Chapter 5

Discussion
5. DISCUSSION

Hair loss or hair thinning has always been a common disorder in clinical dermatology. Parameters like hair density, hair diameter, hair growth rate and anagen/telogen ratio are assessed using phototrichograph based techniques with digital image analysis such as Trichoscan. Human hair serves a variety of purposes, few of which appear to be vital for life like sexual dimorphisms, limited protection of the skin and tactile sensation. Similarly hair of mice is important in sexual attraction. The thick coat of hair in mice serves a vital function by forming a critical thermoregulatory system needed for survival (Muller–Rovet et al., 2001).

Hair growth and the cyclic activity of the hair follicle are timed by a “biological clock” of unknown nature and are the result of as-yet poorly understood tissue interactions (Slominski et al., 1991). The molecular basis for the interesting phenomenon of concerted developmental and pigimentary activity occurring during hair growth in C57/Bl6 mice is still unknown. The hair in mice cycles in a wave like pattern from head to tail as opposed to mosaic pattern in humans (Chase, 1954). Murine models of hair growth offer the opportunity to study large numbers of biologically homogeneous follicle populations, because hair growth in mice is synchronized in contrast to human hair follicles which enter or leave anagen in waves covering large skin areas.

There exists an unmet need for identification of novel hair growth promoters in light of the fact that there are only two drugs, topical minoxidil and oral finasteride approved by the Food and Drug Administration (FDA) for the treatment of alopecia.

Large randomized placebo controlled trials on humans conducted by Upjohn Company for minoxidil, a potassium channel opener showed efficacy in 54% of the treated patients as opposed to 34% in placebo control group. There are significant adverse dermatological effects associated with minoxidil viz. pruritis, dryness, scaling, local irritation, dermatitis (DeVillez, 1990). Finasteride, an oral 5 alpha reductase inhibitor, is known to increase hair growth in patients with male pattern baldness (Androgenetic
alopecia). Large clinical trials conducted by Merck Research Laboratories (MRL clinical study report, 1996; Protocols 089-03, 087-03 and 092) report that 48% of hair regrowth is observed in finasteride recipients in one year. Patients receiving finasteride also showed that it is generally well tolerated, but few patients withdrew the treatment due to drug related sexual disorders. Finasteride is not indicated for use in women (McClellan and Markham, 1999).

Few other drugs either in preclinical development or undergoing Clinical Trials are Dutasteride (Olsen et al., 2006), Spironolactone, small and large peptides like Lys-Pro-Val tripeptide (Mahe, 1998), glyceryl-L-histidyl-L-lysine copper (II) and their derivatives (Pickart, 1993). Bexarotene, capsaicin and hydroxychloroquine, alocicept and roxithromycin are in various phases of clinical trials (www.clinicaltrial.gov, 2007).

Not surprisingly, the demand for drugs that alter hair growth and appearance has led to a multibillion-dollar industry. In United States hair loss sufferers spend more than 3.5 billion dollars in a year for treating hair loss. The American Hair Loss Association recognizes that hair loss is an extremely emotionally distressing disease that makes afflicted patients vulnerable (The Washington Post, 2006).

Now a days, there is tremendous increase in demand for plant products. Hence the present study was initiated for identification of novel plant based drug(s) as a therapy for alopecia. For many of the medicinal plants of current interest, a primary focus of research to date has been in the areas of phytochemistry, pharmacognosy, and horticulture. In the area of phytochemistry, medicinal plants have been characterized for their possible bioactive compounds, which have been separated and subjected to detailed structural analysis. The synergistic or additive pharmacological effect can be beneficial by eliminating the problematic side effects associated with the predominance of a single xenobiotic compound in the body (Tyler, 1999). This theme of multiple chemicals acting in an additive or synergistic manner likely has its origin in the functional role of secondary products in promoting plant survival. Library of plants was selected based on the traditional literature for hair growth promoting activity. Eclipta alba, Hibiscus
rosasinensis and Arnica montana plant/ plant parts were processed for obtaining methanol and ethyl acetate extract. These extracts were further screened for hair growth promoting activity in \textit{in-vivo} and \textit{in-vitro} experiments.

The study of human hair growth and hair disease has been hampered by the difficulty of obtaining sufficient tissue from the intact follicle on which various research techniques were to be performed. To measure hair growth, relevant, easy and inexpensive experimental models were essential. These included \textit{in-vivo} and \textit{in-vitro} models, to reflect major regulatory processes. The dermal component of the hair follicle plays a fundamental role in the induction and maintenance of epithelial differentiation.

Characterized HFDPCs represented a valid \textit{in-vitro} model for screening hair growth promoters. In 1981, