CHAPTER II

REVIEW OF LITERATURE

2.1. Hair ailments

Among the various hair ailments, most commonly known disorders are Male pattern baldness, Female pattern baldness, Telogen effluvium, Anagen effluvium, Seborrheic dermatitis, Dandruff and Head lice infection. Male-pattern hair loss is a genetically determined progressive process that causes a gradual conversion of terminal hair into vellus hair. The role of androgen in Male-pattern hair loss is well established and about 20% of men within the age of 20 are affected with the disorder[90]. William *et al.*[91] studied the effect of testosterone as the main circulating androgen and suggested that the tissue effects of androgens are mediated by binding through to the intracellular androgen receptor. Hoffman[92] classified male pattern hair loss and Norwood[93] noted that the clinical features of Androgenic Alopecia in females differed from common baldness in men. Women presenting with diffuse hair loss, is a very common and challenging problem for dermatologists. The pathogenesis of Male pattern hair loss was described by Deplewski and Rosenfield[94], involves activation of hair follicle cytoplasmic androgen receptors. Both testosterone and dihydrotestosterone (DHT) activate the androgen receptor; however DHT binds five times more strongly than testosterone and DHT is thought to be the principle androgen involved in male pattern hair loss[95].

Bergfeld *et al.*[96] diagnosed female pattern hair loss as the most common cause of alopecia in women. It affects 6-12% of women between the ages of 20 and 30 years, and more than 55% of women older than 70 years. Dinh and Sinclair[57] and Magro[97] characterized female pattern hair loss histologically with increased numbers of miniaturized hair follicles. Androgens and estrogens are the main hormonal regulators implicated in female pattern hair loss and the hair follicles are sensitive to alterations in circulating estrogen and androgen levels as both hormones are synthesized and metabolized locally[98].

Telogen effluvium[99] is characterized by an abrupt onset, rapid, diffuse, self-limited, excessive shedding of normal club hairs, usually seen 2-3 months after triggering events such as severe febrile illness, postpartum, accidental trauma, major surgery, emotional stress, chronic systemic illness, large hemorrhage, and crash diet. According to Chong *et al.*[100] nearly 100-1000 hairs/day may be lost during telogen effluvium and significant reduction in anagen and telogen ratio is observed. Trueb[101] suggested that, in case of Anagen effluvium the proliferating cells of the bulb are affected and the stem cells of the bulge are responsible to re-initiate the follicle growth. The hair follicle resumes normal cycle within a few weeks of cessation. Sperling[102] associated the Anagen effluvium with severe protein malnutrition, exposure to toxic agents, peribulber inflammation such as systemic lupus erythematosus, secondary siphillis etc. Chemotherapy and radiation may also be the cause of Anagen effluvium.

Seborrheic dermatitis is a common hair disorder that typically occurs as erythematous plaques or patches and may vary from mild to dense dandruff,

diffuse, adherent scale affecting areas of the head and trunk where sebaceous glands are prominent, including the scalp[103]. It is associated with genetic, environmental, and general health factors, as well as lipophilic *Malassezia* yeasts[104]. Dawson *et al*.[105] emphasized that the etiology of Seborrheic dermatitis based on three factors: sebaceous gland secretions, microfloral metabolism, and individual susceptibility. Dandruff is a major cosmetic problem and *Pityrosporum ovale* (*Malassezia furfur*) a yeast-like lipophilic fungus, is considered to be the chief cause of the problem[106]. Shuster[107] also suggested a major link between *Malassezia* fungi and dandruff. Various authors reported on dandruff and Seborrheic dermatitis having same symptom severity with the same etiology[108-109]. Dandruff is characterized by patches of loosely adherent flakes, usually accompanied by itching. It also has the clinical feature of small white or gray flakes that accumulate diffusely on the scalp in localized patches like Seborrheic dermatitis but does not exhibit apparent inflammation and is confined to the scalp[110].

Burgess[111] suggested that the head lice infestation is caused by *Pediculus humanus capitis*. According to Chosidow[112], the infestation may be asymptomatic or symptomatic. In symptomatic cases, the itching is found in a highly variable percentage of patients. The itching can be caused both by the bite of lice on the skin and the irritative allergic reaction caused by the deposition of saliva on the scalp. The symptoms occur when the infestation is already old. Pollack *et al*.[113] revealed that the greatest harm associated with head lice results from the well-intentioned but misguided use of caustic or toxic substances to eliminate the lice.

Other than these hair ailments, most severely affected hair disorder is Alopecia areata which is also very common among dermatological problems.

2.2. Alopecia areata

Alopecia areata is a common hair loss disorder characterized by a patchy or diffuse hair loss which may lead to Alopecia totalis or universalis, affects a large numbers of men and women at any age[35]. However, the most common presentation is children and young adults, nearly 30-48% of patients affected before 20 years of age[73]. In recent years, alopecia has been considered as an organ-specific autoimmune disorder, which arises as a result of T-lymphocyte orientation towards the hair follicles[114]. Genetically, it is associated with genes of the major histocompatibility complex (MHC), the interleukin-1 cluster and chromosome 21 in the pathogenesis. Alopecia areata has HLA association with DQB1*03, HLA B-18, and possibly HLA-A2[115]. Loss of hair during active disease is coincidental with an infiltrate of activated CD4+ cells around the hair follicles, along with a CD8+ intra-follicular infiltrate[116].

The histopathologic features of the disease consist of perifollicular lymphocytic infiltrates around anagen hair follicles, consisting of both CD4+ and intra-follicular infiltrates of CD8+ cells. Several studies have shown that within the cascade of pathogenesis of Alopecia areata, cytokines and other molecules that coordinate cyclical hair growth play a crucial role[117]. Christiano *et al.*[118] undertook a genome-wide association study to determine the genetic basis of Alopecia areata. The study revealed several genes controlling the activation and proliferation of regulatory T cells (T_{reg} cells), cytotoxic T lymphocyte associated antigen 4 (CTLA4), interleukin-2/IL-21, IL-2 receptor A (IL-2RA;

CD25) and Eos (also known as Ikaros family zinc finger 4; IKZF4), as well as the human leukocyte antigen (HLA) region. We also find association evidence for regions containing genes expressed in the hair follicle itself (PRDX5 and STX17).

A region of strong association resides within the ULBP (cytomegalovirus UL16-binding protein) gene cluster on chromosome 6q25.1, encoding activating ligands of the natural killer cell receptor NKG2D that have not previously been implicated in an autoimmune disease. ULBP3 in disease pathogenesis, we also show that its expression in lesional scalp from patients with Alopecia areata is markedly up-regulated in the hair follicle dermal sheath during active disease. This study provides evidence for the involvement of both innate and acquired immunity in the pathogenesis of Alopecia areata and the hair follicles are surrounded by an immune infiltrate with activated T-helper cells (T_H cells), cytotoxic Tcells (T_C cells) and natural killer (NK) cells, characterized as a TH1-type inflammatory response[119-120].Genetic factors, infections, psychological factors, autoimmune factors and neuropeptides have been claimed to play a role in its etio-pathogenesis[121].

Apart from metabolic and hereditary causes, Alopecia has also been observed as a major side effect of anticancer, immunosuppressant, and many others drug treatments. In some cases, Alopecia areata is androgen dependent which is known as Androgenic Alopecia. It is a process with continuous miniaturisation of hair follicles in genetically predisposed men and women[34, 122]. In the hair follicle cells, testosterone is converted into the more active androgen dihydrotestosterone (DHT) by 5α-reductase enzyme. The androgens bind to

androgen receptors in the hair follicle, which reduces the anagen phase of the hair cycle. Gradually, over succeeding cycles, the terminal hair converts into a thinner and shorter vellus hair[123-124].

2.3. Synthetic drugs for treating alopecia

According to Wasserman *et al.*[116], no cure or preventive treatment for alopecia has been established; however treatments are directed toward inhibiting the progress of the disease. At present, there are a number of synthetic drugs available in the market for different Alopecia treatment. Minoxidil and finasteride are most widely prescribed drugs. Minoxidil was first used as a vasodilator to treat cardiovascular disorders, however the unexpected side effect of hirsutism lead to its topical use as a hair-growth stimulator[73]. Hypertrichosis was also noted in pediatric patients treated with minoxidil. Mehta *et al.*[125] revealed the use of minoxidil for hair loss from 1970 as an oral medication. Olsen[126] and Rogers and Avram[127] also demonstrated the major side effects of minoxidil treatment in alopecia patients which includes an increase in left ventricular end-diastolic volume, cardiac output, and left ventricular mass, hypertrichosis, allergic contact dermatitis, pruritus etc. have been reported with the use of 2% minoxidil solution. Unfortunately, another potential drawback of minoxidil therapy is the loss of newly grown hair within one to three months after discontinuation of the medicine . So, the long term treatment with local side effects may be a problem with continuing use of minoxidil lotion[128].

