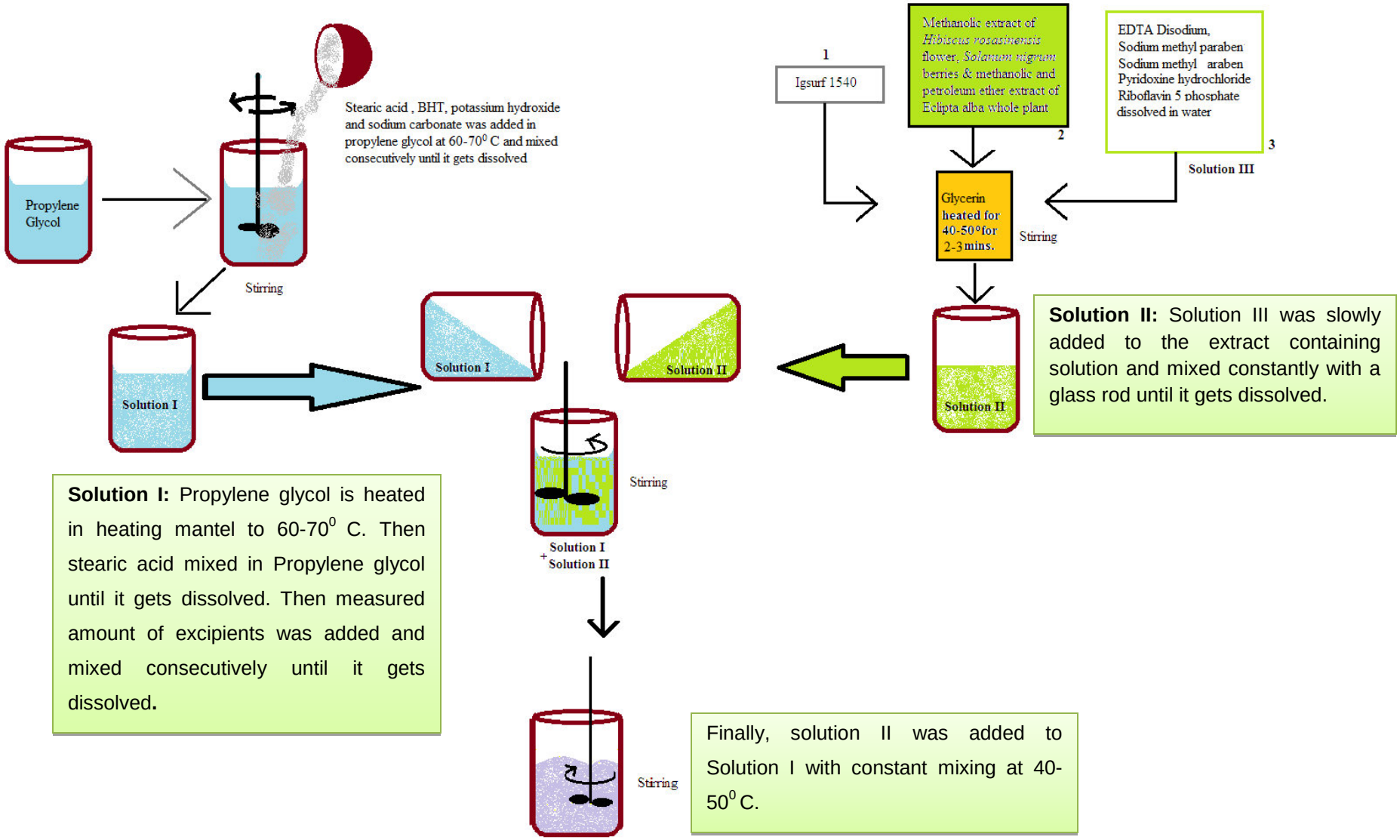


Figure 7.14. Schematic diagram of poly herbal hair cream preparation

7.2.5. **Evaluation of hair cream formulation**

7.2.5.1. *Determination of organoleptic properties*

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

7.2.5.2. *pH*

Accurately weighed 5 g of the sample was dispersed in 45 mL of water. The pH of the suspension was determined at 27°C using digital pH meter.

7.2.5.3. *Homogeneity*

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

7.2.5.4. *Spreadability*

Spread ability may be expressed by the extent of the area to which the topical application spreads when applied to the affected parts on the skin. The therapeutic efficiency of the formulation also depends upon its spreading value. Hence, it was found necessary to determine the spread ability of the formulation. For this purpose, ample (about 3 g) was applied in between two glass slides and they were pressed together to obtain a film of uniform thickness by placing 1000 g weight for 5 minutes. Thereafter a weight (10 g) was added to the pan and the top plate was subjected to pull with the help of string attached to the hook. The time in which the upper glass slide moves over the lower plate to cover a distance of 10 cm is noted. The spread ability (S) can be calculated using the formula.

$$S = m \times L/T$$

Where,S – Spread ability, m- Weight tied to upper glass slide, L- Length moved on a glass slide T- Time taken.The determinations were carried out in triplicate and the average of three readings was recorded.

7.2.5.5. *Smear test*

It was determined by applying the cream on the skin surface of human volunteer. After application of cream, the type of film or smear formed on the skin were checked.

7.2.5.6. *Emolliency*

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

7.2.5.7. *Viscosity*

The viscosity determinations were carried out using a Brookfield Viscometer using spindle number S- 64 at a 20 rpm at a temperature of 25^0C. The determinations were carried out in triplicate and the average of three readings was recorded.

7.2.5.8. *Dilution test*

In this test the emulsion is diluted either with oil or water. If the emulsion is o/w type and it is diluted with water, it will remain stable as water is the dispersion medium" but if it is diluted with oil, the emulsion will break as oil and water are not miscible with each other. Oil in water emulsion can easily be diluted with an aqueous solvent, whereas water in oil emulsion can be diluted with an oily liquid.

7.2.6. *Results and discussion*

The result of the cream formulation is good in appearance, homogeneity and easily spreadable and showed significant inhibition of 5α-reductase enzyme in in-vitro model. Further results also showed that 5α-reductase enzyme inhibition effect of the formulation was better than the effect of marketed hair cream formulation.

Table 7.4. Evaluation parameters of hair gel formulations

Parameters	Results
Organoleptic properties	Yellow colour, odourless cream found.
pH	6.24.
Homogeneity	Homogeneous, smooth and consistent.
Smear	The cream produced non-greasy film on the skin surface.
Emolliency	No residue left.
Viscosity	27015 cps.
Dilution test	O/W type emulsion.

7.3. Preparation and evaluation of hair growth lotion formulation

7.3.1. *Materials and method*

Hibiscus rosa sinensis flower methanolic extract, *Eclipta alba* whole plant methanolic and petroleum ether extract, *Solanum nigrum* berries methanolic extract, isopropyl alcohol, propylene glycol, igsurf 1540, EDTA disodium, purified water.

7.3.2. *Preparation method of hair lotion formulation*

The lotion was prepared using dried *Hibiscus rosa sinensis* flower methanolic extract, *Eclipta alba* whole plant methanolic and petroleum ether extract, *Solanum nigrum* berries methanolic extract, riboflavin 5 phosphate, pyridoxine hydrochloride. The lotion was prepared using Isopropyl alcohol, EDTA disodium, propylene glycol, igsurf 1540 and purified water in quantity to prepare 100 mL lotion. Composition of poly herbal hair growth lotion given below in table 7.5.

Table 7.5. Composition of poly herbal hair growth lotion

Sl. No	Step	Ingredients		Specification	Quantity used on 100 mL basis
1.	I.	Ethyl alcohol 96 vol. % or Isopropyl alcohol		I.P	41.70 mL
2.	II.	Igsurf 1540		I.P	0.5 g
3.		Propylene glycol		I.P	10 g
3.		Hair Complex Aquosum	*Hibiscus rosa sinensis* methanolic extract		1 g
4.			*Eclipta alba* methanolic extract	I.P	0.5 g
5.			*Eclipta alba* pet. ether extract	I.P	0.5 g
6.			*Solanum nigrum* methanolic extract		0.02 g
7.	III.		Riboflavin 5 phosphate	I.P	0.02 g
8.			Pyridoxine hydrochloride	I.P	0.01 g
9.		Purified water		I.P	47.5 mL
10.		EDTA Disodium		I.P	0.1 g
11.	IV.	Perfume		I.P	Q.S

7.3.3. *Active ingredient (label claim)*

Hibiscus rosa sinensis flower 1 *%, Eclipta alba* whole plant 1 %, *Solanum nigrum* plant berries 0.5 % extract*,* riboflavin 5 phosphate 0.02 %, pyridoxine hydrochloride 0.01 %.

Hair Complex Aquosum: Combination of herbal extracts and various types of vitamin B. Used in hair care products.

7.3.4. *Manufacturing method*

Solution I: Measured amount of isopropyl alcohol or ethyl alcohol 96 vol. % was taken in a beaker and closed tightly.

Solution II**:** Propylene glycol is heated in heating mantel to 30-40 ^{0} C for 2-3 minutes. Then igsurf 1540 was taken and added to it with constant stirring until it gets dissolved. Measured amount of *Hibiscus rosa sinensis* flower methanolic extract, *Eclipta alba* whole plant methanolic and petroleum ether extract, *Solanum nigrum* berries methanolic extract is added to the above solution with constant stirring.

Solution III: EDTA disodium, riboflavin 5 phosphate and pyridoxine hydrochloride was added sequentially in a beaker containing 47.5 mL of purified water. Then the solution was slowly added to the solution II and mixed constantly with a glass rod until it gets dissolved.

Finally, solution I was added to the above prepared solution mixture with constant mixing.

Properties of the lotion

Brownish yellow color lotion found.

Figure 7.15. Poly herbal hair growth Lotion.

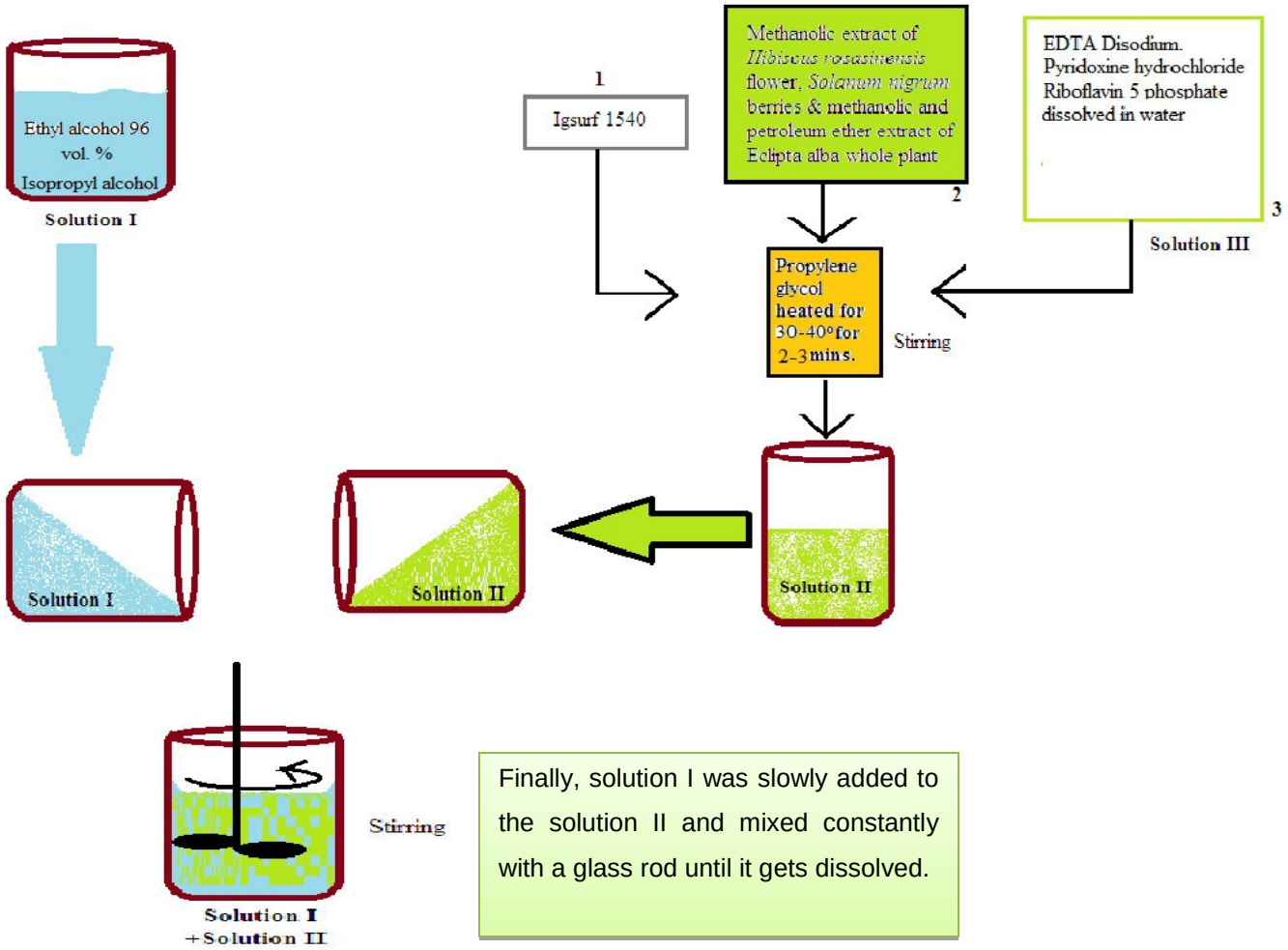


Figure 7.16. Schematic diagram of poly herbal hair lotion preparation

7.3.5. *Evaluation of hair lotion formulation*

7.3.5.1. *pH*

The test solution was prepared by adding 100 mL of distilled water to 5 mL of prepared lotion; it was stirred using a thin glass stirring rod. Next the pH meter is standardized by means of the standard solution provided at room temperature. The electrode of pH meter is immersed into the test solution and beaker is turned slightly to obtained good contact between the test solution and electrode. The meter has auto read system and it automatically signal when stabilized. The pH was recorded.

7.3.5.2. *Visual observation*

Colour, appearance, consistency and odour.

7.3.5.3. *Homogeneity*

The developed lotion was tested for homogeneity by visual inspection, after the lotion has been set in the container, spread on the glass slide for the appearance, tested for the presence of any lumps, flocculates or aggregates.

7.3.5.4. *Drug content*

In this method 1 mL of prepared lotion is mixed with 10 mL of drug soluble or extractable suitable solvent. Then HPLC or HPTLC was carried out. Drug content will be calculated the equation obtained by linear or calibration curve.

7.3.6. **Standardization and quantification of β-sitosterol, quercetin and linoleic acid in Herbal hair growth Lotion by HPTLC**

7.3.6.1. *Equipments and reagents*

The CAMAG HPTLC system consisting of WINCATS software, LINOMAT V automatic sample applicator, and automatic development chamber, scanning densitometer CAMAG scanner 3 and photo documentation apparatus CAMAG reprostar 3 were used. Stationary phase used was aluminum based silica gel plate 60 F_{254} (Merck, Mumbai) with 20 cm x 10 cm in a particle size of 5-10 µm. All the solvents were used of analytical grade.100 µL syringe (HAMILTON, Switzerland) was used for sample application on HPTLC plates. β-sitosterol was purchased from Sigma Aldrich. Linoleic acid was purchased from Sisco Research Laboratories SRL. Quercetin was present in Lab. Methanol, toluene, ethyl acetate of analytical grade were purchased from Merck (Mumbai, India). Whatman's syringe filter (NYL 0.45 µm) was used for the filtration of samples and standard.

7.3.6.2. *Preparation of standard solution*

About 1 mg of β-sitosterol, linoleic acid, quercetin standard was weighed and put in to separate 1 mL eppendorf tubes. 1.0 mL of methanol in added in each 1 mL eppendorf tube and mixed in vortex mixture till the material completely dissolved. It was then filtered through 0.45 µ syringe filter and kept for further study.

7.3.6.3. *Preparation of sample solution*

About 1 g of hair growth lotion formulation was weighed and taken in a centrifuge tube. 10 mL methanol added to the centrifuge tube. Mixed in vortex mixture for 15 minutes and put to ultrasonication bath for 30 minutes. It was then filtered through 0.45 µ syringe filter and kept it in 1mL eppendorf tube for further study.

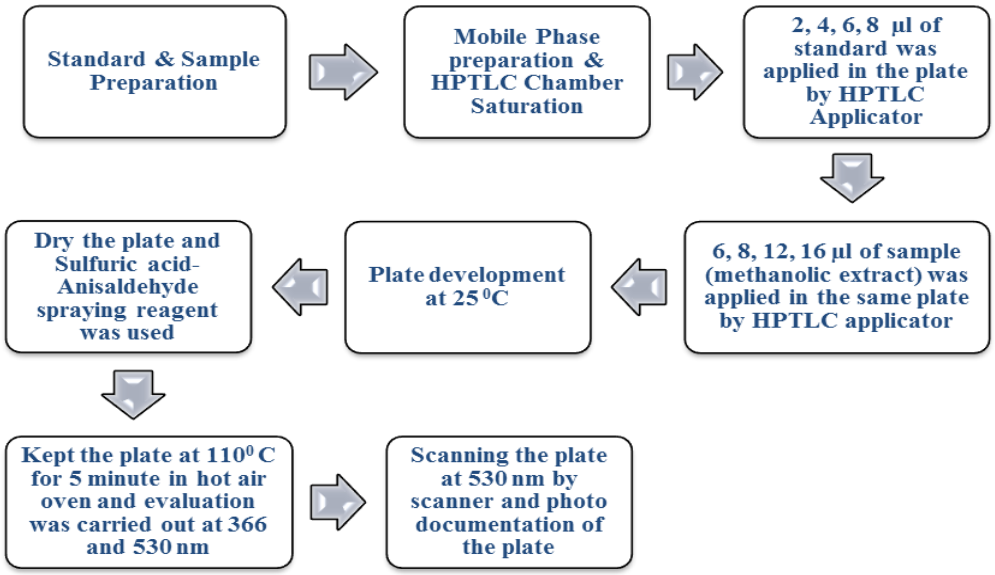


Figure 7.17. Standardization and quantification of β-sitosterol, quercetin and linoleic acid (standards) in herbal hair growth lotion by HPTLC.