Finasteride is a synthetic azo-steroid that has been used for the treatment of androgenetic Alopecia in males. It is a potent and highly selective 5α-

reductase type-2 inhibitor[129]. It binds irreversibly to the 5α-reductase type-2 enzyme and inhibits the conversion of testosterone to DHT by reducing concentrations of scalp and serum DHT[130]. The dose–response curve is non-linear and therefore higher doses do not lead to significantly increased suppression of DHT or clinical benefit[131]. After 24 months of continuous use, 66% of patients experienced very less (10–25%) regrowth of their hair[132] but with the regrowth of hair, finasteride causes a 30% to 50% decrease in prostate specific antigen (PSA). A decreased libido, erectile dysfunction or decreased volumes of ejaculate have been reported as side effects. Finasteride had teratogenic effects in animals on high doses, causing genitourinary abnormalities in male offspring. The concentration of the drug in semen of men who took 1 mg/day was much lower than the concentration associated with teratogenic effects in monkeys[133]. Finasteride is effective for male pattern baldness whereas studies have shown that it is effective in treating female pattern hair loss[134-135].

Other than minoxidil and finasterides, dutasteroid, cimetidine, spironolactone, flutamide, polysorbate 80 and folligen has the unusual properties of working as an anti-androgen by binding to androgen receptor which also known for their various side effects such as hormonal imbalance, decreased male fertility, prohibits pregnancy, obesity etc[74, 136-137]. Off-label uses of cimetidine have been for androgenic alopecia and hirsutism, however, no clinical trials have ever been performed to prove efficacy. The noted side effects that have been reported after using cimetidine include loss of libido, impotence, and gynecomastia[74]. Spironolactone is an alclosterone antagonist also acts as a

weak anti-androgen in blocking the androgen receptor, but also inhibits androgen biosynthesis. The main mechanism of action of spironolactone is inhibition of the interaction between dihydrotestosterone (DHT) and the intracellular androgen receptor[138]. Spironolactone may convert to other active metabolites, via the progesterone 17-hydroxylase enzyme. It may reversibly inhibit adrenal and ovarian cytochrome P-450, which overall will decrease testosterone and DHT. Spironolactone may be effective in preventing hair loss in androgenetic alopecia in women. But as a side effect, use of spironolactone may lead to hyperkalemia. Other interactions have included digitalis glycosides, which may increase absorption of digitalis, leading to increased blood levels[74].

Another drug available in the market is flutamide, which is a non-steroidal anti-androgen that is devoid of other hormonal activity. It most likely acts after converting to 2-hydroxyflutamide, which is a potent competitive inhibitor of DHT binding to the androgen receptor. The mechanism action of flutamide is believed to be potent inhibition of androgen uptake and inhibition of binding of androgens to target tissue. Flutamide has been shown to be effective in the treatment of hirsutism and FPHL in women with hyper-androgenic states[139]. But severe liver problems, persistent abdominal pain are the side effects caused by Flutamide. The drug may be effective *in-vitro* as an anti-androgen but *in-vivo* the rise in plasma testosterone serves to limit its anti-androgenic effects. Noted side effects when taken orally are hepatotoxicity, including progressive liver failure, which limits its usefulness[76]. Corticosteroids are one of the most popular treatments

prescribed by dermatologists. Several methods of corticosteroid administration have been used, notably intralesional injection, topical and systemic therapy which also includes various side effects[140].These drugs also cause various major and minor side effects which reduced their usage.

Polysorbate 80, has been around since 1980 with claims that it may grew hair, however it was not effective in most cases. Like polysorbate 80, folligen is another new treatment containing copper complex and Saw Palmetto as an androgen inhibitor of 5α-reductase. But these drugs have not been thoroughly tested for positive results in double-blind clinical trials[74].

There are various new and novel treatments for use in alopecia. Some of them have gone through rigorous double-blind clinical trial testing with Food and Drug Administration (FDA) approval as to their proven claims, whereas others have yet to do so. Numerous products or surgical procedures are also promoted for the treatment of alopecia as well as improving scalp hair growth. A few of these also include vitamins, amino-acids, scalp massage etc. but their efficacy remains dubious[141]. Other than these drugs Topical, intralesional or systemic corticosteroids, PUVA therapy, cryotherapy, anthralin, topical immunotherapy and cyclosporine are the treatment approaches that induce remissions. The therapeutic effects of most of the treatments are due to an immune-modulatory mechanism. Among these treatment alternatives systemic corticosteroids have been shown to be effective with various harmful side effects[36].

All the synthetic medicinal compounds which have a role in promoting hair growth and treating alopecia are noted in Table 2.1.

Table 2.1 Synthetic compounds for treatment of Alopecia

Sl no.	Synthetic compounds	Drug name	Mechanism of action	Side effects
1	Minoxidil	Mintop, Regain	open potassium channels and increase the proliferation and differentiation of epithelial cells in the hair shaft[74]	hirsutism, local irritation, itching, dryness and erythema[75,126,127]
2	Finasteroid	Propecia	binds irreversibly to the 5α-reductase type-2 enzyme and inhibits the conversion of testosterone to DHT [130,131]	teratogenic effects in animals on high doses, causing genitourinary abnormalities in male offspring, hormonal disorders in male[133]
3	Dutasteroid	Avodart	inhibits both types I and II 5-α reductase isoenzymes[133]	hormonal imbalance, significant reduction in sperm motility[74]
4	Spironolactone	Aldactone	antiandrogen in blocking the androgen receptor, inhibits androgen biosynthesis[138]	hyperkalemia, digitalis glycosides, increase in blood pressure[74]
5	Flutamide	Teva-Flutamide	converts into 2-hydroxy flutamide and acts as potent competitive inhibitor of DHT[139]	miniaturization of hair cycles, hepatic dysfunction and breast tenderness[74]
6	Cyclosporine A	Neoral, Neuro STAT	T cell-specific immuno-suppressant	lymphoma or skin cancer,decrease the functioning of the immune system, high blood pressure and kidney problems[141]

7	Cimetidine	Tagamate	5α-reductase type-2 enzyme inhibitor[74]	loss of libido, impotence and gynecomastia[74]
8	Polysorbate 80	Alkest, Kanarcel	Antiandrogen[74]	Hair grows through cycle miniaturization, not effective for most patients[74]
9	Folligen	Folligen	Contains copper complex with Saw palmetto, anti-androgen against 5α-reductase isoenzymes[133]	not clinically proven in double-blinded clinical test[137]
10	Corticosteroid	Dianabol, cortisone	Immune-modulatory mechanism[140]	Acne, obesity, mild hypertension and lenticular opacities[140]

2.4. Natural Products and their activity against Alopecia areata

Natural products are secondary metabolites which are known for beneficial effects on human health. These molecules are produced in plants with distinct biological properties[142]. It is believed that nearly two third of the world population today still relies on medicinal plants as their primary sources of medicine[143]. Natural products from plants and their various extracts were reported to be active against hair fall related problems in various literatures. A brief review of medicinal plants having anti-alopecic activity is presented below. Natural products and their derivatives have been playing an important role in the development of new drugs for many ailments. During 2000-2012, various synthetic drugs were introduced in the market, however due to various side effects, it lost track. So, people are turning towards natural products related drugs. Different natural products have been reported to have anti-alopecic

activity as reported in a number of literatures[20, 144].

2.5. Application of various plant extracts on hair regeneration

Due to various disadvantages of synthetic drugs, traditional medicinal plants as natural sources of drugs and pharmaceuticals are gaining worldwide interest in modern times[86, 89, 145]. According to Fransworth and Soejarto[146], up to 80% of Indian people use various forms of traditional medicines including Ayurveda. Even the pharmacopias of advanced countries have now derived 25-40% of their medicines from plants. Traditionally known medicinal plants also unequivocally support the hair care industries and about 1,000 plant extracts have been examined for hair care usage. Different researchers from India and other parts of the world have studied on different ethnomedicinal plants and their activity on follicular regeneration and hair growth promotion.

The traditional system of medicine in India, recommended a number of medicinal plants for hair growth promotion and treatment of hair loss. Potential hair growth promoting activity is exhibited by numerous plants which possess anti-androgenic activity i.e. testosterone and 5α-reductase inhibition[147, 148 - 150]. Proanthocyanidine B-2 isolated from grape seed (*Vitis vinifera*) extract showed *in-vitro* hair follicle cells proliferation and *in-vivo* hair cycle conversion from the telogen phase to the anagen phase. So, proanthocyanidin B-2 may act as an active agent for treating androgenic alopecia[147]. Proanthocyanidine B-2 was also isolated from apples (*Malus domestica*) and the effect was studied on the expression of PKC (Protein Kinase C) isozymes in cultured murine hair epithelial cells at different stages of the hair cycle. It is a compound that possesses hair-growth promoting activity by down-regulating or inhibiting

PKC isozymes in hair epithelial cells[42].

Hair growth promoting effect was also studied for dried root extract of *Sophora flavescens* revealed that the extract possesses potent inhibitory effect against type II 5α-reductase. It induces mRNA levels of growth factors such as IGF-1(Insulin-like Growth Factor-1) and KGF (Keratinocyte Growth Factor), suggesting its effect on hair growth through the regulation of growth factors in dermal papilla cells[151]. *In-vivo* and *in-vitro* hair growth promoting activity of leaves and flowers of *Hibiscus rosa-sinensis* was studied by Adhirajan *et al*.[152]. The *in-vivo* study revealed that the petroleum ether extract of leaves has impact on hair follicles and it may improve the hair growth. Many reports are available on *H. rosa-sinensis* leaves as a constituent in the various hair growth promoting formulations[152]. *Asiasari radix* was also studied for hair growth stimulating effect on C57BL/6 and C3H mice model. Ethanol extract of *A. radix* increased the protein synthesis in vibrissae follicle cultures and may proliferate both human keratinocyte cells (HaCaT) and human dermal papillae (DP) cells. Also, the extract induces the expression of VEGF in cultured human dermal papillae cell lines[20].

There are many herbal products available in the market, prepared by combination of one or more plant extracts and widely accepted as hair tonic, hair growth promoter, hair conditioner, hair cleansing agent, antidandruff agents and hair fall related problems[72, 153]. Among them Patanjali Tejas Tailam hair oil (Patanjali ayurved), Zandu ayurvedic hair care products (Zandu), SESA ayurvedic hair oil (Ban Labs. Ltd.), Dabur Amla hair oil (Dabur), Hair and Care hair oil (Marico) are some of the well-known brands available in the

market and their formulation are based on various traditionally ethnomedicinal plants. These products are known for improving hair growth and its quality, without any side effects. Moreover, these products are cost effective compared to the synthetic drugs. Among the known plants used in the formulations of various phytochemical based medicines mainly consist of *Eclipta alba, Asiasari radix, Sophora flavescens, Hibiscus rosa-sinensis, Naringi crenulata, Citrullus colocynthis, Buxus Wallichiana*[20, 151,154-156], *Trichosanthes occidentalis*[157], *Citrullus colocynthis*[72], *Emblica officinalis*[158], *Lastonia inermis*[159].

A polyherbal formulation was developed by Roy *et al*.[160] containing *C. reflexa*, *C. colocynthis* and *E. alba.* These plants are traditionally well known for their hair growth promoting potentials. During their study on animal models (wistar rats), hair growth initiation time was markedly reduced to one third on treatment with the prepared formulation compared with control animals. The time required for complete hair growth was also reduced by 32% compared to normal growth of hair in control animals. Quantitative analysis of hair growth cycle after treatment with the herbal formulation and minoxidil (2%), exhibited greater number of hair follicles in anagen phase. The author also reported on hair growth promoting effect of *C. colocynthis* on albino rats[71]. Hair growth initiation time was significantly reduced to half after treating with the petroleum ether extract of *C. colocynthis* compared with control animals. The time required for complete hair growth was also considerably reduced. The treatment was successful in bringing a greater number of hair follicles (> 70%) in anagen phase compared to standard drug minoxidil (67%). Dhanotia et al.[52]

also studied on *C. colocynthis* promotion of hair growth. Petroleum ether extract of *C. colocynthis* fruit studied on alopecia-induced albino mice. The treatment of androgen induced mice with *C. colocynthis* fruit extract revealed a greater number of hair follicles in anagenic phase than the standard drug finasteride, which described its potency in the treatment of androgen-induced alopecia.

C. reflexa is a traditionally known plant for promoting hair growth. The petroleum ether extract was applied on alopecia induced albino mice by testosterone administration. The extract exhibited promising hair growth–promoting activity by increasing follicular density, anagen / telogen ratio and histological study. Inhibition of 5α-reductase activity of the extract inhibits the conversion of testosterone to dihydro-testosterone which may be useful in treatment of androgen-induced alopecia[161].

Another polyherbal formulation was prepared by Purwal *et al.*[162] containing *E. officinalis*, *B. monnieri*, *T. foenum-graecum*, *M. koenigii* in various concentrations. Hair growth promoting activity was studied for the formulation and the study suggested the increasing hair growth promoting activity of each formulation with increasing concentrations of extracts which showed excellent hair growth promotion in comparison to minoxidil, also enlargement of follicular size and prolongation of the anagen phase. The author suggested that the formulation may be used as an alternative of minoxidil solution.

Biochemically, these crude plant extracts contain different classes and amounts of phytochemicals which principally include flavanoids, tannins, alkaloids, tannoids, phenolics, triterpenoids and β-sitosterol. Various phytoactive

compounds have been reported to be isolated and purified from the source plants and their role in the treatment of hair aliments discussed. Previous reports revealed that flavonoids and triterpenoids possess hair growth promoting activity by strengthening the capillary wall of the smaller blood vessels, improve blood circulation to nourish the hair follicles and thereby promoting hair growth[163]. Many reports connect flavonoids in stimulating telogen to anagen phase and also cause the expressions of some of the growth factors which are known to have stimulatory effects on hair growth, such as insulin-like growth factor-1(IGF-1), vascular endothelial growth factors (VEGF), keratinocyte growth factors (KGF) and hepatocyte growth factors (HGF) [164].

Plant based drugs normally promote hair growth through various modes such as by stimulating hair follicle or scalp metabolism, an acceleration of blood circulation, activation of dermal papilla, anti-testosterone action or increased nutrition to the hair follicles through accelerated blood flow but the mechanism are not yet clear[165-166]. Ghaffar and Semmler[66] and Campli *et al.*[167] described the effect of neem (*Azadirachta indica*) seed extract and tea tree (*Melaleuca alternifolia*) oil against head lice (*Pediculus humanus capitis*). Plant-based compounds such as the flowers bud extract of *Syzygium aromaticu* (clove), *Melia azedarach*, lavender oil, eucalyptus oil, lemon tea tree oil, thymol, and geraniol have been taken into account to limit the emergence and the spread of the parasitic infestation[66, 167-168]. *H. rosa-sinesis* is a well-known medicinal plant and the use of its leaves and floral parts are known to have hair growth promoting and anti-greying properties[166]. *In vitro* study carried out by

Adhiranjan *et al.*[152] revealed that the leaf extract of *H. rosa-sinensis* has direct impact on hair follicles and thus may improve the hair growth. Moreover in India, various herbal products available for enhancing hair growth usually include the extract of various parts of *H. rosa-sinesis*.

E. officinalis, the principal ingredients of various hair tonics prepared of usually contain tannoids (emblicanin A and B, punigluconin, and pedunculagin) and have been reported for their potent antioxidant property due to the presence of flavonoids[169]. Investigations carried out so far revealed that *E. officinalis* has strong immune-suppressive effect, potent superoxide-scavenging activity, enhanced cell survival rate, decreased induction of nitric oxide synthase and significant inhibition of interleukin and gamma-interferons[159, 170]. The leaf extract of the plant *L. inermis* also have been reported as growth accelerator and used to cure the loss of hair. The occurrence of contact dermatitis appears to be extremely rare with the use of *L. inermis*, since its leaf extract reported to have mild anti-inflammatory and anti-allergic effects[158]. Roy *et al.*[72] reported hair growth activity of the stem of *C. reflexa* Roxb through the periodic transformation of hair follicle from Telogen to Anagen phase. Patni *et al*.[158] investigated the hair growth activity contributed by *S. indicum* oil, arial parts of *C. reflexa*, *H. rosa-sinesis*, *A. cepa* and fruits of *E. officinalis*[171].

Thai lanna plants are traditionally known for resolving hair related problems. Efficacy of thai lanna medicinal plant extracts as hair gel for hair loss treatment was studied by Manosroi *et al*.[172]. The hair gel containing thai lanna plants such as ginger, turmeric, chilli and Tong-Pan-Chang extract were applied on non-hereditary alopecia affected volunteers which showed a significant increase in hair density and a decrease in hair loss without any irritation. The study

demonstrated the potentiality of these plants against hair loss. Kumar *et al*.[40] also reported on seventeen thai lanna medicinal plants by determining their potency on 5α-reductase enzyme inhibition and hair growth promotion. Among the seventeen plants, ethanolic extract of *C. tinctorius* is the most active 5α-reductase inhibitor and hair growth promoter, compared to finasteride and minoxidil. The plant extract showed strong relationships between 5α-reductase inhibitory and hair growth promoting activity. Suraj *et al*.[144] reported the petroleum ether extract of the *P. dulcis* plant as consistent hair growth promoter and also significant increase in the length of hair and showed a good percentage of hair follicles in the anagen phase after histological studies. *R. equisetiformis* plant extract contain triterpenes and flavonoids which stimulate the hair growth promotion. The hair growth promoting potentiality of *R. equisetiformis* was reported on albino wistar rats by showing a significant increase in the rate of hair growth and reduction in the diameter of the shaved area[164].

Kawano *et al*.[173] reported on tunisian medicinal plant *E. multiflora* plant extract on stimulating the hair growth cycle by initally stimulating the dermal papilla cells and producing vasodilation. The results suggested that the *E. multiflora* extract promotes dermal papilla cell growth with high activity and induce hair cycle conversion from telogen phase to anagen phase. Jaybhaye *et al*.[174] reported on petroleum ether extract of the seeds of *Tectona grandis Linn* for reducing the hair growth initiation time compared to minoxidil (2%) solution. The study confirms that the petroleum ether extract of the *T. grandis* seeds revitalize the growth of hair compared to 2% minoxidil solution. The remarkable improvement in length of hair follicles supports the hair growth promoting effects of the plant. *R. panax ginseng* is already documented to

possess hair growth activity and widely used to treat alopecia. Application of *F. panax ginseng* extract also significantly increased the proliferation of dermal papillae cells in C57/BL6 mice in dose and time dependent manner. Considerable elongations of anagen phase during hair cycle after treating with *F. panax ginseng* extract was observed. This study indicates the use of *F. panax ginseng* in hair regeneration activity for the treatment of hair loss[175].