7.3.6.4. *Chromatographic conditions*

HPTLC analysis was performed using isocratic technique. Mobile phase was optimized with toluene: ethyl acetate: methanol in a ratio of 5: 3: 2 v/v. The temperature was kept at 25 ^{0}C and mobile phase was developed in a twin trough glass chamber. Standard stock solution of three biomarkers β-sitosterol, quercetin and linoleic acid was applied consequently in the range of 2-8 µL with 2 µL gradual increments. Sample solution was applied 8, 10, 16, 20 µL respectively. All total 18 tracks in HPTLC plate were used for standardization including standard and sample solution respectively in a band wise fashion. After development, plates were dried by hand dryer. The dry plate was treated with sulfuric acid-anisaldehyde spraying reagent. The plate was kept at 110^0 C for 5 minute in hot air oven and evaluation was carried out at 366 and 540.Colored bands were observed at 366 to 530 nm.

7.3.6.5. *Results and discussion*

The percentage content of β-sitosterol, quercetin and linoleic acid in herbal hair lotion methanolic extract was found to be 0.1231, 0.1576, 0.151 % w/w respectively.

R_f value of standard β-sitosterol, quercetin and linoleic acid was found to be 0.72, 0.49, 0.61 respectively. Specificity was confirmed by comparing the R_f of standard and sample (Figure 7.18 – 7.19, 7.20 – 7.21, 7.22 – 7.23).

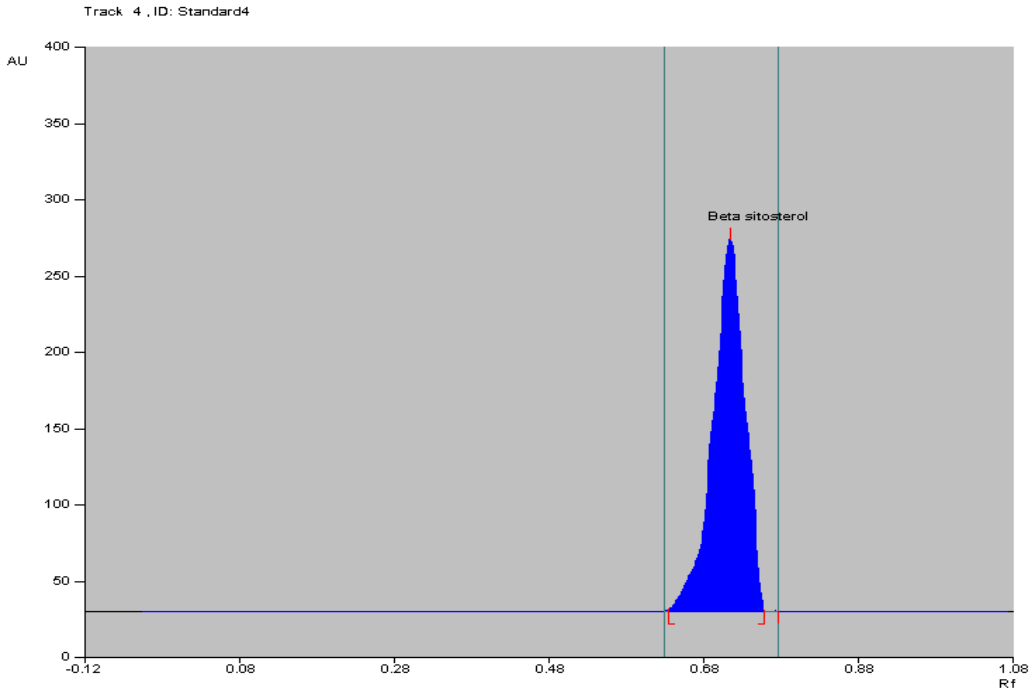


Figure 7.18. HPTLC chromatogram of standard β-sitosterol.

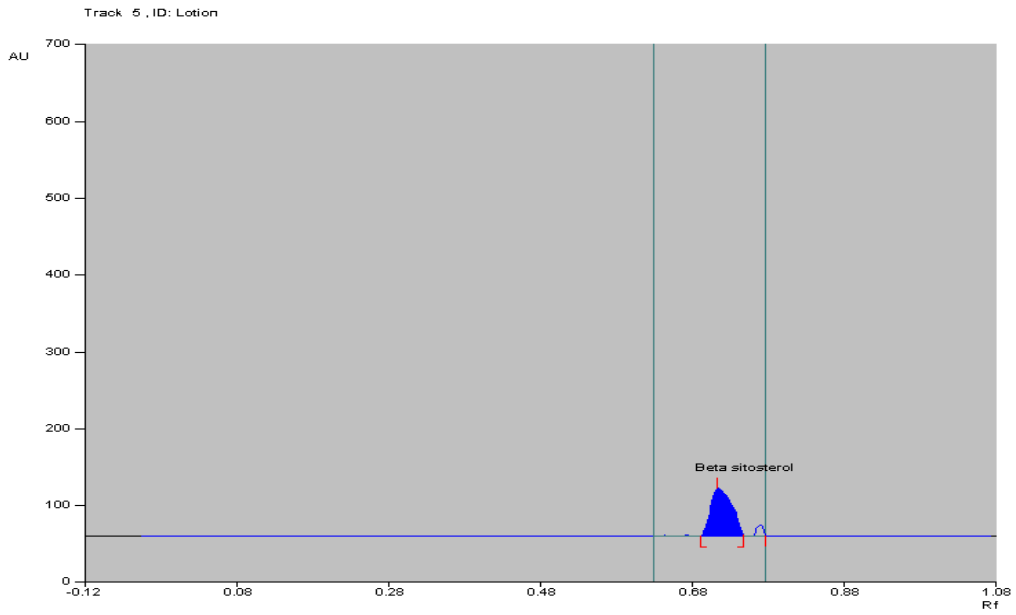


Figure 7.19. HPTLC chromatogram of β-sitosterol present in hair lotion formulation.

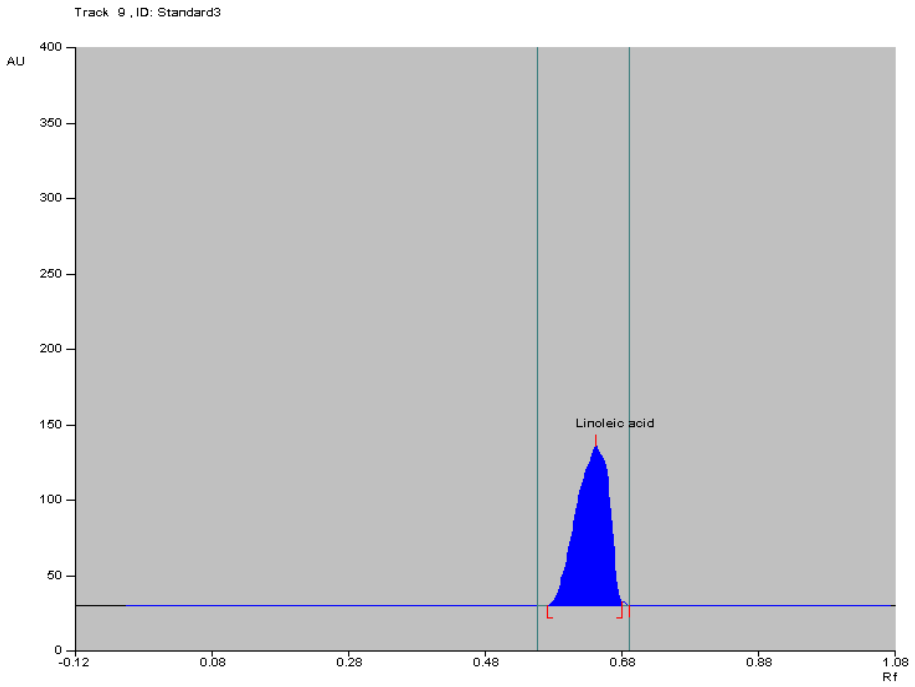


Figure 7.20. HPTLC chromatogram of standard linoleic acid.

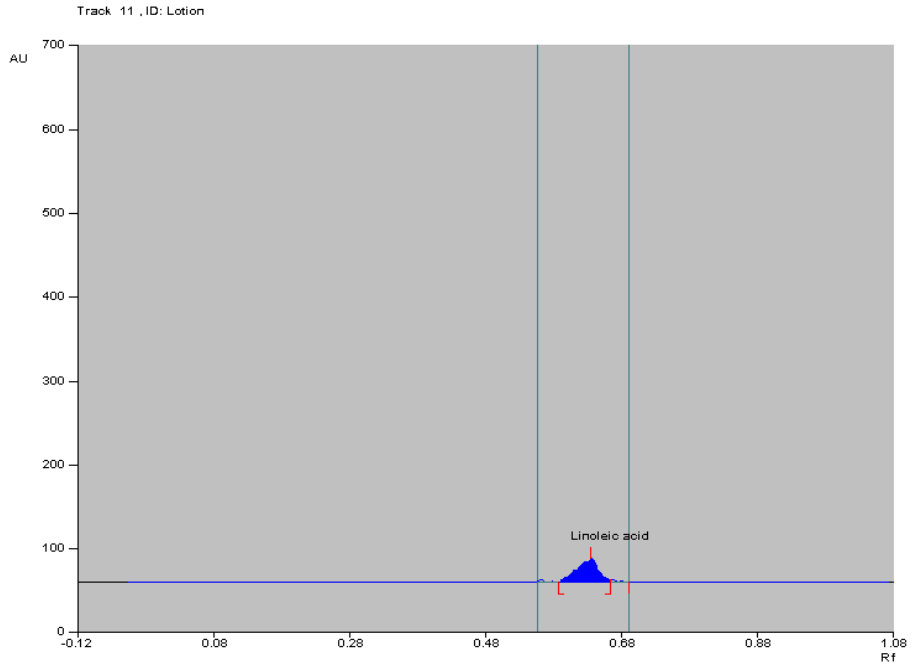


Figure 7.21. HPTLC chromatogram of linoleic acid present in hair lotion formulation.

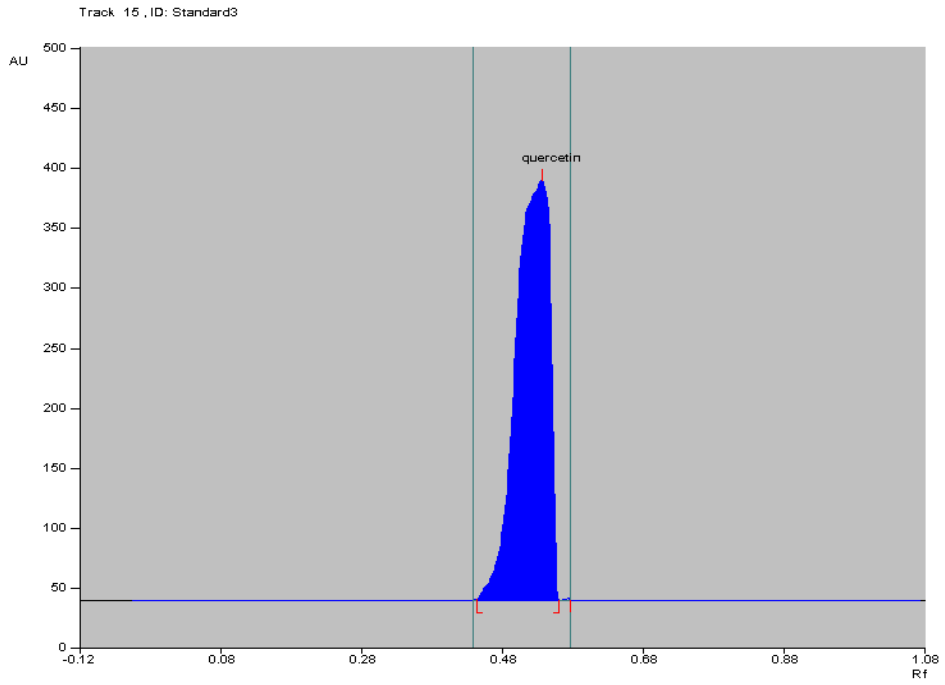


Figure 7.22. HPTLC chromatogram of quercetin.

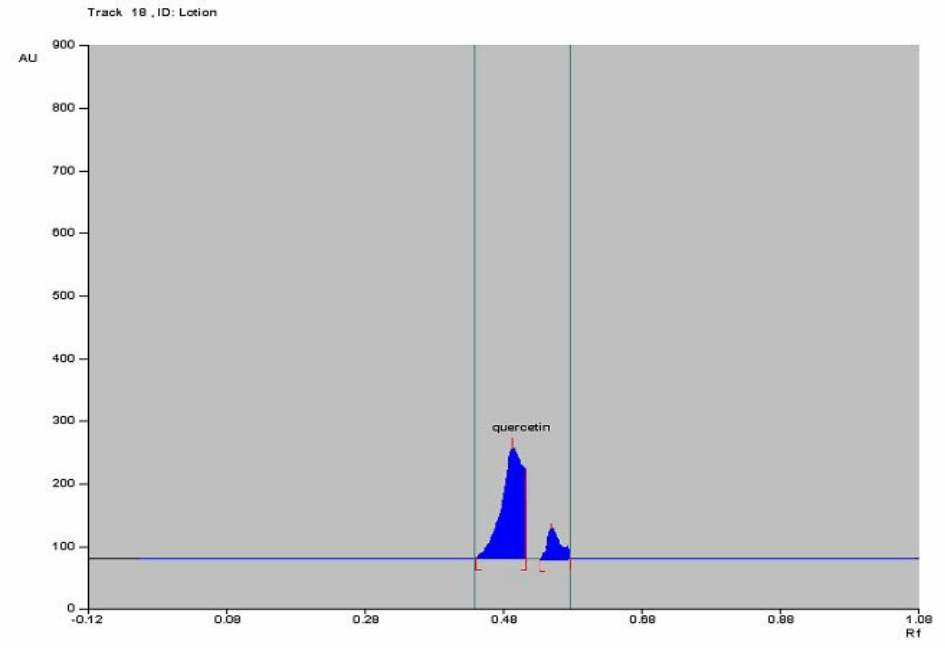


Figure 7.23. HPTLC chromatogram of quercetin present in hair lotion formulation.

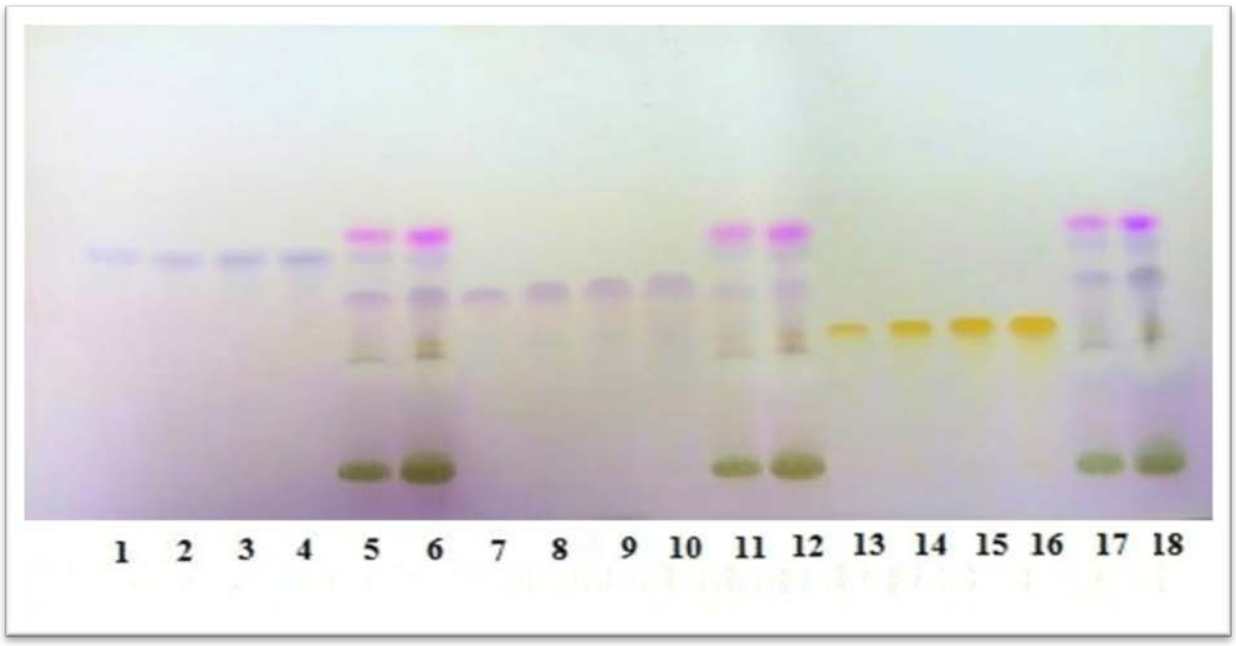


Figure 7.24. Photodocumentaton of hair lotion formulation at 530 nm.