Savali *et al*.[176] studied the hair growth promoting activity of *M. paradisiaca* unripe fruit extract on mice. The effect of the *M. paradisiaca* unripe fruit extract was found to be more significant on hair growth and length compared to standard drug treated animals. The anagen induction by methanolic extract of *M. paradisiacal* and minoxidil treatment is comparable. The result of the study indicated that the *M. paradisiaca* unripe fruit extract have the similar hair growth initiation activity as minoxidil.

N. crenulata is another plant with hair growth promoting potentiality. Presence of terpenoids and flavonoids in the *N. crenulata* plant extracts increases its potentiality in hair growth promotion[156]. The efficiency of *T. cucumerina* aqueous extract on hair growth promotion was experimented on wister rats. It aqueous extract exhibited more substantial effect which was comparable to the 2% minoxidil solution. The author suggested that, the plant extract is rich in flavonoids and saponins, which were potent antioxidant agents. So, the plant might have produced an antioxidant effect which has inducing effect on hair growth. The study showed significant enhancement in the hair growth initiation and completion time after treating with the aqueous extract of *T. cucumerina* as test drug and standard as compared to the control group of animals. A promising hair length was observed for the tested drug which may be due to the

premature switching of follicles from the telogen to anagen phase of hair growth cycle[89]. *E. alsinoides* is traditionally used for its hair growth promoting activity. The study done by Amrita *et al*.[145] revealed that, methanolic extract of the plant was found to be more potent for hair growth promotion and also the initiation and completion of hair growth time was significantly reduced as comparable to minoxidil solution.

The herb *E. alba* is known as "Bhringraj" or "Keshraja" a Sanskrit word that relates the use of herb for providing beneficial effects to the quality of hair. The use of leaves and aerial parts has been traditionally acclaimed for the improvement of hair growth and blackening of hair[177]. β-sitosterol is a well-documented inhibitor of 5α-reductase and present in *E. alba* in appreciable quantity[149]. The extracted juice if taken orally and applied to the scalp blackens the hair[178]. *E. alba* has been reported in various polyherbal formulations[179-185] for hair growth promotion. Hair growth promoting activity of *E. alba* in traditional and published literatures convinced us to explore this plant for the potential hair growth promotions[186]. The petroleum ether extract of *E.alba* was applied on male albino rats which showed improvement of the quality of hair compared to minoxidil treatment. The remarkable improvement in length of hair follicles also supported the traditional information of the herb. Retention of late anagen follicles as well as increase in follicular length and prevention of their miniaturization may therefore be attributed due to 5α-reductase inhibitory activity[155]. The potentiality of *E. alba* on hair growth promotion was also studied by Datta *et al*.[186]. The extract induces the growth of hair follicle from anagen to telogen phase. The activity of the extract was assessed by studying the melanogenesis in resected skin, follicle count in the subcutis, skin thickness

and surrogate markers in vehicle control and extracts treated animals.

Other than the above mentioned plants, *A. barbadensis* also traditionally well known for promoting hair growth and treating hair loss. *A. barbadensis* is known to use for the treatment of brittle hair, controlling hair loss and for improving the hair growth. *A. barbadensis* is a stemless, perennial, drought resisting, succulent plant and has reportedly been used since ancient times for medicinal purposes[188-189]. Inaoka *et al.*[190] reported that aloenin, a major constituent of *A. barbadensis* is responsible for promoting hair growth without irritating the skin.

All the medicinal plants which are used for treating alopecia related hair problems are showed in Table 2.2.

Table 2.2 List of medicinal plants used for treating alopecia

Sl. no	Plant name	Part used	Extracts used
1	*Lowstonia inermis*	Leaves	Aqueous[113]
3	*Cuscuta reflexa*	Whole plant	Petroleum ether[110]
4	*Aloe barbadensis*	Leaf[132, 135]	–
5	*Hibiscus rosa-sinensis*	Fresh leaves, flowers	Petroleum ether[109]
6	*Emblica officinlis*	Fruit[113]	–
7	*Russelia equisetiformis*	whole plant	Methanol[115]
8	*Vitis vinifera*	Seed[98]	–
9	*Sophora flavescens*	dried root	Methanol[100]
10	*Panax ginseng*	Whole plant	95% ethanol[189]
11	*Musa paradisiaca*	unripe fruit	Methanol[190]
12	*Techtona grandis*	seeds	Petroleum ether[188]
13	*Trichosanthes cucumerina*	Leaf	Aquoeus[86]
14	*Asiasari radix*	dried root	Ethanol[20]
15	*Buxus wallichiana*	wood	Methanol[100-103]
16	*Malus domestica*	Fruit[42]	–
17	*Citrullus colocynthis*	Fruit[52]	Petroleum ether[71]
18	*Evolvulus alsinoides*	whole plant	Methanol[96]
19	*Naringi crenulata*	Leaf	Aquoeus[103]
20	*Erica multiflora*	whole plant	70% ethanol[187]
21	*Eclipta alba*	whole plant	95% Methanol[122] , Petroleum ether[102, 121]
22	*Polygonum multiflorum*	leaves, root	Aquoeus, 70% Ethanol[204]
23	*Serenoa repens*	fruits	Aqueous[191]
24	*Tridax procumbens*	whole plant	Ethanol[99]
25	*Ginkgo biloba*	Leaf[114]	–
26	*Thujae occidentalis*	Leaves, fruits	Semen[111]

2.6. Animal models used for the study of hair regeneration

Various species of animals such as mice[192], rats[193], sheep[194] and monkey[195] have been used for hair related studies and the mouse model is most widely reported for hair growth promoting studies due to availability of large database and specific mutants such as nude, hairless, rhino, and severe combined immune-deficient mice[196]. Pigmented C57/BL6 mice are one of the most commonly used strains as their truncal pigmentation is entirely dependent on their follicular melanocytes. The epidermis in this species lacks melanin-producing melanocytes and melanin production is strictly coupled to anagen phase of hair growth. The strict coupling of follicular melanogenesis and hair follicle cycling thus leads to characteristic changes in skin pigmentation during anagen development[197-199].

The C57/BL6 model has been widely reported for evaluation of cyclosporin A[200], oligopeptide[201], capsaicin[202], pyrrolidine derivative[203] and tellurium which is an immune-modulator for hair growth promoting activity[204]. Datta *et al*.[186] reported the potentiality of *E. alba* extract as hair growth promoter on C57/BL6 mice. Six-week-old female C57BL/6 mice were also used to study the hair growth promoting effect of *S. flavescens and P. multiflorum* extract[205, 151]. *F. panax ginseng* extract showed hair regeneration in C57BL/6 mice[175]. Six-week-old female C57BL/6 and C3H mice were used to evaluate the hair growth promoting study of *A. radix* plant extract[20]. Male Sprague Dawley rats and seven-week old male C56/BL6 Mlac mice were another animal model used for the study of 5α-reductase inhibition and hair growth promotion of some Thai plants traditionally used for hair treatment[40].

Other than C57/BL6 mice model, wistar strain albino rats, C3H/He mice, swiss albino mice are also a very commonly used animal model for studying hair growth and regenerations[71, 154-55, 205]. Wistar albino rats of either sex were used by Suraj *et al*.[144] to study the *in-vivo* hair growth activity of *P. dulcis* seeds for its hair growth activity. The same animal model was also used by Roy *et al*.[160] to determine and evaluation of a polyherbal formulation containing *C. reflexa*, *C. colocynthis* and *E. alba* for hair growth–promoting activity. Swiss albino mice were used for hair growth promotion evaluation of *M. paradisiaca* unripe fruit extract[176]. *T. grandis* seed extract was evaluated for hair growth promotion on albino mice[174]. Uno[195] studied the quantitative evaluation of hair growth potential of minoxidil on macaque monkey and fuzzy rats by determining the percentage transformation of hair follicles from telogen to anagen. In another study, Uno and Kurata[206] reported that the topical application of fuzzy rat with minoxidil, diazoxide and copper peptide produced a conversion of short vellus hairs to long terminal hairs and an enlargement of the follicular size with prolongation of anagen phase by enhancing the rate of cell proliferation.

Sandhya *et al.*[89] evaluated the potentiality of aquoeus leaf extract of *T. cucumerina* Linn on hair growth promotion in wistar albino rats. *In-vivo* and *in-vitro* evaluation of hair growth potential of *H. rosa-sinensis* was studied on female wistar albino rats[152]. Evaluation of hair growth activity of *B. wallichiana* extract was successfully experimented in wistar albino rats[154]. To determine the hair growth promoting effect of *C. colocynthis* extract, alopecia was introduced in male swiss albino mice using testosterone[52]. Effect of *C. colocynthis* extract on hair growth promotion was also studied by Roy *et al*.[72].

In- vivo evaluation of hair growth potential of fresh leaf extracts of *N. crenulata* was determined on wistar albino mice[156]. A scientific investigation of *E. alsinoids* as hair growth promoter was studied on albino rats[145]. Takahashi *et al.*[147] chose isolated proanthocyanidine from grape seed extracts and 8-weeks old C3H/HeS1C mice whose hair cycle is in the telogen phase, for *in-vivo* study to convert the hair cycle using proanthocyanidine. Female wistar albino rats were used to evaluate hair growth promoting effect of *R. equisetiformis*[164]. Similarly, Kim *et al.*[207] reported the promotion effect on hair growth of Acankoreoside J from *Acantho panax koreanum* and Park *et al.*[208] studied the hair growth-promoting effect of *Aconiti ciliare* tuber extract mediated by the activation of Wnt/b-catenin signalling. Also, Park *et al.*[205] reported on the hair growth-promoting activities of *P. multiflorum* root extract and the mechanism of action. *P. multiflorum* distributed in northeast Asia, is a well-known traditional Chinese herbal medicine. Hair growth promoting activity of *P. multiflorum* roots is well known medicine and many studies have shown that it has a strong effect on hair growth and hair colour. A recent study demonstrated that an active component, 2, 3, 5, 4'-tetrahydroxystilbene-2-O-b-D-glucoside from *P. multiflorum* induced melanogenesis in melanocytes[209].

3. Materials and Methods

3.1. Collection of plants

In the present investigation, *E. alba* and *A. barbadensis* plants were collected from the Medicinal Plants garden at Tezpur University, Tezpur, Assam, India. *A. barbadensis* plantlets were cultivated in pots with sand and less water-absorbing soil for a period of 30 days with regular pulverization. *E. alba* plantlets were grown in water absorbing soil for a period of 15 days till plants attained maturity and its seeds were also used for cultivation in higher quantity.

3.2. Morpho-phenological study of the plants

The important morphological characters such as plant height, leaf shape, arrangement, type of inflorescence, flower colour and type of seeds were studied for both the selected plants and recorded. Height and length of the plant, size of leaf were measured using measuring tape and scale was used to measure length petiole, flowers diameter etc. Colour chart was used to describe the flower colour. Data were collected from five sample plants. The phenological data such as flower initiation, flowering period, seed formation etc were studied and recorded.

3.3. Genomic study of the plants

3.3.1. DNA isolation

Fresh young leaves were used for the isolation of DNA from the selected plant

species. The leaves were collected in the morning, washed with distilled water repetitively, placed in between moist tissue papers and then stored in darkness at room temperature. The experiment was done according to the protocol given by Doyle and Doyle[210] with some modifications.

3.3.1.1. Equipment used

Autoclave

Mortar and pestle

Mettler electronic balance

Micropipettes – (2-20 µl, 20-200 µl and 200-1000 µl) with tips

Polypropylene tube (25 ml)

Microcentrifuge tube (1.5 ml)

Microwave oven

Incubator (37°C)

Sorvall RC 5B Plus centrifuge

Bench top centrifuge (Hettich Zentrifugen, MIKRO 12-24)

Magnetic stirrer

Shaking hot water bath

Speed vacuum (Maxi dry plus, Hoefer Pharmacia Biotech Inc., USA)

Gel Doc system (BIO RAD Gel Doc 1000)

Vertical Gel Apparatus

UV/VIS Spectrophotometer (Beckman DU® 530 Life Sciences)

3.3.1.2. Reagents and chemicals

Tris-Cl pH 8.0 (1.0 M): Tris base 121.1 g was dissolved in 800 ml of dH_2O. The pH was adjusted to 8.0 by adding concentrated HCl. The solution was allowed to cool to room temperature. The volume was adjusted to 1 liter and sterilized by autoclaving. The solution was stored at room temperature.

EDTA pH 8.0 (0.5 M): Na EDTA.$2H_2O$ 186.1 g was dissolved in 700 ml of dH_2O. The pH was adjusted to 8.0 with 10 M NaOH (~ 50 ml). The volume was adjusted to 1 liter and sterilized by autoclaving. The solution was stored at the room temperature.

NaCl (5.0 M): NaCl 292 g was added to 900 ml of dH_2O and the volume was adjusted to1.0 liter.

CTAB (2.5%): CTAB 2.5 g was added to 80 ml of distilled water and dissolve properly. Volume was adjusted to 100 ml with distilled water.

Chloroform: Isoamyl alcohol (24:1 v/v): Isoamyl alcohol 4 ml was added to 96 ml of chloroform and mix properly.

β -mercaptoethanol (Himedia)

Bromophenol blue (Himedia)

Ethidium bromide (Himedia)

Isopropanol (Merck)

RNase (Bangalore Genei)

Agarose (Himedia)

Hind III digested λ DNA molecular weight marker (Banglore Genei, India)

3.3.1.3. Buffers

DNA extraction was performed using extraction buffer, high salt TE buffer and TAE buffer.

3.3.1.3.1. Extraction buffers (100 ml)

100 mM Tris-Cl (pH 8.0): From 1M Tris.Cl solution, 10 ml was added to the extraction buffer to make the concentration 100mM.

25 mM EDTA: From the stock EDTA solution (0.5 M), 5 ml was added to the extraction buffer.

1.5 M NaCl: From 5M NaCl solution, 30 ml was added to the extraction buffer to make 1.5M concentration of NaCl.

2.5% CTAB: 2.5 gm CTAB was added to the extraction buffer and dissolve properly.

0.2% β -mercaptoethanol (v/v): 200 µl of β–mercaptoethanol was added to the extraction buffer just before the experiment.

After adding all the compositions, volume was made upto 100 ml with distilled water.

3.3.1.3.2. High salt TE buffer (100 ml)

1 M NaCl: From the 5M NaCl solution, 20 ml was added to the high salt TE buffer.

10 mM Tris-Cl (pH 8.0): From the 1M Tris-Cl solution, 1 ml was added to the TE buffer to make the Tris-Cl concentration 10mM.

1 mM EDTA: 200 µl of 0.5M EDTA was added to the TE buffer solution to make the final concentration of EDTA as 1mM in the solution.

3.3.1.3.3. TAE buffer

24.2 g Tris-Cl dissolved in 57.1 ml glacial acetic acid and 10.1ml EDTA was added into the solution and pH was adjusted to 8.0. Volume was made upto 100ml with distilled water.

3.3.1.4. Loading dye and fluorochrome

Bromophenol blue and ethidium bromide were used as loading dye and fluorochrome, respectively for DNA visualization during and after electrophoresis.

3.3.1.5. Bromophenol blue (6x, 4.0 ml)

Bromophenol blue	10 mg
Xylene cyanol	10 mg

Glycerol 1.2 ml (autoclaved)

3.3.1.6. Ethidium bromide (10 mg/ml)

Ehidium bromide 100 mg

Sterile dH_20 10 ml

Stored at 4^oC in darkness

3.3.1.7. DNA extraction protocol

The CTAB based DNA isolation protocol described by Doyle and Doyle[210] was used and standardized with slight modification.

1. Preheated CTAB extraction buffer at 60^oC in a water bath.
2. Fresh leaves weighing 1 g of the selected plants was ground into fine powder in liquid nitrogen in a chilled mortar.
3. The powder was transferred directly to a 25 ml polypropylene tube and added 2/3 of freshly prepared preheated extraction buffer and mixed by gentle inversion to slurry.
4. The sample was incubated at 60^oC in a water bath for 2 h with occasional mixing to avoid aggregation of the homogenate.
5. Extract once with chloroform-isoamyl alcohol (24:1), mixing gently but thoroughly. This produces two phases, an upper aqueous phase which contains the DNA, and a lower chloroform phase that contains some degraded proteins, lipids, and many secondary compounds. The interface

between these two phases contains most of the "junk"--cell debris, many degraded proteins, etc.

6. The extract was centrifuged at 6,000 rpm in a Sorvall RC-5B Plus centrifuge for 10 min at 25°C to get rid of the junk. The upper aqueous phase was transferred to a clean polypropylene tube and the process was repeated twice to clear the aqueous phase.
7. An aliquot of 3 ml of 5 M NaCl was added to the aqueous phase and mixed properly by gentle inversion without vortexing.
8. An ice cold isopropanol (0.6 volumes) were added to the mixture and the mixture was incubated at 4°C overnight to precipitate the nucleic acid.
9. The sample was centrifuged at 8,000 rpm in the centrifuge for 10 min at 4°C.
10. The supernatant was poured off and the pellet was washed with 80% ethanol and carefully transferred to a clean micro-centrifuge tube. The pellet was again washed with 80% ethanol.
11. The pellet was air-dried and dissolved in 0.5 ml of high salt TE buffer.
12. 5 µl of RNase was added to the sample and incubated at 37°C for 1h.
13. After incubation, the sample was extracted with equal volume of chloroform: isoamyl alcohol (24:1). The aqueous layer was transferred to a fresh 1.5 ml microcentrifuge tube and added 2 volumes of pre-cooled ethanol.
14. The sample was then centrifuged at 7,500 x g (10,000 rpm) for 10 min in a bench top centrifuge (Hettich Zentrifugen, MIKRO 12-24) at room

temperature (25-30°C) to precipitate the DNA.

15. The pellet was rinsed with 80% ethanol, dried in speed vacuum (Maxi dry plus, Hoefer Pharmacia Biotech Inc., USA) and resuspended in 200 µl of high salt TE buffer.