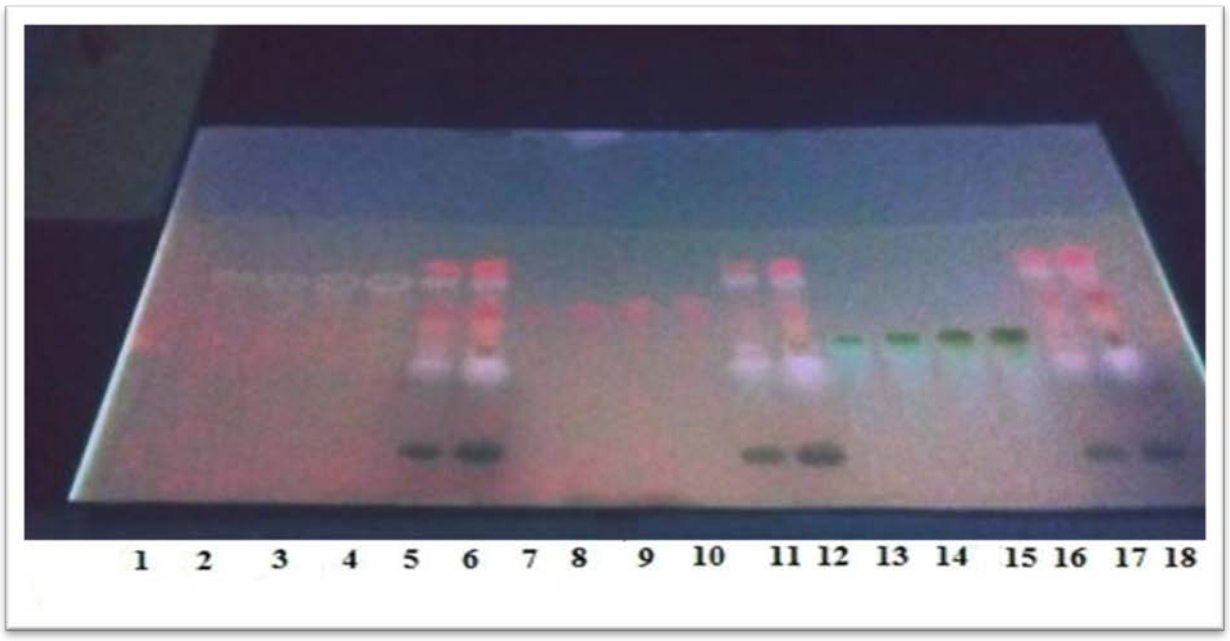


Figure 7.25. Photodocumentaton of hair lotion formulation at 366 nm.

Track no: 1, 2, 3, 4 - Standard β-sitosterol, track no: 7, 8, 9, 10 - linoleic acid, track no: 13, 14, 15, 16 - quercetin, track no: 5, 6, 11, 12, 17, 18 - lotion formulation.

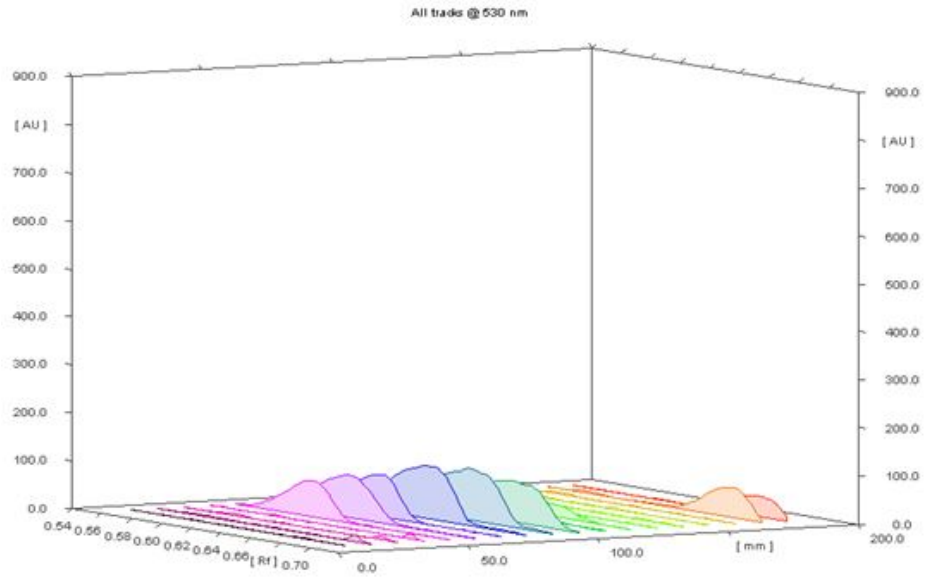


Figure 7.26. 3D chromatogram of herbal hair lotion with β-sitosterol, quercetin and linoleic acid marker at 530 nm.

7.3.7. *Results and discussion*

The result of the lotion formulation is good in visual observance, homogeneity and easily spreadable and showed significant inhibition of 5α-reductase enzyme in in-vitro model. Further HPTLC method was done for quantification of plant biomarkers used in formulation. The results also showed that 5α-reductase enzyme inhibition effect of the formulation was better than the effect of marketed hair lotion formulation.

Table 7.6. Evaluation parameters of hair gel formulations

Parameters	Results
pH	6.75
Visual observation	Brownish yellow colour liquid found.
Homogeneity	Homogeneous, smooth and consistent. No aggregates found.
Drug content	β-sitosterol, quercetin and linoleic was found to be 0.1231, 0.1576, 0.151 % w/w respectively.

7.4. Preparation and evaluation of β-sitosterol hair growth gel formulation

7.4.1. *Materials and method*

β-sitosterol, carbopol 940, propylene glycol, polyvinyl pyrrolidine, DMDM hydration, potassium sorbet, sodium benzoate, triethanolamine, EDTA disodium, igsurf 1540, Water.

7.4.2. *Preparation method of hair gel formulation*

The gel was prepared using pure β-sitosterol. The gel was prepared using carbopol 940 (1%), propylene glycol, DMDM hydration, potassium sorbet, sodium benzoate, EDTA disodium, Triethanolamine and Purified water in quantity to prepare 100 g gel. Water required for the formulations was divided into two parts. In one parts the exact amount of extract was dissolved and to this calculated quantity of igsurf 1540, DMDM hydration, potassium sorbet, sodium benzoate, and EDTA disodium and propylene glycol was added and in other part, carbopol 940 was dissolved and to this solution EDTA disodium, polyvinyl pyrrolidine was added. Both of these solutions were mixed in beaker and triethanolamine was added to the mixture drop wise to obtain gel consistency. Composition of β-sitosterol hair growth gel given below in table 7.7.

Table 7.7. Composition of β-sitosterol hair growth gel

Sl. No		Ingredients	Specification	Quantity used on 100 g basis
1.	I.	β-sitosterol (pure)	USP/EP/BP	0.02 g
2.		DMDM Hydration	I.P	0.3 g
3.		Potassium sorbet	I.P	0.1 g
4.		Sodium benzoate	I.P	0.3 g
5.		Propylene glycol	I.P	15 g
6.		Igsurf 1540	I.P	0.5 g
7.		Kollidon 30	I.P	0.2 g
8.		Purified water	I.P	38 g
9.	II.	Carbopol 940	I.P	1.0 g
10.		EDTA Disodium	I.P	0.5 g
11.		Purified water	I.P	40.0 g
12.	III.	Triethanolamine		Q.S.

7.4.3. *Active ingredient (label claim)*

β-sitosterol (pure) 0.02%.

7.4.4. *Manufacturing method*

Solution I: EDTA disodium mixed in 40 mL water until it gets dissolved. Then measured amount of carbopol 940 added and mixed for 30 minutes and let swell during one hour.

Solution II: Take 15 g of Propylene glycol in a 50 mL beaker and heated 40-50°C in heating mantel for 4-5 minutes. Then Igsurf 1540 was taken and added to it with constant stirring. Measured amount β-sitosterol is added to the above solution with constant stirring.

Polyvinyl pyrrolidine (Kollidon 30), DMDM Hydration, potassium sorbet, sodium benzoate, was added sequentially in a beaker containing 38 g of purified water. Then the solution was slowly added to the β-sitosterol containing mixture into 50 mL beaker with constant stirring.

Solution III: Solution II was added to Solution I with constant mixing. Then neutralized to skin pH (6.5-6.8) with triethanolamine by constant stirring for 10 minutes.

Properties of the gel

Light color transparent gel.

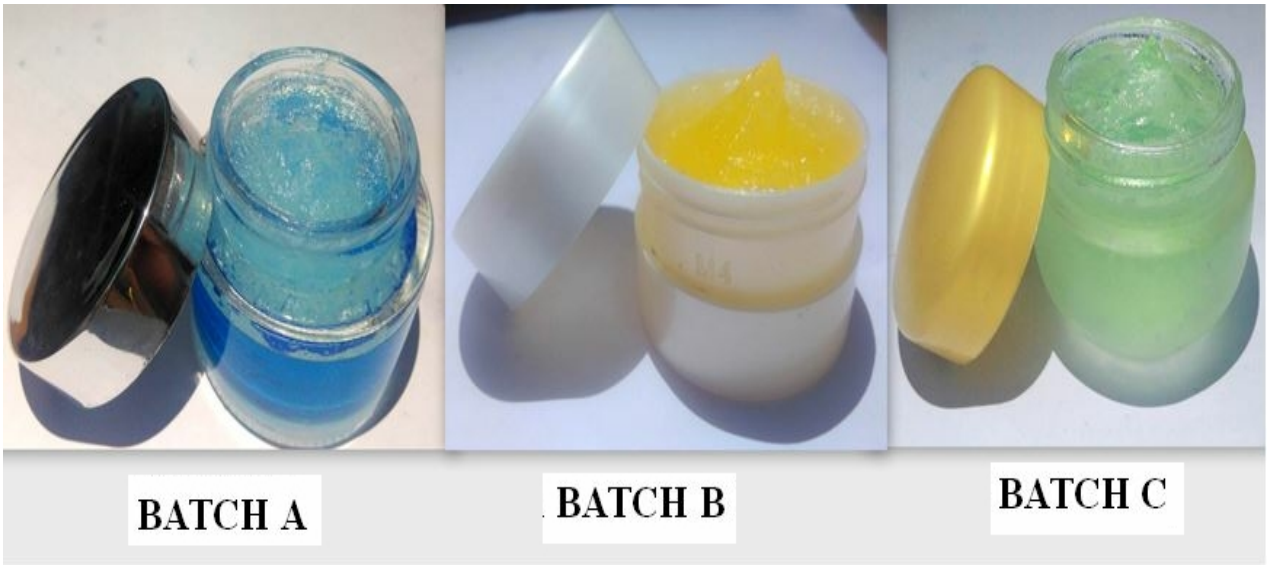
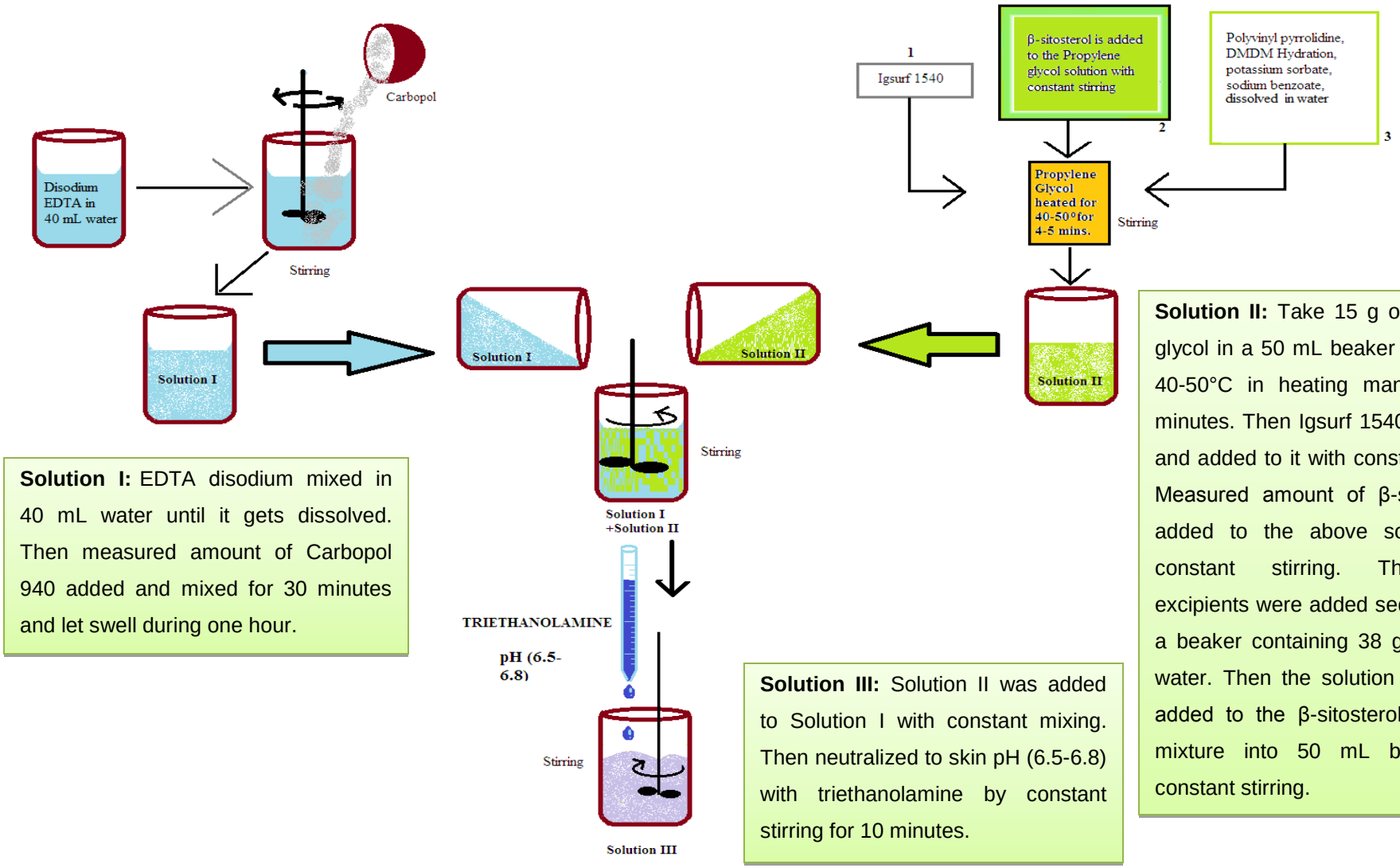


Figure 7.27. Batch A, Batch B, Batch C represent 3 different batches of β-sitosterol gel formulation for evaluation of process validation by checking different critical parameters of gel. In each batch same manufacturing method and condition is maintained. Different colours are added for different batch to identify easily.

In Batch A brilliant blue colour is used for making light blue. In Batch B tartrazine yellow colour is used for making light orange and for Batch C brilliant blue and tartrazine yellow mixed together in 7:3 w/w ratio for making green colour. FD&C colouring agents are used in formulations.



Solution I: EDTA disodium mixed in 40 mL water until it gets dissolved. Then measured amount of Carbopol 940 added and mixed for 30 minutes and let swell during one hour.

Solution II: Take 15 g of Propylene glycol in a 50 mL beaker and heated 40-50°C in heating mantel for 4-5 minutes. Then Igsurf 1540 was taken and added to it with constant stirring. Measured amount of β-sitosterol is added to the above solution with constant stirring. Then other excipients were added sequentially in a beaker containing 38 g of purified water. Then the solution was slowly added to the β-sitosterol containing mixture into 50 mL beaker with constant stirring.

Solution III: Solution II was added to Solution I with constant mixing. Then neutralized to skin pH (6.5-6.8) with triethanolamine by constant stirring for 10 minutes.

Figure 7.28. Schematic diagram of β-sitosterol hair gel preparation

7.4.5. **Evaluation of hair gel formulation**

7.4.5.1. *pH*

The pH of various gel formulations was determined by using digital pH meter. The measurement of pH of each formulation was done in triplicate and average values were calculated.

7.4.5.2. *Viscosity*

Brookfield viscometer is used for the measurement of viscosity of the prepared gel. The Brookfield viscometer is rotated at 50 rpm by spindle no.64. Each reading was taken after equilibrium of the sample at the end of two minutes. The samples were repeated three times.

7.4.5.3. *Spredibility*

It was determined by wooden block and glass slide apparatus. Weights about 20g were added to the pan and the time was noted for upper slide (movable) to separate completely from the fixed slides. Spredibility was then calculated by using the formula:

$$S = M.L / T$$

Where, S = Spredibility, M = Weight tide to upper slide, L = Length of glass slide, T = Time taken to separate the slide completely from each other. The therapeutic efficacy of a formulation also depends upon its value.

7.4.5.4. *% of drug content*

In this method 1g of prepared gel is mixed with 10 mL of drug soluble or extractable with methanol. Aliquots or different concentrations are prepared by suitable dilutions after filtration the stock solution and the absorbance is measured. Drug content will be calculated the equation obtained by linear or calibration curve.

7.4.5.5. *Physical stability*

The gel formulations were evaluated in terms of physical character like phase separation & change in colour, odour & rheological parameters.

7.4.5.6. *Homogeneity*

All developed gels were tested for homogeneity by visual inspection after the gels have been set in the container. They were tested for their appearance and presence of any aggregates.

7.4.5.7. *Skin irritation test*

Test for irritation was performed on human volunteers. For each gel, five volunteers were selected and 1.0 g of formulated gel was applied on an area of 2 square inch to the back of hand. The volunteers were observed for lesions or irritation.

7.4.6. Standardization and quantification of β-sitosterol, in β-sitosterol hair growth gel by HPTLC

7.4.6.1. *Equipments and reagents*

The CAMAG HPTLC system consisting of WINCATS software, LINOMAT V automatic sample applicator, and automatic development chamber, scanning densitometer CAMAG scanner 3 and photo documentation apparatus CAMAG reprostar 3 were used. Stationary phase used was

aluminum based silica gel plate 60 F_{254} (Merck, Mumbai) with 20 cm x 10 cm in a particle size of 5-10 µm. All the solvents were used of analytical grade.100 µL syringe (HAMILTON, Switzerland) was used for sample application on HPTLC plates. β-sitosterol was purchased from Sigma Aldrich. Methanol, toluene, ethyl acetate of analytical grade were purchased from Merck (Mumbai, India). Whatman's syringe filter (NYL 0.45 µm) was used for the filtration of samples and standard.

7.4.6.2. *Preparation of standard solution*

About 1 mg of β-sitosterol standard was weighed and put in to separate 1 mL eppendorf tube. 1.0 mL of methanol in added in each 1 mL eppendorf tube and mixed in vortex mixture till the material completely dissolved. It was then filtered through 0.45 µ syringe filter and kept for further study.

7.4.6.3. *Preparation of sample solution*

About 1 g of 3 β-sitosterol gel formulations (Batch-A, Batch-B, Batch-C) was weighed and taken in three separate centrifuge tube. 10 mL methanol added to each centrifuge tube. Mixed in vortex mixture for 15 minutes and put to ultrasonication bath for 30 minutes. It was then filtered through 0.45 µ syringe filter and kept it in 1 mL eppendorf tube for further study.

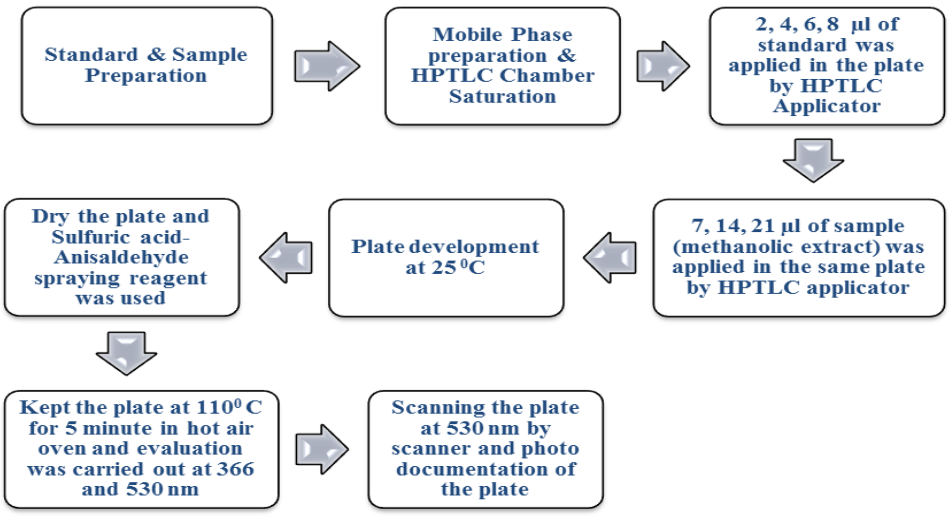


Figure 7.29. Standardization and quantification of β-sitosterol (standards) in Herbal hair growth Gel (sample) by HPTLC.

7.4.6.4. *Chromatographic conditions*

HPTLC analysis was performed using isocratic technique. Mobile phase was optimized with toluene: ethyl acetate: methanol in a ratio of 5: 3: 2 v/v. The temperature was kept at 25 ^{0}c and mobile phase was developed in a twin trough glass chamber. Standard stock solution of three

biomarkers was applied consequently in the range of 2-10 µL with 2 µL gradual increment. Sample solution was applied 7, 14, 21 µL of Batch-A, Batch-B and Batch-C respectively. All total 14 tracks in HPTLC plate were used for standardization including standard and sample solution respectively in a band wise fashion. After development, plates were dried by hand dryer. The dry plate was treated with sulfuric acid-anisaldehyde spraying reagent. The plate was kept at 110^0 C for 5 minute in hot air oven and evaluation was carried out at 366 and 540.Colored bands were observed at 366 to 530 nm.

7.4.6.5. *Results and discussion*

The percentage content of β-sitosterol in hair growth gel formulation was found to 97.58, 97.51 and 97.98 % w/w respectively in three different batch named Batch-A, Batch-B and Batch-C. This was determined by a calibration curve with the equation of Y = 3813.444*X + 435.851 (correlation coefficient = 0.9469 and standard deviation = ± 8.40%) as shown in Fig 7.30 where X represents amount of β-sitosterol and Y represents area under the curve.

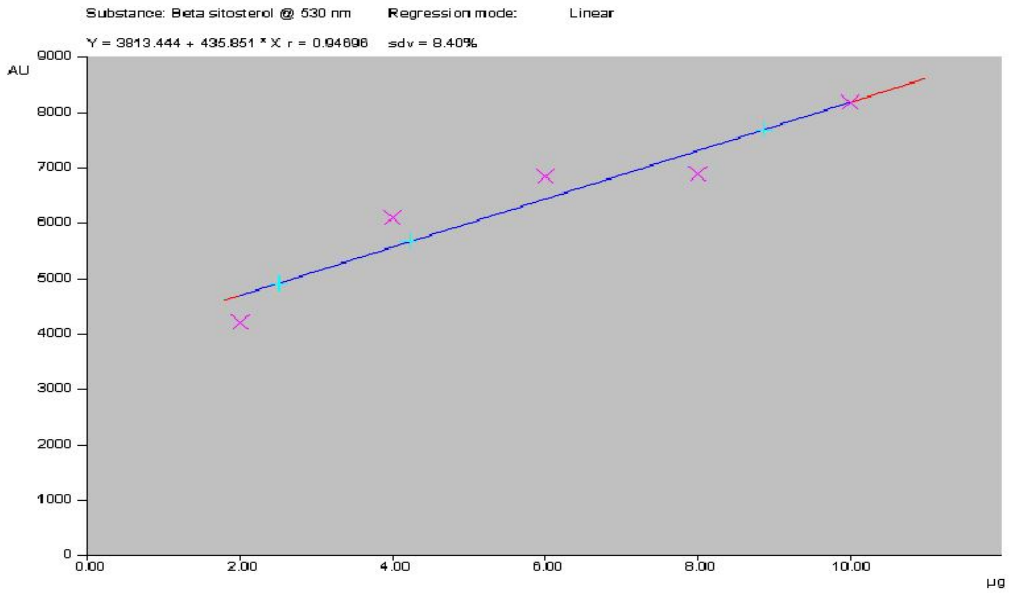


Figure 7.30. Calibration curve of β-sitosterol.

R_f value of standard β-sitosterol was found to be 0.66. Specificity was confirmed by comparing the R_f of standard and sample (Figure 7.31 – 7.34).

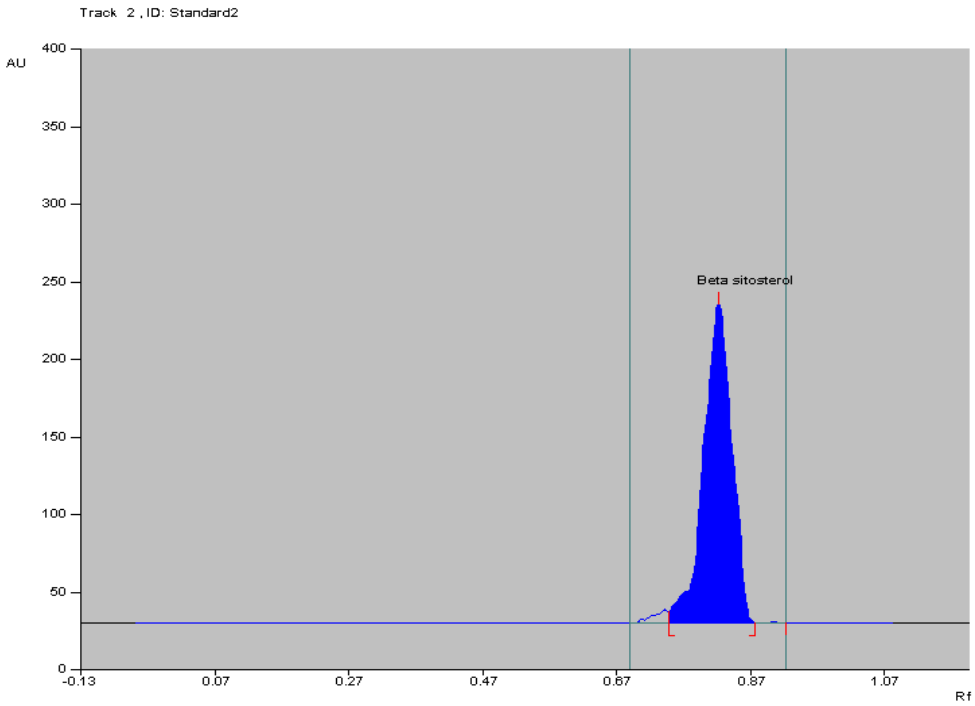


Figure 7.31. HPTLC chromatogram of standard β-sitosterol.

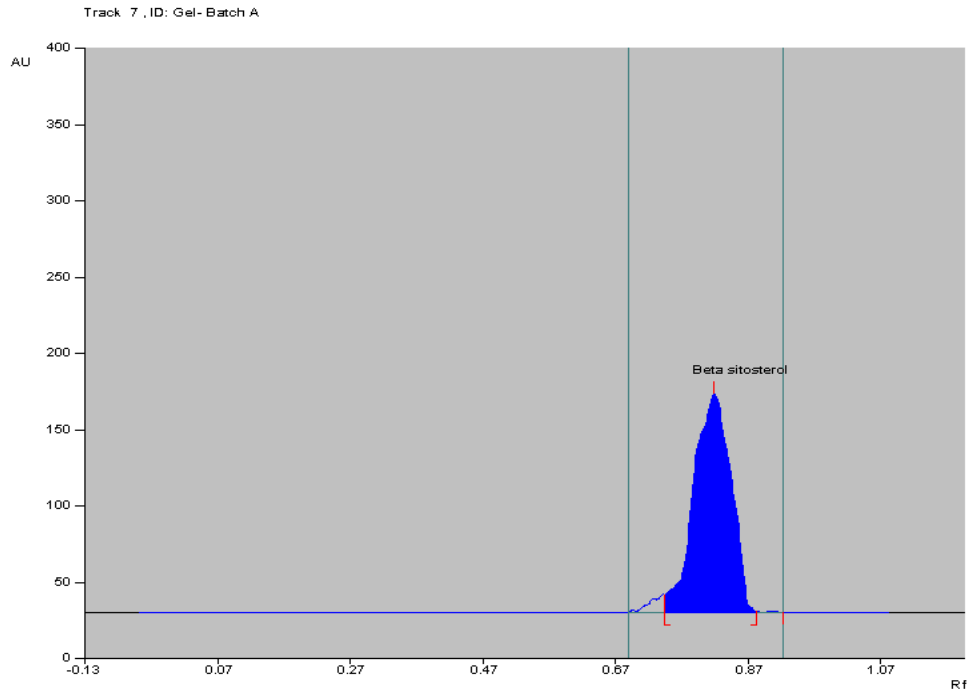


Figure 7.32. HPTLC chromatogram of β-sitosterol present in hair gel formulation Batch A.

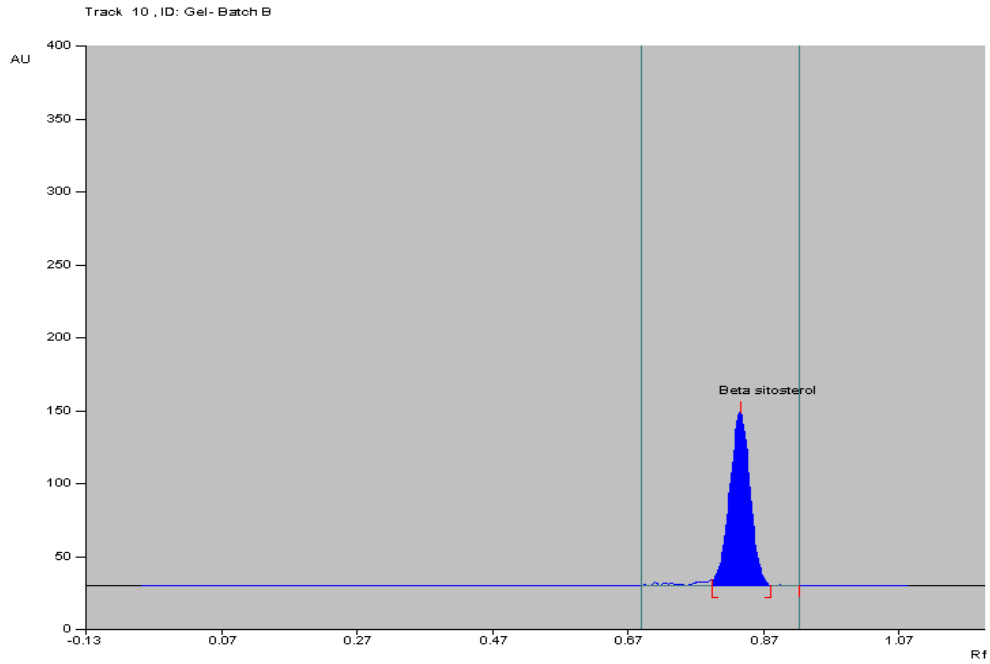


Figure 7.33. HPTLC chromatogram of β-sitosterol present in gel formulation Batch B.

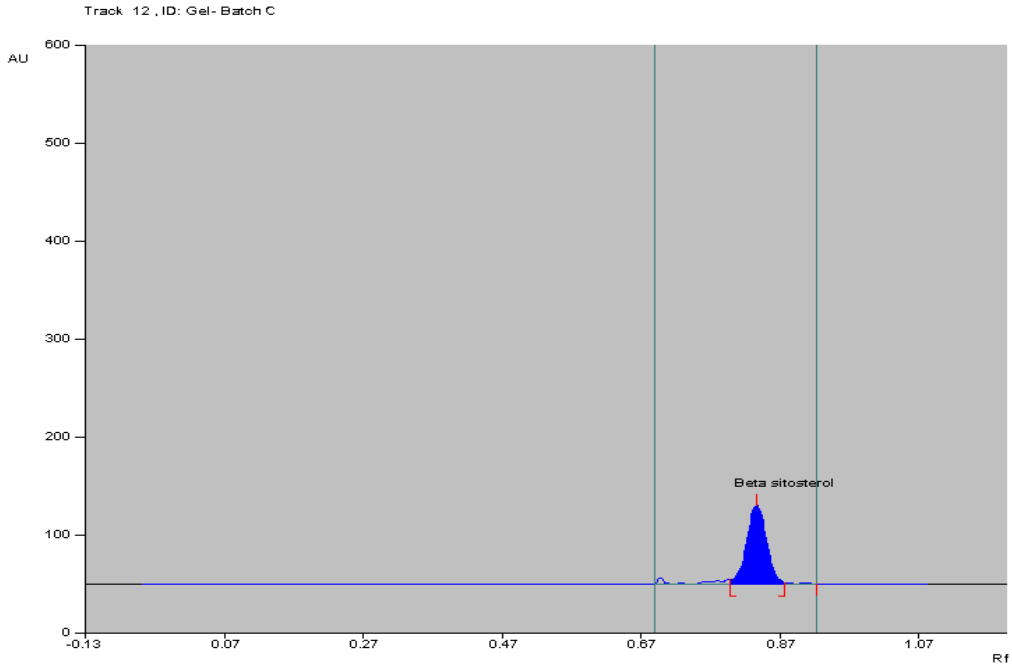


Figure 7.34. HPTLC chromatogram of β-sitosterol present in hair gel formulation Batch C.

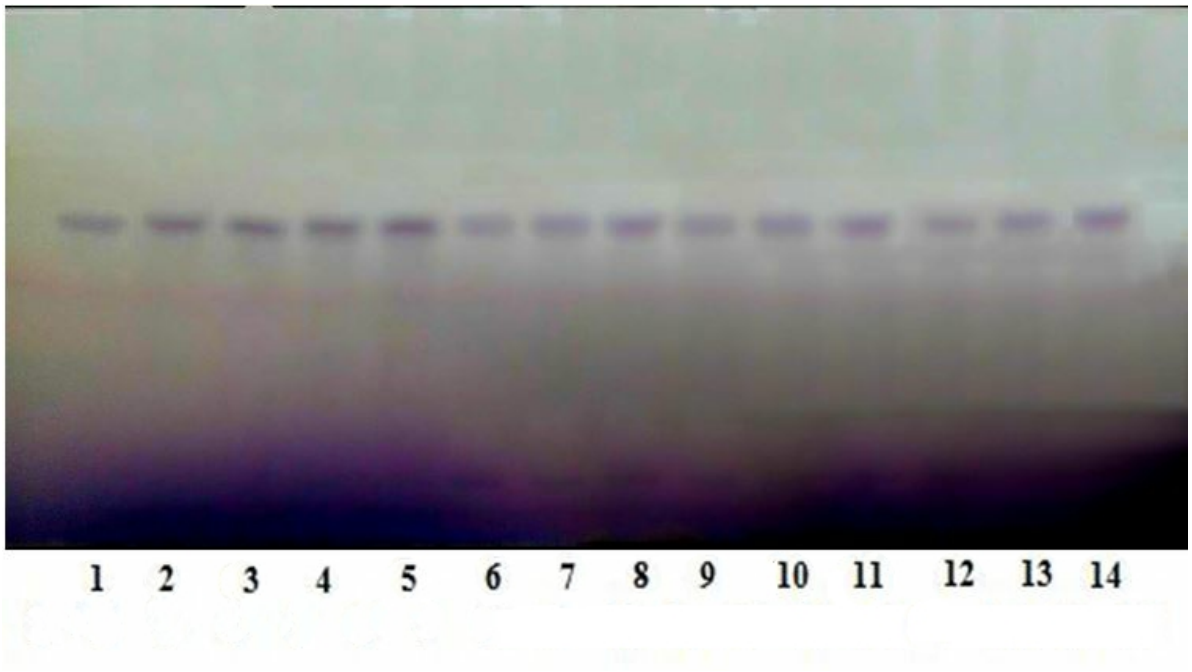


Figure 7.35. Photodocumentaton of β-sitosterol gel at 530 nm.