3.3.1. 8. Modification of the protocol

The chloroform: isoamyl alcohol (24:1) washing step was performed twice to clear the aqueous phase of the extract. Before addition of ice-cold isopropanol, 3 ml of 5M NaCl solution was added to the sample to precipitate the DNA.

3.3.1.9. Purity and yields of the isolated DNA from selected plants

The concentration and the purity of the isolated DNA were measured by taking the reading at 260 nm and 280 nm in a UV/VIS spectrophotometer (Beckman DU® 530 Life Sciences) against blank and diluted sample. Isolated DNA sample 5 µl was taken in a quartz cuvette and made up the volume to 1 ml by adding double distilled water. Since 1 OD (optical density) corresponds to 50 µg of double stranded (ds) DNA/ml, the following calculation was done to determine the concentration of DNA:

$$\text{DNA concentration } (\mu g/ml) = (OD_{260}) \times (\text{dilution factor}) \times (50\ \mu g/ml)$$

The ratio of absorbance of DNA solution at 260 nm/280 nm is a measure of the purity of DNA sample and it should be in between 1.75 to 2.00.

3.3.2. Genome size determination

Genome size of the plants was determined by using flow cytometry according to the procedure described by Otto[211] with minor modifications. Otto I and Otto II buffer solutions were used for the same.

3.3.2.1. Preparation of Otto I buffer

4.2 gm of citric acid monohydrate (0.1 M) and 1ml 0.5% (v/v) Tween 20 was dissolved in 100 ml distilled water. The volume was adjusted to 200 ml with distilled water and kept at $4^{o}C$ for further use.

3.3.2.2. Preparation of Otto II buffer

28.65 g of Na_2HPO_4, $12H_2O$ (0.4 M) was dissolved in 100 ml of distilled water. The volume was adjusted to 200 ml with distilled water and kept at $4^{o}C$ for further use.

3.3.2.3. Stain or flurochrome

Propidium Iodide 50 µg/ml

RNase 50 µg/ml

3.3.2.4. Procedure

1. 20 mg of fully grown young leaves were collected from a selected plant, washed thoroughly and chopped with a razor blade in 0.5 ml of ice cold Otto I buffer in a Petri dish.
2. Then added another 0.5 ml of ice cold Otto I buffer and mixed thoroughly

with a pipette.

3. The suspension was filtered through a 42 µm nylon mesh and incubated the sample for 5 min with occasional shaking.
4. Otto II buffer 2 ml was added to the sample along with the stained solution (200 µl of stock solution) and stored at room temperature for about 15 min.
5. The sample was analyzed in a FACS Calibur flow cytometer (Becton Dickinson, USA) for relative DNA content of isolated nuclei. The instrument was calibrated using FACS COMP software.
6. Garden pea (*Pisum sativum*) was used as the external reference standard. The use of an internal reference standard gave poor reading of results in peak quantities, probably resulting from interference between the staining solutions and the genome of pea and the selected species. For this reason external reference standard was used and controlled every 3 samples to check the calibration of the flow cytometer.
7. The gain of the instrument was adjusted so that G_o/G_1 peak of pea (reference standard) was positioned at channel 200.
8. The nuclear DNA content of the plant samples was estimated according to the equation:
9. 2C nuclear DNA content of the sample = (9.09 x G_o/G_1 peak mean of the sample) / G_o/G_1 peak mean of pea. The means of nuclear DNA content were calculated for each sample and analyzed as a single value.

3.4. Isolation of phyto-compounds from the selected plants

Fresh tender leaves of *E. alba* and *A. barbadensis* were used for the isolation of active compounds. Young leaves from both the plants were collected.

3.4.1. Isolation of phyto-compounds from *E. alba*

3.4.1.1. Preparation of crude extracts

The tender leaves of *E. alba* were washed thoroughly with distilled water and shade-dried. Dried leaves were ground into powder and 400 g of the powder was extracted with 800 ml of methanol in a soxhlet apparatus for 12 h. The solvent was evaporated to dryness from the extract in a rotary evaporator. The procedure yielded 8.23 g of brown methanol extract. The crude extract was partitioned between equal volumes of ethylacetate and methanol. Both the parts (4.08 g each) were concentrated and taken for further analysis.

3.4.1.2. Fractionation of crude extracts

The methanol dissolved fraction was chromatographed with column size 20 cm x 14 mm and the column was packed with silica gel (60-120 mesh size) and methanol. Elution was done with water - methanol in increasing polarity and at 70:30 (v/v) solvent gradients and the compound Ea 1 was collected.

The crude ethylacetate extract was chromatographed with column size 32 cm x 19 mm and it was packed with silica gel (100-200 mesh size) and hexane. The compound was eluted successfully with 0.5-10% ethylacetate in hexane with a flow rate 1ml/min. The fraction eluted with 2% ethylacetate (2:98, v/v) was

afforded as Ea 2.

3.4.1.3. Thin layer chromatography

Compound Ea 1 was also subjected to thin layer chromatography (TLC) using chloroform: toluene (7:3) solvent system followed by Iodine spraying and heating. The R_f value was calculated for Ea 1. The preparative TLC was performed on the TLC plate having size 20 cm x 20 cm and coated with silica gel. TLC plates were visualized by Iodine spray, followed by heating. The iodine visible portions were scraped, then dissolved in the same solvent system and eluted with a column where the silica gel retained and the dissolved compound passed through. After evaporation of the solvent the fraction afforded the compound Ea 1 (56.2 mg).

Similarly, the compound Ea 2 was subjected to thin layer chromatography (TLC) using hexane: ethylacetate (3:7) solvent system followed by anisaldehyde spraying. The R_f value was calculated for the compound. The preparative TLC was performed on the TLC plate having size 20 cm x 20 cm and coated with silica gel. The TLC plates were visualized after spraying with anisaldehyde reagent. The visible portions were scraped, dissolved in the same solvent system and eluted with a column where the silica gel retained and the dissolved compound passed through. After evaporation of the solvent the first fraction afforded the compound Ea 2 (22.5 mg).

3.4.2. Isolation of phyto-compounds from *A. barbadensis*

3.4.2. A) Preparation of crude extracts

Tender leaves of *A. barbadensis* were collected, washed thoroughly with distilled water and shade-dried. Dried leaves were powdered and 500 g of it was extracted with 1000 ml of methanol in a soxhlet apparatus for 20 h. The solvent was evaporated to dryness from the extract in a rotary evaporator. The procedure yielded 6.77 g of dark brown methanol extract.

3.4.2. B) Fractionation of crude extracts

The crude methanol extract was eluted with column size 32 cm x 19 mm and it was packed with silica gel (100-200 mesh size) and hexane. The extract was separated using 0.5-10% ethylacetate in hexane with a flow rate 1ml/min. The fraction eluted with 2% ethylacetate (2:98, v/v), afforded the compound Av 3**.** From the same extract, another compound Av 4 was eluted by using 4% ethylacetate in column chromatography.

3.4.2. C) Thin layer chromatography

The fraction Av 3 was subjected to TLC using hexane: ethylacetate (3:7) solvent system followed by anisaldehyde spraying. The R_f value was calculated for the compound. The preparative TLC was performed on the TLC plate having size 20 cm x 20 cm and coated with silica gel. TLC plates were visualized by anisaldehyde spray. The anisaldehyde visible portions were scraped, then dissolved in the same solvent system and eluted with a column where the silica gel retained and the dissolved compound passed through. After

evaporation of the solvent the first fraction afforded the compound Av 3 (43.2 mg).

To isolate the compound Av 4, TLC was done using hexane: ethylacetate (3:7) solvent system followed by anisaldehyde spraying and heating and R_f value of the compound was calculated. The compound spotted portions were scraped, then dissolved in the same solvent system and eluted with a column where the silica gel retained and the dissolved compound passed through. After evaporation of the solvent, 51.4 mg of compound Av 4 was collected.

3.5. Identification of isolated compounds

The isolated compounds from the plant species were subjected to high performance liquid chromatography (HPLC), infrared (IR) spectroscopy and mass spectroscopy. Proton and carbon nuclear magnetic resonance (NMR) spectra were also studied for their identification and structure elucidation.

3.5.1. High performance liquid chromatography (HPLC)

Purification of the isolated compounds from *E. alba* and *A. barbadensis* was done in HPLC using Ascentis Reverse Phase-Amide column with the dimension of 10 cm×10 mm I.D. and particle size 10 µm (Supelco, USA). The HPLC analysis of the compound was performed using liquid chromatography (Waters, model 600E) with a 486 UV variable wavelength detector and Novapack® column C-18 (5 µm, 150×3.9 mm). The mobile phase consisted of a gradient mixture, methanol/water (70:30, v/v). The solution was degassed in an ultrasound bath and filtered under vacuum through a membrane (Millipore,

PVDF). The flow was 1.0 ml/min and the sensitivity was 0.001 AUFS. The absorption spectra of the compounds were measured at 280 nm for purification purpose. All three compounds were separated using two solvent gradient systems: solvent A, 100% MilliQ water and solvent B, 100% Methanol (HPLC grade). The separated compounds were collected by repetitive injections. The collected compounds were run in the same gradient to confirm their elution time. The solvent gradient used for performing HPLC is shown in Table 3.1.