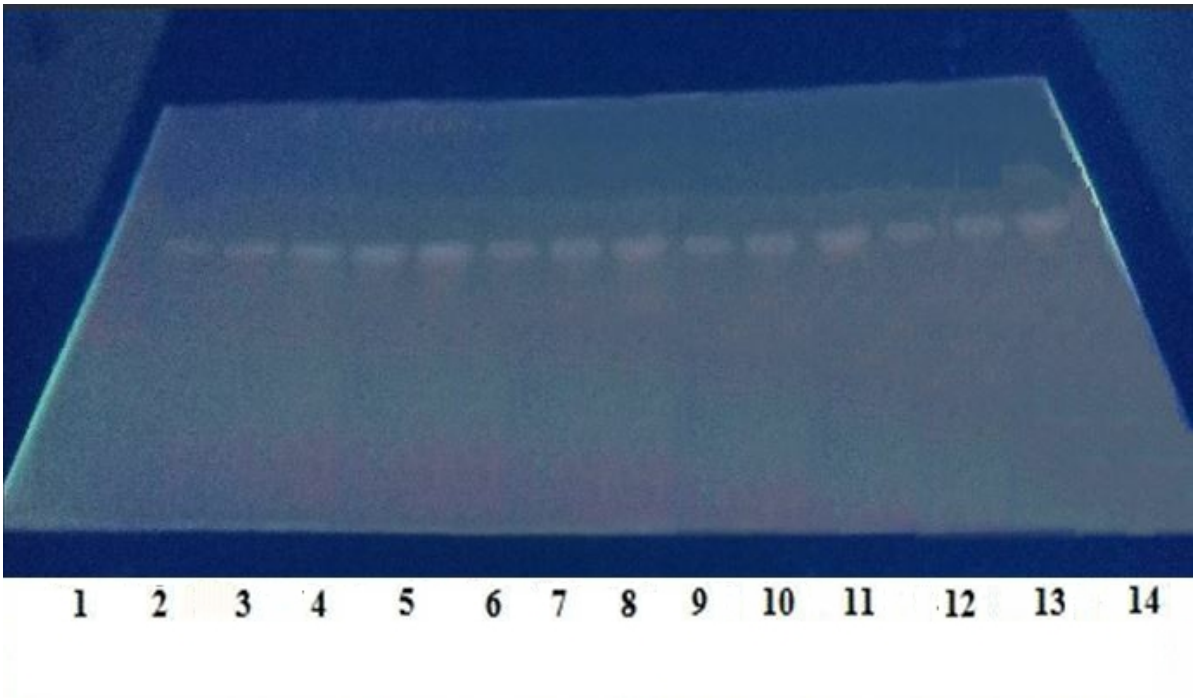


Figure 7.36. Photodocumentaton of β-sitosterol gel at 366 nm

Track no: 1, 2, 3, 4, 5- Standard β-sitosterol, track no: 6, 7, 8 – Batch-A, track no: 9, 10, 11 – Batch-B, track no: 12, 13, 14 – Batch-C.

7.4.7. *Standardization Herbal hair gel by HPLC*

HPLC is a method to standardize and identify the chemical component which is expected to be present in a medicinal plant or any herbal formulation. This is done from regulatory perspective to ensure the quality, efficacy, safety and quantify the herbal drugs present in a plant or herbal formulation. Thus it provides a very reliable way of determining the purity and percentage content of the active biomarker in the herbal formulations. In this chapter the standardization and quantification of β-sitosterol biomarker in three batches (Batch-A, Batch-B, Batch-C) of β-sitosterol hair gel by HPLC. The label claim of active ingredient β-sitosterol presents 0.02 % in gel. This process ensures the manufacturing process validation of the gel.

7.4.7.1. *Equipments and Reagents*

RP-HPLC system (Shimadzu Prominence, Kyoto, Japan) equipped with two Shimadzu LC-20 AD UFLC reciprocating pumps, a variable Shimadzu SPD-M20A Prominence PDA detector and a Rheodyne manual injector with a loop size of 20 µL was used. The peak area was calculated with LC solution software. The analysis was carried out in isocratic condition using a C_{18} reverse phase column having dimension of 250 mm (length) × 4.6 mm (width) with a particle size of 5 µm (Phenomenex-Luna C_{18}, Torrance, CA, USA). β-sitosterol was purchased from sigma Aldrich. Methanol, acetonitrile analytical grade were purchased from Merck (Mumbai, India). All other solvents were used of analytical grade. Whatman's syringe filter (NYL 0.45 µm) was used for the filtration of samples and standard.

7.4.7.2. *Preparation of standard solution*

Standard stock solution: About 1 mg of β-sitosterol standard was weighed and put in to separate 1 mL eppendorf tube. 1.0 mL of methanol in added in 1 mL eppendorf tube and mixed in vortex mixture till the material completely dissolved. Volume was made with methanol to obtain standard stock solution of concentration 1000 µg/mL. It was then filtered through 0.45 µ syringe filter and kept for further study. Further dilutions were made as required with methanol for calibration curve.

Working standard solution: Take 20 µL of standard stock solution in 1 mL eppendorf tube and add 980 µL fresh methanol in the tube to make 20 µg/mL.

7.4.7.3. *Preparation of sample solution*

About 1 g of β-sitosterol gel was weighed and taken in a centrifuge tube. 10 mL methanol was added to the centrifuge tube. Mixed in vortex mixture for 15 minutes and put to ultrasonication bath for 30 minutes. It was then filtered through 0.45 µ syringe filter and kept it in 1 mL eppendorf tube for further study. Sample solution obtained concentration was 20 µg/mL β-sitosterol.

7.4.7.4. *Chromatographic conditions*

HPLC analysis was performed using isocratic technique. Mobile phase was optimized with of Acetonitrile and Methanol in the ratio of 70:30 v/v respectively. The temperature was kept at 25 ^{0}c and standard and sample was inject 20 µL. Standard stock solution of biomarker was applied consequently in the range of 2.5 to 15 µL with 2.5 µL gradual increment for calibration curve. Sample solution was applied 20 µL. Stationary phase used RP C18 (250 x 4.6, 5 µm). Detection was carried out at 210 nm wavelength. Flow rate was adjusted to 1 mg/mL.

Table 7.8. HPLC Method Parameters

HPLC System	**:**	Shimadzu HPLC with rheodyne injector
Pump	:	Binary pump
Detector	:	PDA Multi-wavelength detector
Stationary phase	**:**	RP-C18 (250 mm × 4.6 mm i.d. particle size 5 µm) column
Mobile phase	**:**	Acetonitrile: Methanol (70:30 v/v)
Detection wavelength	**:**	UV detector, 210 nm.
Flow rate	**:**	1 mL/minute
Sample size	**:**	20 µL

7.4.7.5. **Results and discussion**

The percentage content of β-sitosterol in β-sitosterol hair gel Batch-A, Batch-B, Batch-C was found to be 97.62, 97.59, 97.85 % w/w respectively. This was determined by a calibration curve with the equation of Y = 2649.8*X + 228397 (correlation coefficient = 0.9923) as shown in Fig 11.1 where X represents amount of β-sitosterol and Y represents area under the curve.

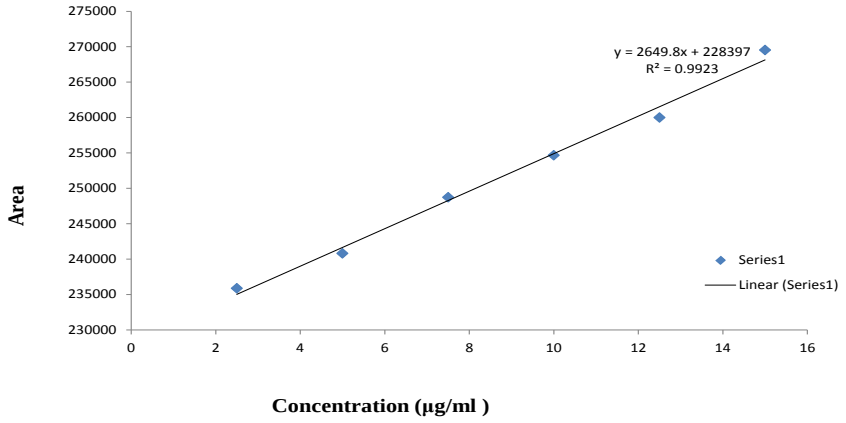


Figure 7.37. Calibration curve of β-sitosterol.

Retention time (R_t) value of standard β-sitosterol was found to be 2.3 minute. Specificity was confirmed by comparing the R_t of standard and sample (Figure 7.38 – 7.41).

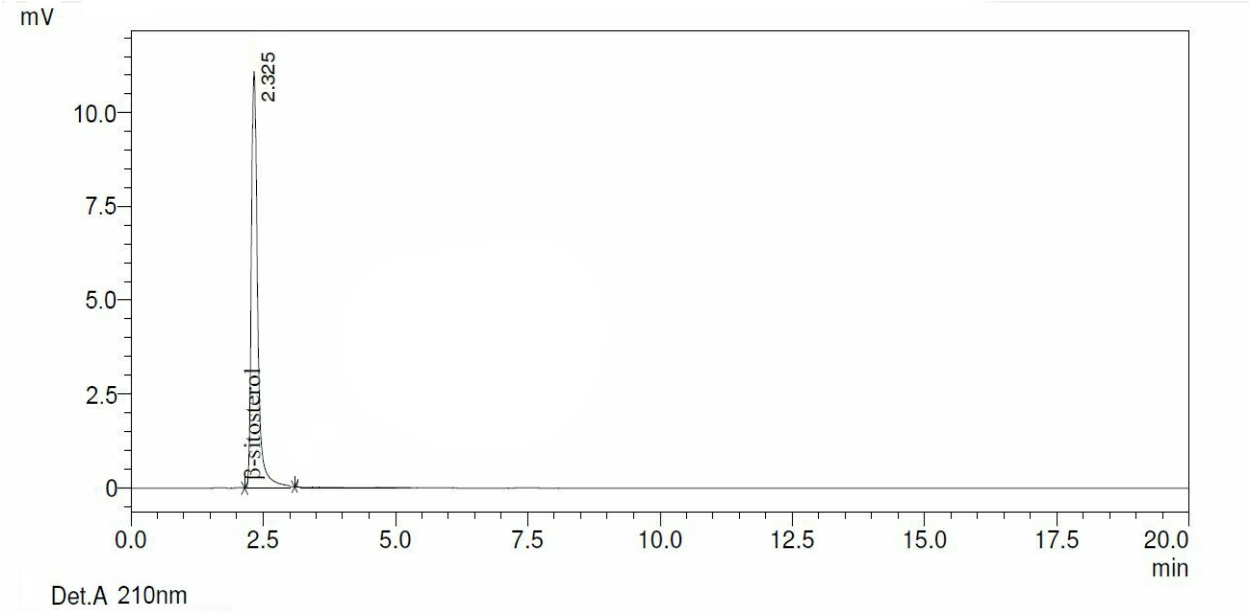


Figure 7.38. Chromatogram of β-sitosterol.

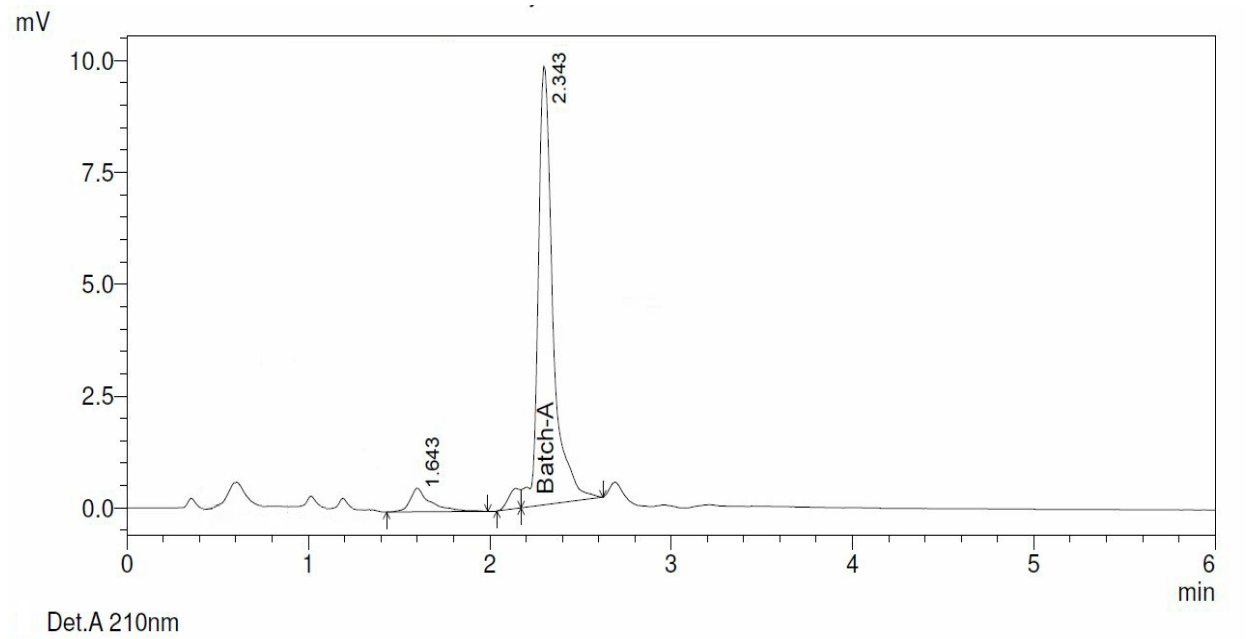


Figure 7.39. Chromatogram of β-sitosterol hair gel Batch-A.

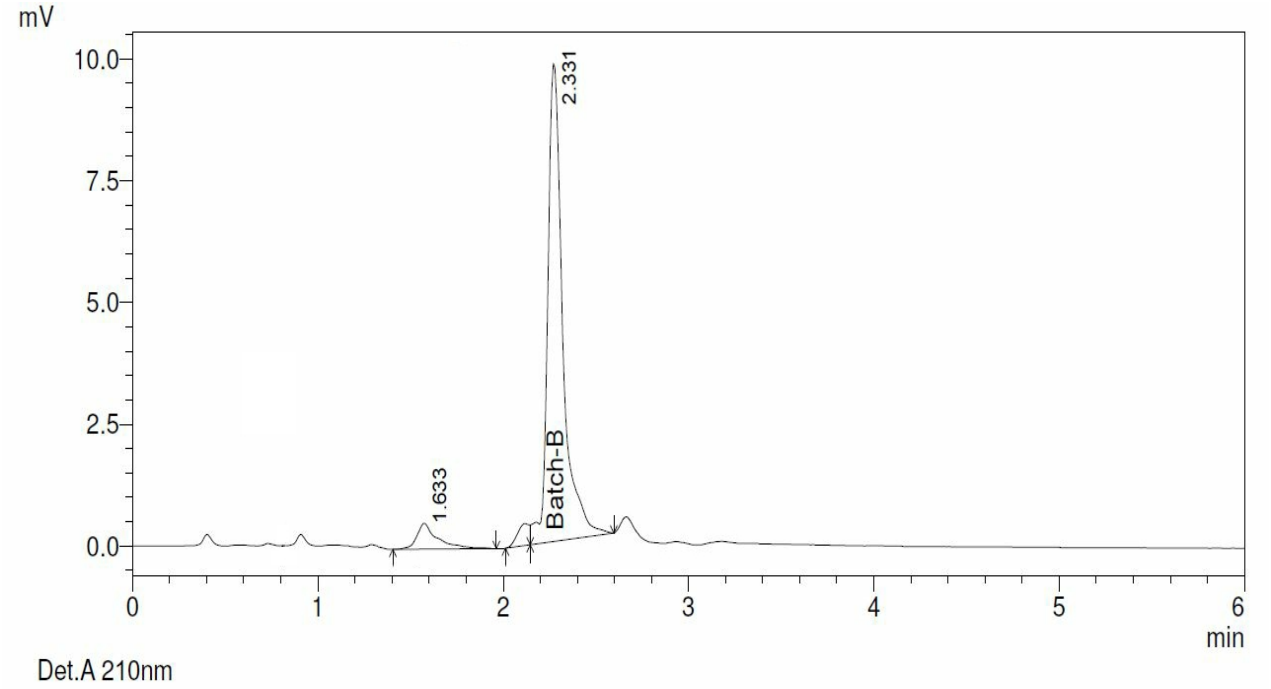


Figure 7.40. Chromatogram of β-sitosterol hair gel Batch-B.

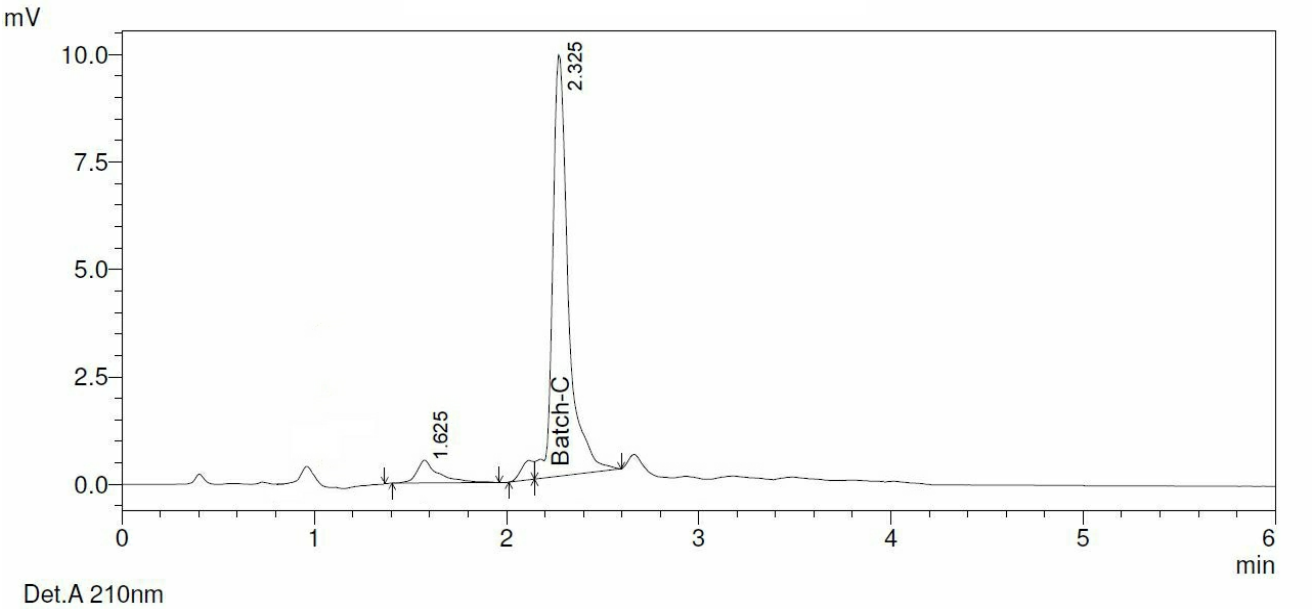


Figure 7.41. Chromatogram of β-sitosterol hair gel Batch-C.

7.4.8. **Results and discussion**

The result of the gel formulation is good in appearance, homogeneity and easily spreadable and showed significant inhibition of 5α-reductase enzyme in in-vitro model. Further HPTLC method

was done for quantification of plant biomarkers used in formulation. The results also showed that 5α-reductase enzyme inhibition effect of the formulation was better than the effect of marketed hair gel formulation. Batch A, Batch B, Batch C represent 3 different batches of β-sitosterol gel formulation. By checking different critical parameters of three different batches gel, we can evaluate the manufacturing process method validation and the process reproducibility. HPLC method was also done for quantification of plant biomarkers and correlate with HPTLC data.

Table 7.9. Evaluation parameters of β-sitosterol hair gel formulation

Sl. no	**Parameters**	**Results**			**(Mean ±Std. Dev)**
		Batch A	**Batch B**	**Batch C**	
1.	pH	6.65	6.67	6.61	6.64 ± 0.03
2.	Viscosity	4736	4735	4735	4735.33 ± 0.577
3.	% of drug content HPTLC	97.58	97.51	97.98	97.69 ± 0.25
4.	% of drug content HPLC	97.62	97.59	97.85	97.68 ± 0.14
5.	Physical appearance	Light blue, odourless, have a good consistency.	Light orange, odourless, have a good consistency.	Light green, odourless, have a good consistency.	-
6.	Homogeneity	Homogenous, no aggregates found.	Homogenous no aggregates found.	Homogenous no aggregates found.	-
7.	Skin irritation test	Skin compatible, no irritation found.	Skin compatible, no irritation found.	Skin compatible, no irritation found.	-
8.	Spredibility (g-cm/sec)	10.56	10.47	10.52	10.51 ± 0.04

Chapter – 8

Comparative evaluation of 5α-reductase inhibitory potential of some marketed product with prepared formulations

8.1. Comparative evaluation of 5α-reductase inhibitory potential of Alova marketed gel with prepared herbal gel formulations

8.2. Comparative evaluation of 5α-reductase inhibitory potential of Himalaya Anti-hair fall marketed cream with prepared cream formulation

8.3. Comparative evaluation of 5α-reductase inhibitory potential of Foligain marketed hair lotion with prepared lotion

8.1. Comparative evaluation of 5α-reductase inhibitory potential of Alova marketed gel with prepared herbal gel formulations

8.1.1. *Reagent used*

Testosterone was obtained from marketed formulation Testoviron Depot injection (100 mg/mL), Finasteride was obtained from marketed formulation Fincar tablet (contains 5 mg Finasteride in each tablet), NADPH was purchased from Sisco research laboratories (SRL) and Tris buffer were purchased from Sigma-Aldrich. Ethylenediamine tetraacetic acid (EDTA), sodium Phosphate and sucrose were purchased from Merck, Mumbai. Methanol, ethanol (95%), ethyl acetate and petroleum ether (60–80°C) (analytical grade) were purchased from Merck, Mumbai. All other chemicals used in the study were of analytical grade.

8.1.2. *Reagent and chemical preparation*

8.1.2.1. *Tissue homogenization medium*

0.0372 g EDTA, 10.95 g sucrose and 0.760 g tri-sodium phosphate dodecahydrate dissolved in 100 mL water. pH was adjusted to 6.5.

8.1.2.2. *Preparation of NADPH solution*

Stock NADPH solution was prepared by dissolving of 0.0083 g NADPH in 10 mL methanol (1000µM). 1.54 mL NADPH stock solution was diluted with 68.46 mL methanol for preparation of 22µM working solution (70 mL).

8.1.2.3. *Preparation of testosterone solution*

Stock solution: Testosterone stock was prepared by dissolving 28 µL of testosterone solution (injection) in 9972 µL methanol. 28 µL of testosterone solution contains 0.0028 g of testosterone in 10 mL methanol (1000 µM).

Working solution: 3.52 mL stock was diluted with 43.48 mL methanol for preparation of 75µM working solutions (46 mL).

8.1.2.4. Preparation of finasteride solution

Stock solution: 0.1151 g finasteride cursed power (tablet) was dissolve in 10 mL methanol. 0.1151 g finasteride cursed power contains approximate 0.0037 g of pure finasteride. Sonication was carried out for 1 hour and vortexed for 15 min. Then filtration was carried out by 0.45 µ syringe filter, and filtrate was collected in two separate eppendorf tube.

Working solution: 2 µL of stock finasteride solution diluted with 1998 µL methanol to obtained 1 µM working solutions.

8.1.2.5. *Preparation of Tris HCl*

7.88 g tris HCl dissolved in 100 mL water.

8.1.3. *Methods of preparation 5α-reductase solution*

8.1.3.1. *Isolation of 5α-reductase enzyme solution*

Rats were anesthetized with diethyl ether. Testes were quickly removed. Isolated testis was wash with 0.9% normal saline. Testes were minced in small piece and homogenized with medium (20 mM sodium phosphate, pH 6.5, containing 0.32 M sucrose and 1 mM EDTA). The homogenate was centrifuged at 4000 rpm (716 g) for 15 min. the Supernatant was used as source of enzyme. Protein concentration in the supernatant was determined by Bradford method of protein estimation (Nahata & Dixit, 2013).

8.1.3.2. *Estimation of protein concentration*

8.1.3.2.1. *BSA standard curve*

1 mg/mL stock BSA solution was prepared in deionized water. Two fold serial dilutions were prepared from stock with the concentrations of 0.5, 0.25 and 0.125 mg/mL. Different concentrations of prepared BSA solution (5 µL) were added to 96 well micro plates. 200 µL of Bradford reagents were added to the BSA solutions. Absorbance was measured at 592 nm. Standard curve was plotted with the concentrations of standards against the absorbance at 592 nm.

8.1.3.2.2. *Protein concentration of testis solution*

5 µL of rat testis solutions were added to micowell plate. 200 µL of Bradford reagent was added into the test solution. Absorbance was measured at 592 nm. Concentration of the protein was determined from BSA standard curve.

8.1.3.2.3. *Optimum protein concentration for the assay*

Protein concentration of isolated testis was found 16.631 mg/mL. Further the solution was diluted to 100 µg/mL by tissue homogenization medium for enzyme assay.

8.1.4. *Preparation of NADPH stranded curve*

Standard curve of NADPH was prepared in methanol at 340 nm using concentration of 1, 3, 5, 8, 10, 12, 15 and 20 µg/mL. Equation of straight line was $y = 0.0244x + 0.00056$, and the correlation coefficient (r^2) was = 0.9978 indicating good linearity between absorbance and concentration.

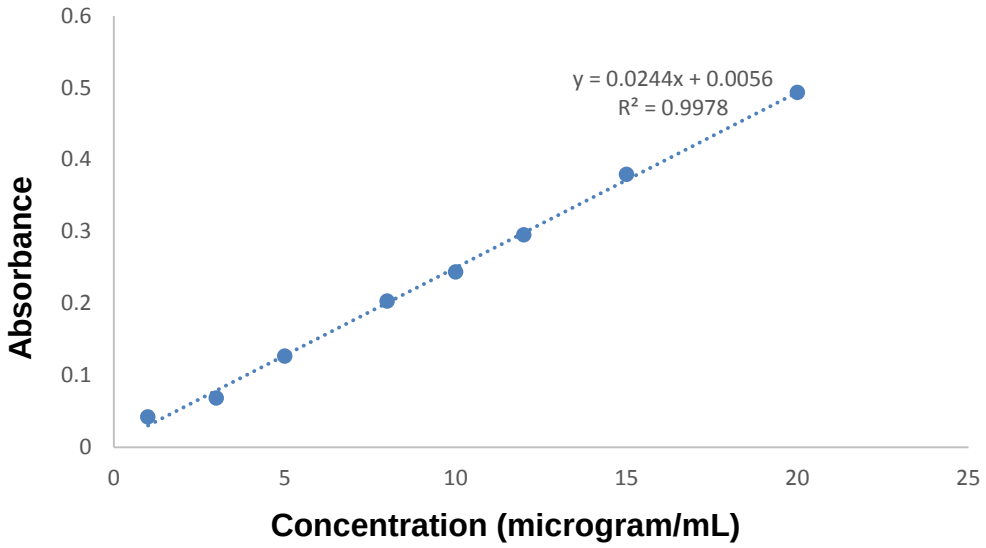


Figure 8.1. Calibration curve of NADPH.

8.1.5. *Sample preparation of herbal hair gel*

About 1 g of marketed hair gel (Alova) was taken in each of two separate centrifuge tubes. To one of the centrifuge tubes 10 mL methanol was added and to the other 10 mL of ethyl acetate was added. Sonication was carried out for 1 hour and each sample was vortexed for 15 min. Then filtration was carried out by 0.45 µ syringe filter for each sample, and filtrate was collected in two separate eppendorf tube. After that 10 µL of each methanolic and ethyl acetate extract was taken in centrifuge tubes and volume was made upto was carried 10 mL by respective parent solvent to make 100 µg/mL concentration. Similar procedure was carried out for the prepared herbal hair gel (A) and β-sitosterol gel (B) formulation.

For this research work two different solvents namely methanol and ethyl acetate were selected for purpose of extraction. Methanol was selected so as to extract out the polar active ingredients whereas ethyl acetate was used in order to extract the non-polar active ingredients used in the formulation.

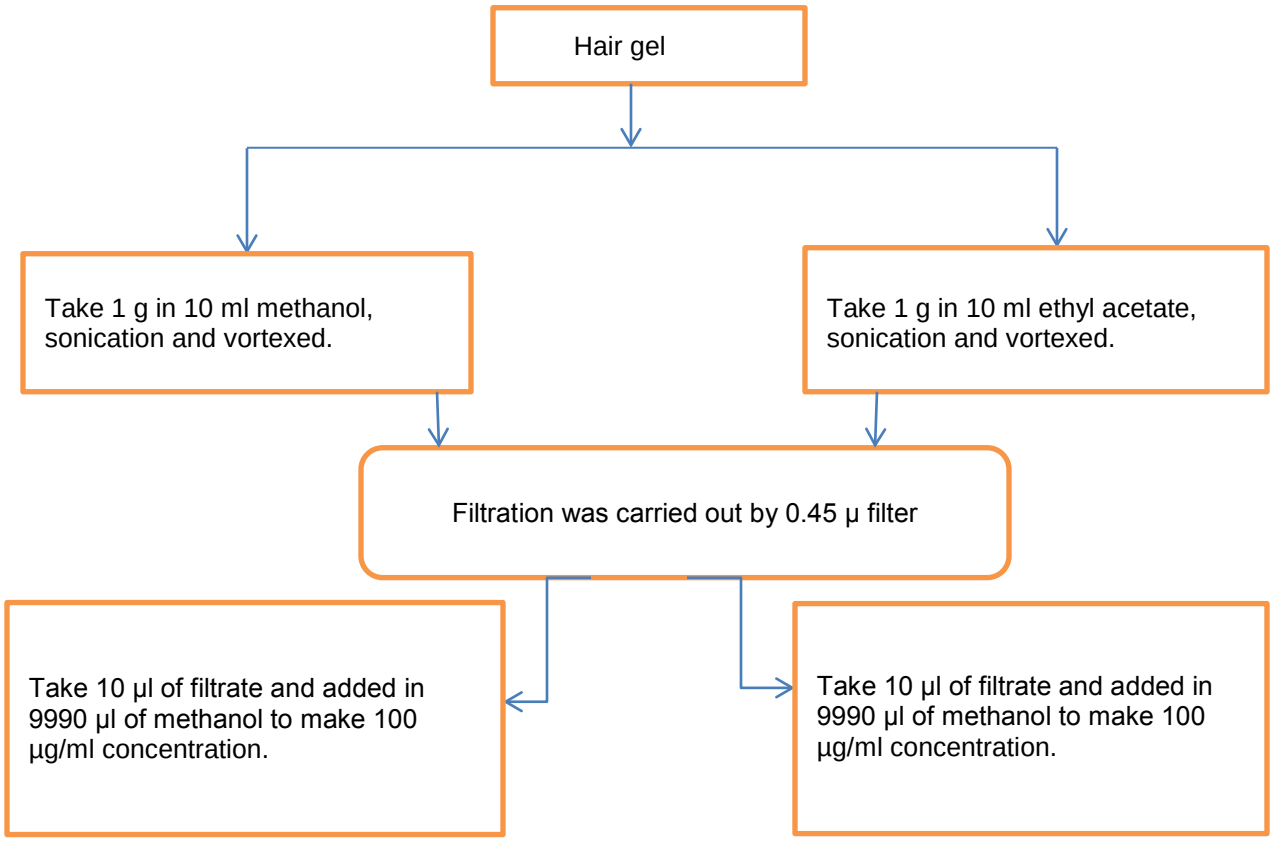


Figure. 8.2. Sample preparation of herbal hair gel.

Prepared herbal gel (A) **Marketed herbal hair gel** **Prepared β-sitosterol gel (B)**

Figure. 8.3. Marketed and prepared hair gel formulations.

8.1.6. *Assay procedure of 5α-reductase inhibition of herbal gel formulations (methanolic and ethyl acetate extract)*

5α-reductase inhibition assays were performed based on the method described by Nahata & Dixit, 2013. In brief, rat testis homogenate containing enzyme, T, NADPH and test samples were mix together. The detail reaction mixtures have been described in Table 8.1. All the reaction mixtures were incubated at 37 °C for 30 min. Absorbance was measured spectrophotometrically at 340 nm. Corresponding concentration of NADPH of the test samples were calculated from the NADPH standard curve. Residual amount of NADPH in the reaction mixture were determined. % scavenging of NADPH was determined from the NADPH concentration. Blank absorbance was deducted from the test samples to get net absorbance of NADPH. The 5α-reductase inhibition was calculated for individual test substances which reflect the original efficacy of the test substances against the enzyme. % inhibition of 5α-reductase was determined by % NADPH scavenging potential.

Table 8.1. Enzyme, substrate and coenzyme mixture

Sample ID	Methanol (mL)	Tris HCL (mL)	NADPH (mL)	Enzyme (mL)	Finasteride / (mL)	Test sample (mL)	Vortex and incubate at 37°C for 10 min	Testosterone (mL)	Vortex and incubate at 37°C for 30 min	Total volume (mL)
Blank Control	4	4	3	1						12
Negative control	2	4	3	1				2		12
Finasteride		4	3	1	2			2		12
Test samples		4	3	1		2		2		12

Absorbance taken at 340 nm

Net absorbance of test = (Test Absorbance – Blank Absorbance)

Calculate the NADPH concentration in each tube from NADPH standard curve prepared previously.

Percentage inhibition: = (100 – [(54.78- net NADPH concentration of test)/54.78) × 100]

8.1.7. *Result and discussion*

For this research work two different solvents namely methanol and ethyl acetate were selected for purpose of extraction. Methanol was selected so as to extract out the polar active ingredients whereas ethyl acetate was used in order to extract the non-polar active ingredients used in the formulation. Comparative evaluation of 5α-reductase inhibitory potential of marketed gel formulation (Alova) against prepared herbal hair gel (A) and β-sitosterol gel (B) formulation was done. Prepared poly herbal hair gel methanolic extract was found to have maximum inhibition activity. Whereas Beta sitosterol ethyl acetate extract showed better inhibition than its methanolic extract. Methanolic extract of marketed gel Alova showed better inhibition than its ethyl acetate extract so it can be concluded most of the active ingredient used in Alova gel is polar in nature. Comparative percentage inhibition of gels given in table 8.2 and represented by bar graph given below.

Table 8.2. Percentage inhibition of herbal gel formulations

Sl. no	Sample	Concentration	Percentage of inhibition
1.	Negative control		4.8989
2.	Positive control (finasteride)	0.2 µM	17.462
3.	Alova gel methanolic extract	100 µg/mL	16.863
4.	Alova gel ethyl acetate extract	100 µg/mL	9.9055
5.	Prepared poly herbal hair gel methanolic extract	100 µg/mL	21.352
6.	Prepared poly herbal hair gel Ethyl acetate extract	100 µg/mL	12.524
7.	Beta sitosterol methanolic extract	100 µg/mL	15.591
8.	Beta sitosterol ethyl acetate extract	100 µg/mL	16.788

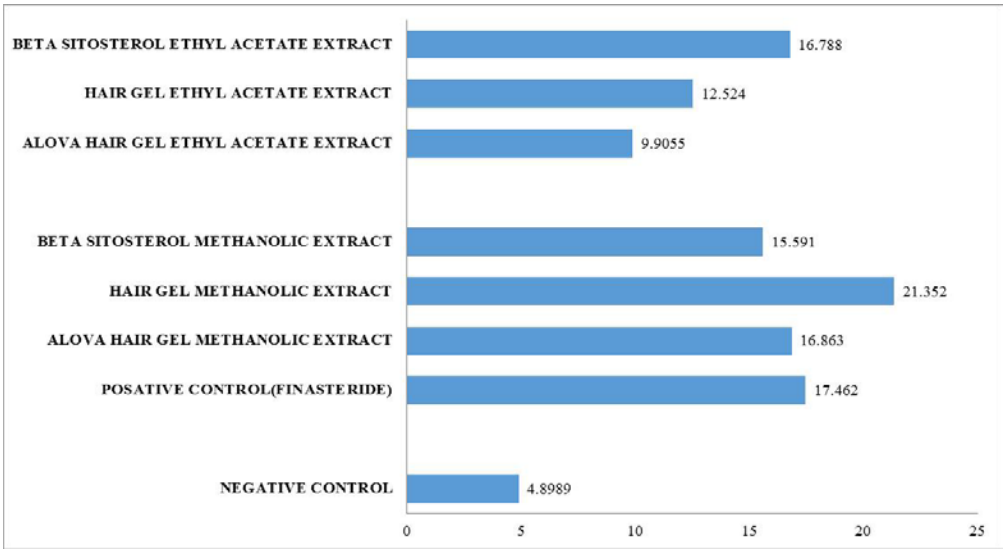


Figure 8.4. Comparative evaluation of 5α-reductase inhibition of hair gel formulations.