Table 3.1 HPLC solvent gradient system

Time (min)	Flow (ml/min)	% A	%B
0	2	100	0
5	2	80	20
10	2	70	30
15	2	60	40
20	2	50	50
25	2	40	60
30	2	30	70
35	2	20	80
40	2	0	100
45	2	100	0
48	2	100	0

3.5.2. Fourier Transformation Infrared Spectroscopy (FTIR)

FTIR spectra of all the isolated compounds were recorded using KBr pellet in Nicolet Impact 410 FT-IR Spectrometer. From each sample, 5 mg was prepared by dispersing the sample uniformly in a matrix of dry KBr, compressed to form an almost transparent disc. The spectra showing functional groups were used to study the composition of the compounds. IR spectra were collected from 400 – 4,000 wave numbers (cm^{-1}).

3.5.3. Mass spectroscopy

The mass spectra of the compounds were recorded in a Micromass Tof-Spec 2E instrument using nitrogen 337 nm lasers with 4-nanosecond pulse and mass Lynx 4.1 SCN 714 in SAIF, Central Drug Research Institute, Lucknow, India. Each compound was dissolved in methanol (MeOH) was used as the matrix. A minimum of 1-2 mg sample was taken for the mass spectra analysis.

3.5.4. Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR spectra of the isolated and purified compounds were scanned on Varian Mercury 400 Spectrometer operating at 400 MHz for ^{1}H and ^{13}C nuclei, respectively. The duterated chloroform ($CDCl_3$) was used as solvent and tetramethyl silane (TMS) as the internal standard. For ^{1}H NMR spectra 5 mg of sample and for ^{13}C NMR spectra, 30-35 mg sample were taken for analysis.

3.6. Biological characterization of the isolated compounds

3.6.1. Antibacterial assay of the isolated compounds

The purified compounds were subjected to antibacterial assay using three pathogenic bacteria and one yeast.

3.6.1.1. Test organisms

The standard strains of Microbial Type Culture Collection (MTCC) were obtained from the Department of Molecular Biology & Biotechnology, Tezpur University, Assam, India. The same was used to assess the antibacterial potential of the plant compounds. Bacterial strains *Bacillus subtilis* (MTCC 619), *Klebsiella pneumoniae* (MTCC 109), *Escherichia coli* (MTCC 739), *Pseudomonas aeruginosa* (MTCC 7815) and *Stapphylococcus aureus* (MTCC 737) were used as the test organisms.

3.6.1.2. Media

The bacterial test pathogens were cultured and maintained in nutrient agar (NA) medium. For antibacterial activity test, Mueller-Hinton (MH) agar medium was used. The composition of the medium is presented below:

3.6.1.2.1. Nutrient Agar medium

Compositions	g/l
Peptone	10.0
Beef extract	10.0

Sodium chloride	5.0
Agar	12.0

pH of the medium was adjusted to 7.3

3.6.1.2.2. Muller Hinton agar medium

Compositions	g/l
Beef infusion	300.0
Casein acid hydrolysate	17.5
Starch	1.5
Agar	17.0

pH of the medium was adjusted to 7.2

3.6.1.3. Determination of antibacterial activity

The antibacterial activity of the isolated and purified compounds of *E. alba* and *A. barbadensis* were evaluated by well diffusion method[212]. Stock cultures were maintained at 4ºC on nutrient agar medium. Active cultures were prepared by transferring a loop full of cells from each stock culture to test tubes containing Mueller-Hinton broth (MHB) and incubated at 37ºC for 24 h without agitation. The disc diffusion method was used to determine the antibacterial activity of the isolated compounds from *E. alba* and *A. barbadensis*. *In-vitro* antibacterial activity was screened by using Mueller Hinton Agar medium. The MHA plates were prepared by pouring 15 ml of

molten media into sterile petri dishes. The plates were allowed to solidify for 20 min and then 200 µl of the test microbes in the log phase of growth (10^6-10^8 cells as per McFarland standard) were seeded on the surface of Mueller Hinton agar medium using a micropipette and spreaded all over the medium using a sterile glass spreader. With the help of a sterile cork borer wells having 6 mm diameter each were made on Mueller Hinton agar plates. The tested compounds (Ea 1, Ea 2, Av 3 and Av 4) were dissolved in sterilized DMSO (10% v/v) and introduced into one of the wells. As the 10% DMSO (v/v) had no detectable effect on bacterial growth, compounds at concentrations of 0.1 $g.ml^{-1}$ were prepared in 10% DMSO (v/v). Streptomycin sulphate (1 $mg.ml^{-1}$) was taken as a positive control and 10% DMSO (v/v) as negative one. After the incubation of the plates at 37˚C for overnight period, bacterial growth was determined by measuring the diameter of inhibition zone using a transparent metric ruler.

The microbroth dilution method was performed to determine the minimum inhibitory concentration (MIC). The *E. alba* and *A. barbadensis* isolated compounds were dissolved and diluted in Luria Bertani (LB) broth, seeded in a 96-well culture plate and then inoculated with a fresh bacterial inoculum. Inoculated microplates were incubated at 37°C for 24 h. Each compound concentration was tested in duplicates for each organism. The viability of the treated cells was determined by MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium) assay and the absorbance was measured at 570 nm using a microtitre plate reader (Bio-Rad Model 680; Hercules, California) for

bacterial strains. The MIC was determined as the lowest concentration of each purified compounds required inhibiting the growth of each organism. The mean and standard deviation of triplicates for each treatment were calculated.

3.6.2. Antifungal assay of the purified compounds

The compounds from *E. alba* and *A. barbadensis* were subjected to antifungal assay using different fungal strains.

3.6.2.1. Test organisms

The fungal strains used in the present investigation were obtained from the Department of Molecular biology and Biotechnology, Tezpur, Assam, India. The fungal strains are *Candida albicans* (MTCC 227) and *Fusarium oxysporium* (MTCC 284).

3.6.2.2. Media used

Potato Dextrose Agar medium (PDAM)

Compositions	g/l
Potato infusion	4.0 (Infusion from 200 g potatoes)
Dextrose	20.0
Agar	15.0

pH of the medium was adjusted to 7.3

3.6.2.3. Determination of antifungal activity

Fungal cultures were maintained at room temperature in PDAM. Active cultures of the fungal strains were prepared by seeding a loopful of fungi into PD broth and incubated without agitation for 48 h at 25ºC. The culture was diluted with PD broth to achieve the optical density corresponding to 2.0×10^5 spores/ml.

The disc diffusion method was also used to screen for antifungal properties. In vitro antifungal activity was screened by using PDA media. The PDA plates were prepared by pouring 15 ml of molten media into sterile petriplates. The plates were allowed to solidify for 10 min and 200 µl of the test strains were introduced into media and allowed to spread with the help of sterile glass spreader. The plates were incubated at room temperature for 10 min. A sterile cork borer of 5 mm diameter was used to make wells on each plate and filled with 200 µl of each plant isolated fractions. These were carried out in triplicate for each fungal strain. The plates were incubated at 25ºC for 96 h and the resulting zone of inhibition was measured using a transparent metric ruler. Each set of seeded plates were compared for confirmation. Amphitericin ($1 mg.ml^{-1}$) was used as positive while 10% DMSO (v/v) was kept as negative control.

3.6.3. Antioxidant assay

The purified compounds were studied for free radical scavenging as well as antioxidant activity. The free radical scavenging capacity of the isolated

compounds was measured *in-vitro* by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay according to the method described by Brand-Williams *et al.*[213].

3.6.3.1. Chemicals and Reagents used

DPPH was purchased from Signa-Aldrich (St. Louis, MO). Ethanol was purchased from Merck Co. (Germany), Mumbai. All the chemicals are of analytical grade and used as received.

3.6.3.2. Determination of free radical scavenging activity

The stock solution was prepared by dissolving 4 mg DPPH in 50 ml absolute methanol and stored in a dark coloured bottle at 4°C until required. An aliquot of 3.0 ml DPPH solution was mixed with 100 µl of each phyto-compound at various concentrations (3.12-100µg/ml). The reaction mixture was incubated in the dark for 30 min at room temperature and absorbance was observed at 517 nm. The absorbance of 3.0 ml DPPH solution as control was measured in each compound. The scavenging activity was estimated based on the percentage of DPPH radical scavenged as per the following equation:

Scavenging percentage (%) = (Control absorbance-Sample absorbance) / Control absorbance × 100

Compound concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotted as scavenging percentage against compound concentration. Quarcetin and gallic acid were taken as standards. All tests were carried out in triplicate.

3.6.4. Assessment of cell cytotoxicity on murine macrophage cell line (RAW 264.7)

Murine macrophage cell line was obtained from the Defence Research Laboratory, DRDO, Solmara, Tezpur, Assam, India. The cells were maintained in Dulbecco's minimum essential medium (DMEM) containing 2 $mM.l^{-1}$glutamine, 1.5 $g.l^{-1}$ sodium bicarbonate ($NaHCO_3$), 0.1 mM non-essential amino acid and 1.0 mM sodium pyruvate, supplemented with 10% (v/v) fetal bovine serum and 1% antibiotic antimycotic solution (1,000 U.ml-1 penicillin G). Cells were maintained at 37°C in a saturated-humidity atmosphere.