8.2. Comparative evaluation of 5α-reductase inhibitory potential of Himalaya Anti-hair fall marketed cream with prepared cream formulation)

8.2.1. *Sample preparation of herbal hair cream*

About 1 g of marketed Himalaya anti hair fall cream was taken in each of two separate centrifuge tubes. To one of the centrifuge tubes 10 mL methanol was added and to the other 10 mL of ethyl acetate was added. Sonication was carried out for 1 hour and each sample was vortexed for 15 min. Then filtration was carried out by 0.45 µ syringe filter for each sample, and filtrate was collected in two separate eppendorf tube. After that 10 µL of each methanolic and ethyl acetate extract was taken in centrifuge tubes and volume was made upto was carried 10 mL by respective parent solvent to make 100 µg/mL concentration. Similar procedure was carried out for the prepared herbal hair cream formulation.

For this research work two different solvents namely methanol and ethyl acetate were selected for purpose of extraction. Methanol was selected so as to extract out the polar active ingredients whereas ethyl acetate was used in order to extract the non-polar active ingredients used in the formulation.

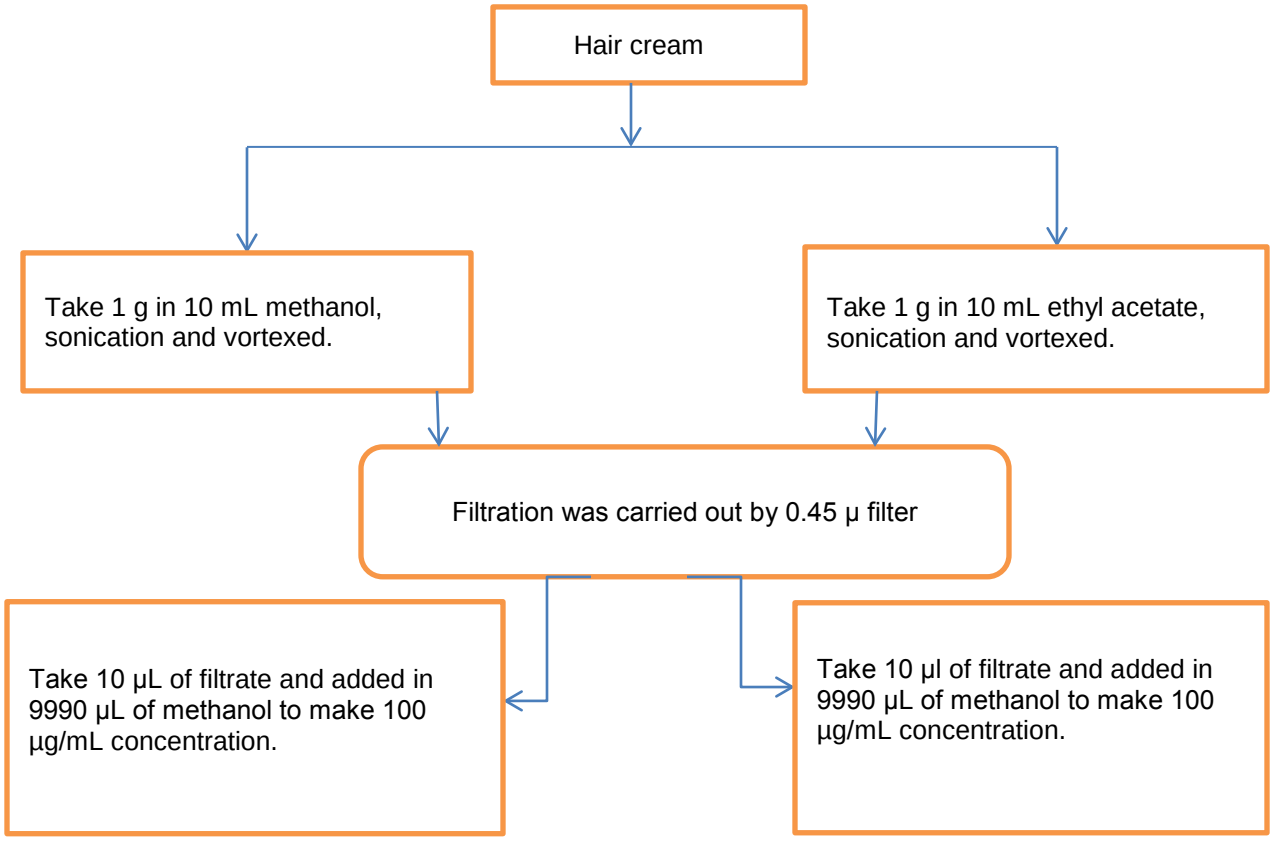


Figure. 8.5. Sample preparation of herbal hair cream.

Prepared herbal cream **Marketed herbal hair cream**

Figure. 8.6. Marketed and prepared hair gel formulations.

8.2.2. *Assay procedure of 5α-reductase inhibition of herbal cream formulations (methanolic and ethyl acetate extract)*

5α-reductase inhibition assays were performed based on the method described by Nahata & Dixit, 2013. In brief, rat testis homogenate containing enzyme, T, NADPH and test samples were mix together. The detail reaction mixtures have been described in Table 8.1. All the reaction mixtures were incubated at 37 °C for 30 min. Absorbance was measured spectrophotometrically at 340 nm. Corresponding concentration of NADPH of the test samples were calculated from the NADPH standard curve. Residual amount of NADPH in the reaction mixture were determined. % scavenging of NADPH was determined from the NADPH concentration. Blank absorbance was deducted from the test samples to get net absorbance of NADPH. The 5α-reductase inhibition was calculated for individual test substances which reflect the original efficacy of the test substances against the enzyme. % inhibition of 5α-reductase was determined by % NADPH scavenging potential.

Net absorbance of test = (Test Absorbance – Blank Absorbance)

Calculate the NADPH concentration in each tube from NADPH standard curve prepared previously.

Percentage inhibition: = (100 – [(54.78-net NADPH concentration of test)/54.78) × 100]

8.2.3. **Result and discussion**

For this research work two different solvents namely methanol and ethyl acetate were selected for purpose of extraction. Methanol was selected so as to extract out the polar active ingredients whereas ethyl acetate was used in order to extract the non-polar active ingredients used in the formulation. Comparative evaluation of 5α-reductase inhibitory potential of marketed cream formulation (Himalaya Anti-hair fall) against prepared herbal hair cream formulation was done. Prepared poly herbal hair cream ethyl acetate extract was found to have maximum inhibition activity, whereas methanolic extract showed relatively less inhibition. Methanolic extract of marketed Himalaya Anti-hair fall cream showed better inhibition than its ethyl acetate extract so it can be concluded most of the active ingredient used in Himalaya cream is polar in nature. Comparative percentage inhibition of creams mentioned in table 8.3 and represented by bar graph given below.

Table 8.3. Percentage inhibition of herbal cream formulations

Sl. no	Sample	Concentration	Percentage of inhibition
1.	Negative control		4.8989
2.	Positive control (finasteride)	0.2 µM	17.462
3.	Himalaya Anti-hair fall methanolic extract	100 µg/mL	11.402
4.	Himalaya Anti-hair fall ethyl acetate extract	100 µg/mL	4.0699
5.	Prepared poly herbal hair cream methanolic extract	100 µg/mL	19.407
6.	Prepared poly herbal hair cream ethyl acetate extract	100 µg/mL	22.026

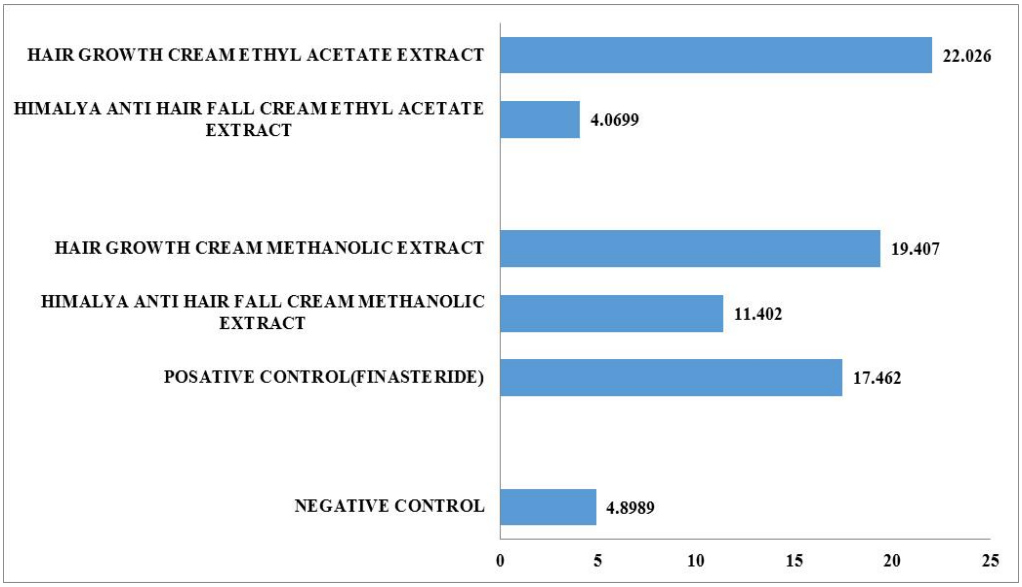


Figure 8.7. Comparative evaluation of 5α-reductase inhibition of hair cream formulations.

8.3. 5α-reductase Comparative evaluation of 5α-reductase inhibitory potential of Foligain marketed hair lotion with prepared lotion

8.3.1. *Sample preparation of herbal hair lotions*

About 1 g of marketed Foligain marketed hair lotion was taken in each of two separate centrifuge tubes. To one of the centrifuge tubes 10 mL methanol was added and to the other 10 mL of ethyl acetate was added. Sonication was carried out for 1 hour and each sample was vortexed for 15 min. Then filtration was carried out by 0.45 µ syringe filter for each sample, and filtrate was collected in two separate eppendorf tube. After that 10 mL of each methanolic and ethyl acetate extract was taken in centrifuge tubes and volume was made upto was carried 10 mL by respective parent solvent to make 100 µg/mL concentration. Similar procedure was carried out for the prepared herbal hair formulation.

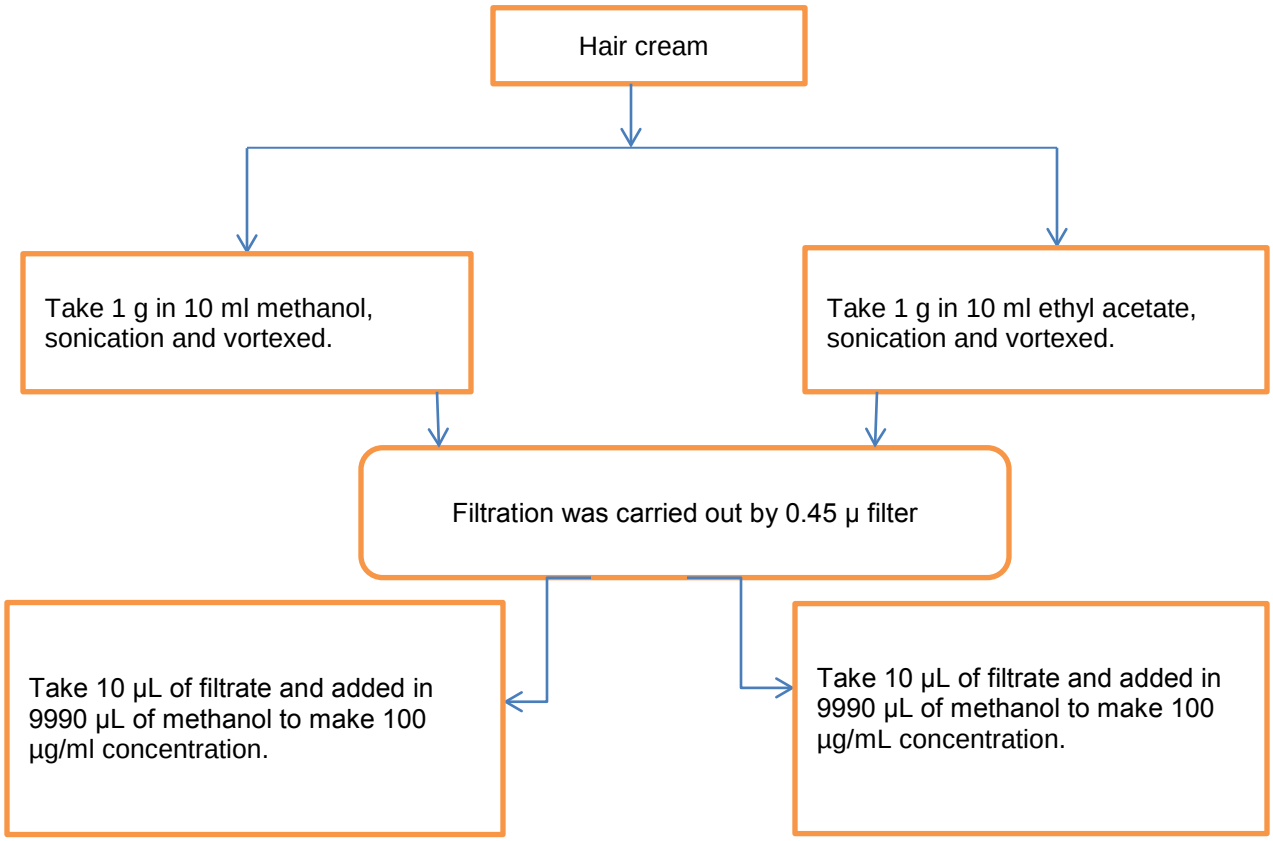
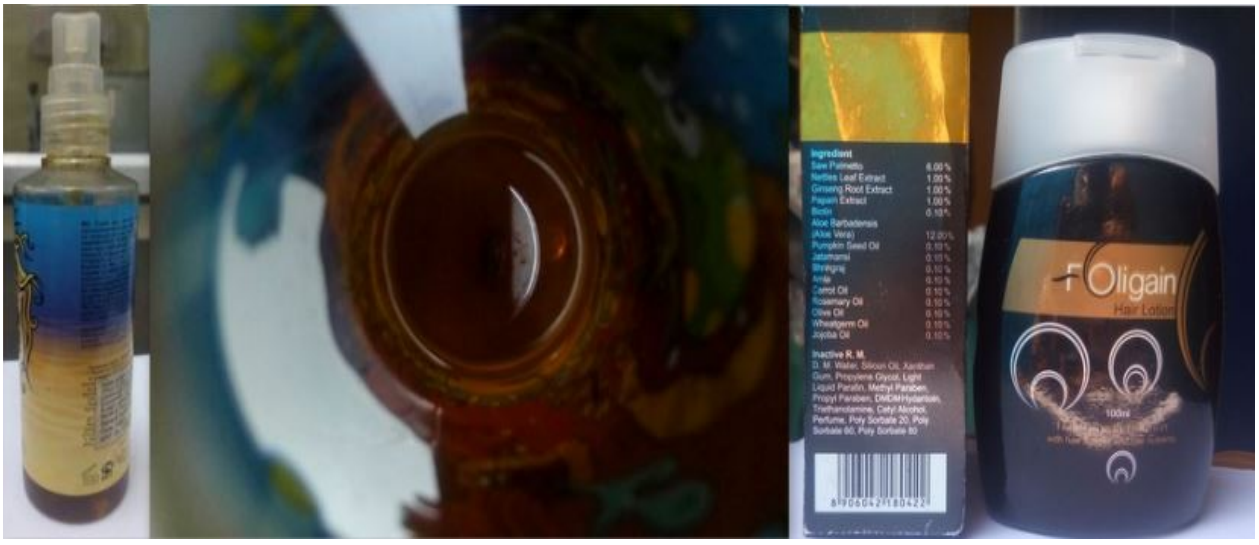


Figure. 8.8. Sample preparation of herbal hair lotion.

Similar procedure was carried out for sample preparation of marketed and prepared herbal lotion formulation.



Prepared herbal lotion **Marketed herbal hair lotion**

Figure. 8.9. Marketed and prepared hair lotion formulations.

8.3.2. **Assay procedure of 5α-reductase inhibition of herbal lotion formulations (methanolic and petroleum ether extract)**

5α-reductase inhibition assays were performed based on the method described by Nahata & Dixit, 2013. In brief, rat testis homogenate containing enzyme, T, NADPH and test samples were mix together. The detail reaction mixtures have been described in Table 8.1. All the reaction mixtures were incubated at 37 ^{o}C for 30 min. Absorbance was measured spectrophotometrically at 340 nm. Corresponding concentration of NADPH of the test samples were calculated from the NADPH standard curve. Residual amount of NADPH in the reaction mixture were determined. % scavenging of NADPH was determined from the NADPH concentration. Blank absorbance was deducted from the test samples to get net absorbance of NADPH. The 5α-reductase inhibition was calculated for individual test substances which reflect the original efficacy of the test substances against the enzyme. % inhibition of 5α-reductase was determined by % NADPH scavenging potential.

8.3.3. *Result and discussion*

For this research work, two different solvents namely methanol and ethyl acetate were selected for purpose of extraction. Methanol was selected so as to extract out the polar active ingredients whereas ethyl acetate was used in order to extract the non-polar active ingredients used in the formulation. Comparative evaluation of 5α-reductase inhibitory potential of marketed lotion formulation (Foligain lotion) against prepared herbal hair lotion formulation was done. Prepared poly herbal hair lotion ethyl acetate extract was found to have maximum inhibition activity, whereas methanolic extract showed relatively less inhibition. Methanolic extract of marketed Foligain lotion showed better inhibition than its ethyl acetate extract so it can be concluded most of the active ingredient used in Himalaya cream is polar in nature. Comparative percentage inhibition of lotions was shown in table 8.4 and represented by bar graph given below.