To quantitatively measure cell toxicity, MTT (3-[4, 5-dimethylthia-zole-2-yl]-2, 5-diphenyl tetrazolium bromide) dye conversion assay was used (Mossman, 1983). For the MTT viability studies, murine macrophage cell line RAW 264.7 was cultured at a density of 1×10^4 cells per well in a 100 µl volume of cell culture medium (DMEM supplemented with 10% fetal bovine serum) in a 96-well cell culture plate. After 24 h, cultured cells were treated with a series of different concentrations (20, 40, 60, 80 and 100 $\mu g.ml^{-1}$) of Ea1, Ea 2, Av 3 and Av 4 dispersed in 100 µl per well DMEM without serum and phenol red, and incubated further for 4 hours with MTT dye. After incubation 100 µl of dimethylsulfoxide (DMSO) was added to each well to dissolve blue formazan precipitate, and absorbance was measured at 570 nm using a microtitre plate reader (Bio-Rad Model 680; Hercules, California). All experiments were performed in quadruplets. The cell viability was expressed as a percentage of

the control by the following equation: Cell viability (%) = Absorbance of control cells -Absorbance of treated cells/ Absorbance of control cells × 100

3.7. Inducing alopecia on animal model using warfarin

The protocol for experimentation was approved by the Central Animal Resources, Defence Research Laboratory, Defence Research and Development Organization, Tezpur, Assam, India. Approval to carry out these studies was obtained from the Institutional Animal Ethics Committee.

3.7.1. Animal husbandry and maintenance

Adult 12 wistar strain albino rats (5–8 weeks, both male and female) weighting 150-180 gm were placed in polypropylene cages with free access to standard laboratory diet (Pranav Agro Industries Limited, Sangli, Maharastra, India) and provided municipal water ad libitum. Each individual animal was clinically examined and identified by fur marked with picric acid. The females were nulliparous and not pregnant at the time of experiment. Animals were grouped and housed in an environmentally controlled room with temperature of 22ºC ± 3ºC and 40-70% relative humidity with a 12-hour light–dark cycle and ventilation of 15-21 air changes/h for an acclimatization period of 7 days to laboratory conditions prior to the beginning of the experiment in order to adjust to the new environment and to overcome stresses incurred during their transit. Only healthy animals were assigned for these studies.

3.7.2. Alopecia induction

For the experiment, 18 wistar albino rats were taken and they were subjected to warfarin treatment to induce alopecia. Standardization of warfarin drug was done after various trials to induce alopecia in the rats. The rats were dosed with 3, 2.5, 2, 1.7 and 1.5 mg/kg warfarin to induce hair loss. Among them, warfarin dose 1.7 mg/kg showed patchy hair loss after 2 months of continuous medication without any harmful side-effects or mortality, which lead to severe alopecia in the albino rats. After 2 months of continuous dosing, medication was stopped.

3.8. Application of isolated phyto-compounds on alopecia induced animal model

After inducing hair loss in wistar albino rats by using warfarin drug, all the animals were kept for 1 month in normal food, water and normal temperature to observe if any hair re-growth occurs. The animals were divided into 6 groups, 3 animals in each group. Group I was kept as control, group II was studied with standard drug minoxidil and rest four groups were treated with tested compounds i.e. Ea 1, Ea 2, Av 3 and Av 4. Histopathology was done for the warfarin treated albino rats to check any follicular regeneration. The purified phyto-compounds Ea 1 and Ea 2 from *E. alba* and AV 3 and Av 4 from *A. barbadensis* were dissolved in alcohol to prepare 2% solution and applied topically in the alopecia affected area for 15 days. The standard drug minoxidil solution (2%) was applied on the rats as positive control. Qualitative, haematological, serum biochemistry and histological study were carried out for

both treated and control animal skin to differentiate changes between plant purified compounds and minoxidil treated and control animals in follicular and dermal levels.

3.8.1. Acute dermal irritation study

The acute dermal irritation/corrosion study was carried out in accordance to the OECD Guideline 404 method[214]. Twelve healthy rabbits with intact Primary irritation index (PII) were used. It was then classified according to Draize method using PII scoring as non-irritant (if PII <0.5), slightly irritant (if PII <2), moderately irritant (if PII < 2.5), and severely irritant (if PII >5). For each animal, the dermal response scores (sum of the scores for erythema and edema formation) at 24, 48, and 72 h after the removal of the patches were summed up, and divided by 3 to obtain a mean irritation score per time point. The mean scores at 24, 48, and 72 h were summed and derived the average to obtain the PII.

3.8.2. Qualitative study

Qualitative hair growth was evaluated by observation of two parameters: hair growth initiation time and hair growth completion time i.e. minimum time taken to cover the denuded skin region with new hair completely. Hair growth initiation and completion time were recorded for each group of animals and compared with the positive control minoxidil 2% solution and control. Also the average length and weight of hair was recorded and compared for each group of animals.

3.8.3. Haematological study

All animals were fasted overnight prior to necropsy and blood collection (14th day of topical application of phyto-compound treatment). Blood samples were collected through orbital sinus vein puncture technique from retro orbital sinus of rats by 75 mm heparinized capillary tube (Haematocrit capillary, Himedia Laboratories Private Limited, Mumbai, Maharastra,India). The blood samples were collected in non-vacuum blood collection tubes containing K3 EDTA (Peerless Biotech Pvt Ltd,Chennai, Tamil Nadu, India) and analyzed within 60 minutes. The hematological parameters including white blood cell (WBC) count, WBC differential counts, like lymphocyte (Lym), monocyte (Mon), neutrophill (Neu), eosinophil (Eo), and basophil (Ba) counts, red blood cell (RBC) count, mean corpuscular volume (MCV), hematocrit (Hct), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), hemoglobin (Hb) concentration, and platelet (Pct) were examined by Automatic Hemato analyzer (MS-4) (Melet Schloesing Laboratories, Osny, France).

3.8.4. Study of serum

Blood for clinical chemistry was placed in the vacuum blood collection tubes (Peerless Biotech Pvt Ltd) devoid of anticoagulant (serum tube) and allowed to clot at room temperature. Blood samples were centrifuged within 60 min at 3,000 rpm (604 g) for 10 min after collection and then the serum was separated. Serum biochemistry parameters including low-density lipoprotein (LDL), high-density lipoprotein (HDL), glucose (GLUC), urea (UREA), total protein (TP),

uric acid (UA), triglycerides (TGL), cholesterol (CHOL), creatinine (CRE), alanine aminotransferase (ALT/SGPT) and aspartate aminotransferase (AST/SGOT) were analyzed by Coralyzer-100 (Tulip Diagnostics Pvt Ltd, Goa, India) with the help of commercially available biochemical kits.

3.8.5. Histological study

3.8.5.1. Chemicals

Formalin, paraffin wax, ammonia, Hematoxylene stain, Eosin stain, Mount DPX etc were purchased from Sigma-aldrich (St.MO), absolute alcohol and xylene were purchased from Merck Co. (Germany). All chemicals were of analytical grade.

3.8.5.2. Preparations of reagents

3.8.5.2.1. Acid-alcohol solution

One ml of concentrated Hydrochloric (HCl) acid was dissolved in 70% alcohol to prepare the acid-alcohol solution.

3.8.5.2.2. Mayer's albumin

Egg albumin 1 part was mixed with 1 part of glycerol and then added water in such a way that eggs get dissolved on shaking. Added thymol as preservative and the total volume of the solution was 2.0 ml.

3.8.5.3. Staining Protocol

1. After harvesting tissue from the control and the treated animals, a part

tissue was collected and washed in 0.9% (w/v) saline (3 times) and put in 10% formalin with 1.0 ml ammonia for 24 h.

2. Formalin was decanted and tissues were kept in 70% alcohol with 1.0 ml ammonia for 2 h.
3. Alcohol 70% was decanted and tissues were kept in 80% alcohol for 2 h.
4. 80% alcohol was decanted after 2 h and the tissues were kept in 90% alcohol for 2 h.
5. 90% alcohol was decanted after 2 h and tissues were kept in 100% alcohol for 2 h.
6. Decanted 100% alcohol and added xylene (100%) till the tissues become transparent and hard in appearance.
7. Infiltration of the tissue was done by putting the cleared tissues in melted paraffin for 2 h by using infiltration cassette. Infiltration was done twice for each tissue.
8. Embedding was done by using melted paraffin in 'O' spare ring which used for block making. Allowed the paraffin to solidify and then put the tissue to get the transverse section. Allowed the block to solidify properly on a cooling plate.
9. After solidification for 2 h, the block was ready for trimming and sectioning.
10. Trimmed cuboid of the block in such a way that on one side, the tissue in

the front which could be the face for sectioning.

11. Took the trimmed block for making sections that were overlaid on water.
12. Sections were cut using automatic microtome instrument.
13. Keep the tissue sections on the slide, and then coated with Mayer’s albumin.
14. Once the sections were layered on the slide, they were kept on the slide warmer plate so that the tissue could be stretched properly. Now the slides were ready for staining.