Table 8.4. Percentage inhibition of herbal lotion formulations

Sl. no	Sample	Concentration	Percentage of inhibition
1.	Negative control		4.8989
2.	Positive control (finasteride)	0.2 µM	17.462
3.	Foligain lotion methanolic extract	100 µg/mL	11.402
4.	Foligain lotion ethyl acetate extract	100 µg/mL	4.0699
5.	Prepared poly herbal hair lotion methanolic extract	100 µg/mL	19.407
6.	Prepared poly herbal hair lotion ethyl acetate extract	100 µg/mL	22.026

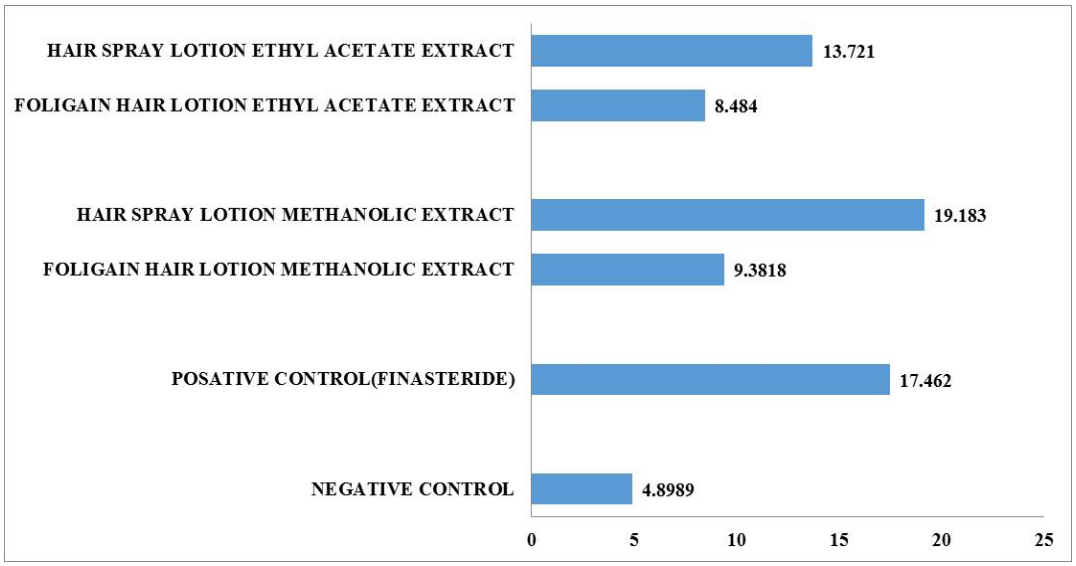


Figure 8.10. Comparative evaluation of 5α-reductase inhibition of hair lotion formulations.

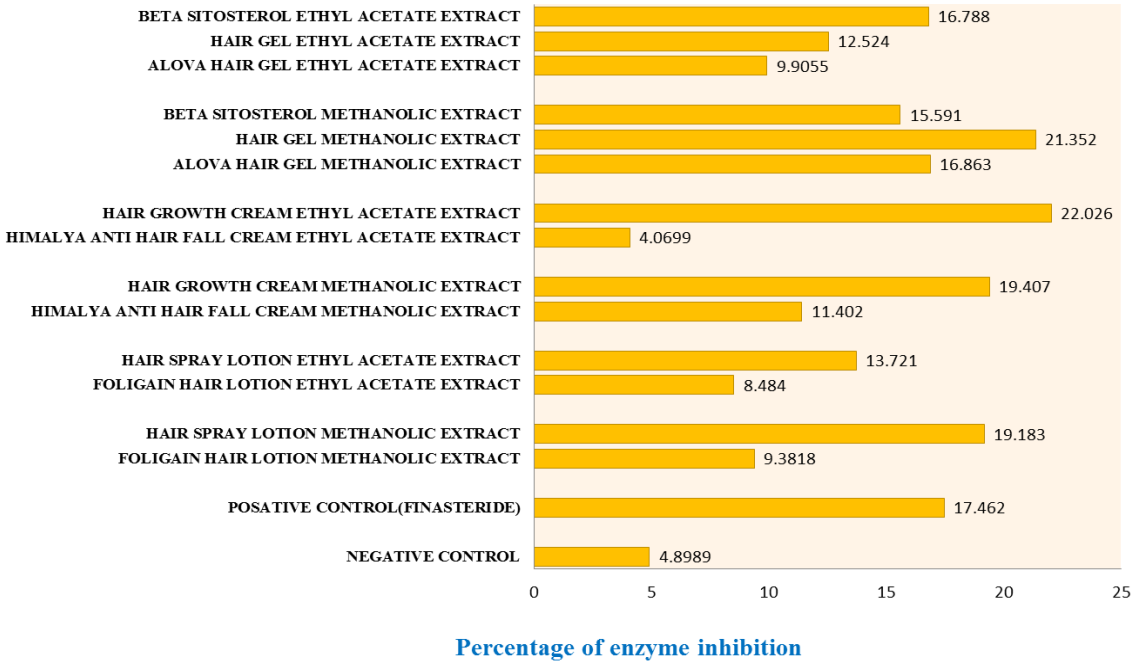


Figure 8.11. Comparative evaluation of 5α-reductase inhibition of all hair formulations.

Chapter - 9

Discussion and conclusion

9.1. Discussion

9.2. Conclusion

9.1. Discussion

β-sitosterol, quercetin and linoleic acid were used as biomarkers for standardization of *Eclipta alba*, *Hibiscus rosa-sinensis* flower *and Solanum nigrum* berries respectively, since they are already being reported to be found in the mentioned plant parts. Specificity was confirmed by comparing the R_f of standard and sample.

In our research work we have found that by HPTLC, percentage content of β-sitosterol in *Eclipta alba* methanolic extract was 0.10% and petroleum ether extract was 4.65% respectively suggesting petroleum ether as a better extracting solution. The calibration curve obtained had the equation of Y = 3447.759*X - 1121.100 (correlation coefficient = 0.9686 and standard deviation = ± 5.20%). R_f value of standard β-sitosterol was found to be 0.66. HPLC study showed the percentage content of β-sitosterol in *Eclipta alba* petroleum ether extract was found to be 4.67 % w/w. This was determined by a calibration curve with the equation of Y = 2649.8*X + 228397 (correlation coefficient = 0.9923). Retention time (R_t) value of standard β-sitosterol was found to be 2.325 minute.

The percentage content of linoleic acid in *Solanum nigrum* berries methanolic extract was found to be 9.21 % w/w. This was determined by a calibration curve with the equation of Y = 3141.508*X + 1366.840 (correlation coefficient = 0.9954 and standard deviation = ± 5.46%). R_f value of standard linoleic acid was found to be 0.56.

Quercetin content present in methanolic extract of *Hibiscus rosa-sinensis* flower was found to be 0.30 % w/w. This was determined by a calibration curve with the equation of Y = 3303.952*X + 2991.844 (correlation coefficient = 0.9960 and standard deviation = ± 2.22%). R_f value of standard quercetin was found to be 0.52. This was confirmed by HPLC method where the percentage content of quercetin in methanolic extract of *Hibiscus rosa-sinensis* flower was found to be 0.37 % w/w. The calibration curve had equation of Y = 95635*X - 66568 (correlation coefficient = 0.9931). R_t value of standard quercetin was found to be 5.034 minute.

The enzyme inhibition results showed significant IC_{50} values of 200.54 ± 14.51, 168.42 ± 11.99, 149.99 ± 19.42, 219.21 ± 8.75, 45.76 ± 3.07, 119.00 ± 1.68, 123.01 ± 3.36 (µg/ml) for *Eclipta alba* methanolic extract and petroleum ether extract (whole plant), *Hibiscus rosa-sinensis* flower methanolic extract, *Solanum nigrum* plant berries methanolic extract, β-sitosterol, quercetin, linoleic acid biomarkers respectively. Among them, *Hibiscus rosa-sinensis* and *Eclipta alba* petroleum ether extract have found potential 5α-reductase inhibition activity in respect to standard drug Finasteride (IC_{50} value: 202.59 ± 2.26) (ng/ml).

The levels of heavy metals in all the plant samples were within the prescribed limits as determined by AAS study.

Hair growth gel, cream and lotion were prepared by using plant materials with vitamin B. The formulations were evaluated and results indicated the formulations were good in appearance,

homogeneity and easily spreadable and showed significant inhibition of 5α-reductase enzyme in in-vitro model by comparing with some marketed formulation. Further HPTLC and HPLC method was done for quantification of plant biomarkers used in formulation. The results also showed that 5α-reductase enzyme inhibition effect of the formulation was better than the effect of marketed hair gel formulation.
Finally, pure β-sitosterol (0.02%) gel was prepared. Reproducible results were obtained by evaluation of 3 different batches. The results also showed that 5α-reductase enzyme inhibition effect of the formulation was better than the effect of marketed hair gel formulation. Less quantity β-sitosterol (0.02 %) was used in gel formulation. If the concentration of β-sitosterol was optimized properly in gel formulation, then it will give better result in enzyme inhibition studies. Further, HPLC method was also done for quantification of plant biomarkers and correlate with HPTLC data.

Future scope

The therapeutic uses of 5α-reductase inhibitor are prevailing now a day in the cosmetic and medicinal industries due to their preventive effect on alopecia disorder. 5α-reductase inhibitors reduce the dihydrotestosterone (DHT) biosynthesis, thus helping to reduce alopecia. Based on the present investigation the following studies may be pursued further.

- Stability studies need to be focused for the formulations prepared.
- In-vivo studies are needed to be performed so as to determine side effects of the formulations if present, time required to get desired hair growth effect.
- Prepared formulations can be evaluated for their pharmacokinetic and pharmacodynamics profiles in animals and humans to best of their advantages.
- Inclusion of more effective ingredients in formulation.
- Better optimization of formulation prepared by utilizing more parameters for evaluation.

9.2. Conclusion

The traditional knowledge with its holistic and systematic approach supported through experimental base can serve as an innovative and powerful discovery of natural 5α-reductase inhibitor. The work was performed and represented the lead finding for 5α-reductase inhibitors from Indian Medicinal Plants. Experimental results revealed that all the plant extracts showed potential 5α-reductase inhibition activity. *Eclipta alba* and *Hibiscus rosa-sinensis* have promising cosmetic application. *Solanum nigrum* berries methanolic extract also showed equipotent 5α-reductase inhibition activity compared to reference standard. Based on these findings, we suggest that these plants extract could potentially be a useful for alopecia. This study provides

newer insight for treatment and controlling baldness disorder as well as cosmetic application of the plant. Hair growth gel, cream and lotion were prepared by using plant materials with vitamin B. The formulations were evaluated and results indicated the formulations were good in appearance, homogeneity and easily spreadable and showed significant inhibition of 5α-reductase enzyme in in-vitro model by comparing with some marketed formulation. Further HPTLC and HPLC method was done for quantification of plant biomarkers used in formulation. The results also showed that 5α-reductase enzyme inhibition effect of the formulation was better than the effect of marketed hair gel formulation.

Chapter - 10

Summery

10.0. **Summery**

5α-reductase, also known as 3-oxo-5α-steroid 4-dehydrogenases involved in steroid metabolism. The enzyme 5α-reductase is responsible for conversion of testosterone (T) to dihydrotestosterone (DHT). Testosterone gets reduced by the enzyme 5α-reductase. Inhibition of DHT formation serves as a strategy for the treatment of alopecia and prostate hyperplasia by proposed mechanism (Raynaud et al., 2002; Roh et al., 2002). There are synthetic medicines, such as e.g. finasteride, dutasteride, alfatradiol but these compounds have been reported to have their adverse effects which necessitate the interest in finding better 5α-reductase inhibitors from natural resources.

It has been observed that 5α-reductase inhibitors have important role in pharmaceutical and cosmetics formulation that may be used to prevent or treat alopecia. 5α-reductase exists widely in animals, and over expression of the enzyme is involved in the formation of alopecia and prostate hyperplasia. Plants secondary metabolites like flavonoids such as kaempferol, quercetin, sterol and anthocyanin show good inhibitory activity of 5α-reductase.

Plant used for 5α-reductase inhibitory activity described in chapter 2. Standardization of this plant material by HPTLC, HPLC and AAS was performed in chapter 5. HPTLC study showed the percentage content of β-sitosterol in *Eclipta alba* methanolic and petroleum ether extract was found to be 0.10% and 4.65% respectively. This was determined by a calibration curve with the equation of Y = 3447.759*X - 1121.100 (correlation coefficient = 0.9686 and standard deviation = ± 5.20%). R_f value of standard β-sitosterol was found to be 0.66. Specificity was confirmed by comparing the R_f of standard and sample. The percentage content of linoleic acid in *Solanum nigrum* berries methanolic extract was found to be 9.21 % w/w. This was determined by a calibration curve with the equation of Y = 3141.508*X + 1366.840 (correlation coefficient = 0.9954 and standard deviation = ± 5.46%). R_f value of standard linoleic acid was found to be 0.56. Specificity was confirmed by comparing the R_f of standard and sample. Quercetin content present in methanolic extract of *Hibiscus rosa-sinensis* flower was found to be 0.30 % w/w. This was determined by a calibration curve with the equation of Y = 3303.952*X + 2991.844 (correlation coefficient = 0.9960 and standard deviation = ± 2.22%). R_f value of standard quercetin was found to be 0.52.

HPLC study showed the percentage content of β-sitosterol in *Eclipta alba* petroleum ether extract was found to be 4.67 % w/w. This was determined by a calibration curve with the equation of Y = 2649.8*X + 228397 (correlation coefficient = 0.9923). Retention time (R_t) value of standard β-sitosterol was found to be 2.325 minute. Specificity was confirmed by comparing the R_t of standard and sample. The percentage content of quercetin in methanolic extract of *Hibiscus rosa-sinensis* flower was found to be 0.37 % w/w. This was determined by a calibration

curve with the equation of Y = 95635*X - 66568 (correlation coefficient = 0.9931). R_t value of standard quercetin was found to be 5.034 minute. Specificity was confirmed by comparing the R_t of standard and sample.

The AAS study was done to identify the presence of seven metals, including heavy metals, the quantitative determinations were carried out using standard calibration curve obtained by the standard solution of metals having optimal detectable concentration ranges. The concentration of the metals obtained in plant materials were expressed in terms of parts per million. The levels of heavy metals in all the plant samples were within the prescribed limits.

In this context, this frame work was designed to evaluate the 5α-reductase inhibitory potential of three medicinal plants by *in-vitro* enzyme assay in chapter 6. The enzyme inhibition results showed significant IC_{50} values of 200.54 ± 14.51, 168.42 ± 11.99, 149.99 ± 19.42, 219.21 ± 8.75, 45.76 ± 3.07, 119.00 ± 1.68, 123.01 ± 3.36 (µg/mL) for *Eclipta alba* methanolic extract and petroleum ether extract (whole plant), *Hibiscus rosa-sinensis* flower methanolic extract, *Solanum nigrum* plant berries methanolic extract, β-sitosterol, quercetin, linoleic acid biomarkers respectively. Among them, *Hibiscus rosa-sinensis* and *Eclipta alba* petroleum ether extract have found potential 5α-reductase inhibition activity in respect to standard drug Finasteride (IC_{50} value: 202.59 ± 2.26) (ng/mL). *Solanum nigrum* plant berries methanolic extract, β-sitosterol, quercetin, linoleic acid biomarkers respectively. Among them, *Hibiscus rosa-sinensis* and *Eclipta alba* petroleum ether extract have found potential 5α-reductase inhibition activity in respect to standard drug Finasteride (IC_{50} value: 202.59 ± 2.26) (ng/mL).

In chapter 7, it describes about preparation and evaluation of hair growth gel, cream and lotion was performed by using plant materials with vitamin B. The result of the formulations is good in appearance, homogeneity and easily spreadable and showed significant inhibition of 5α-reductase enzyme in in-vitro model by comparing with some marketed formulation. Further HPTLC method was done for quantification of plant biomarkers used in formulation. In hair gel formulation β-sitosterol, quercetin and linoleic acid was found to be 0.1377, 0.120 and 0.379 % w/w respectively. In hair lotion β-sitosterol, quercetin and linoleic was found to be 0.1231, 0.1576, 0.151 % w/w respectively. *In-vitro* results also showed that 5α-reductase enzyme inhibition effect of the formulation was better than the effect of marketed hair gel formulation.

Finally, pure β-sitosterol (0.02%) gel was prepared. Batch A, Batch B, Batch C 3 different batches of β-sitosterol gel formulation was manufactured. By checking different critical parameters of three different batches gel, we can evaluate the manufacturing process method validation and the process reproducibility. The result of the gel is good in appearance, homogeneity and easily spreadable and showed significant inhibition of 5α-reductase enzyme in in-vitro model. Further HPTLC method was done for quantification of plant biomarkers used in formulation. The results also showed that 5α-reductase enzyme inhibition effect of the

formulation was better than the effect of marketed hair gel formulation. HPLC method was also done for quantification of plant biomarkers and correlate with HPTLC data.

In chapter 8, comparative evaluation of 5α-reductase inhibitory potential of some marketed product with prepared formulations was done. Among them, prepared hair cream and gel found more prominent activity in *in-vitro* result. Besides β-sitosterol gel was found good result enzyme inhibition then marketed products. Less quantity β-sitosterol (0.02 %) was used in gel formulation. If the concentration of β-sitosterol was optimized properly by giving more concentration in gel formulation, then it will give better result.

This dissertation work deals with development and evaluation of 5α-reductase inhibitory activity of several plants and their formulations. *Eclipta alba, Hibiscus rosa-sinensis, Solanum nigrum* was showed potential 5α-reductase inhibitory activity in the order of *Hibiscus rosa-sinensis* > *Eclipta alba* > *Solanum nigrum.* Besides three plant markers name β-sitosterol, linoleic acid, quercetin also evaluated 5α-reductase potential activity and showed higher potential activity in order of β-sitosterol > quercetin > linoleic acid.

Four different formulations were made by using *Eclipta alba, Hibiscus rosa-sinensis, Solanum nigrum plant* extracts, riboflavin 5 phosphate, pyridoxine hydrochloride (vitamin B) and β-sitosterol. This formulation containing *Eclipta alba, Hibiscus rosa-sinensis, Solanum nigrum plant* extracts with riboflavin 5 phosphate and pyridoxine hydrochloride showed highest inhibitory activity. Activity of the formulation in giving higher activity in the order of cream > gel > lotion > β-sitosterol. These formulations may be further explored for pilot scale studies and industrial applications.

Chapter - 11

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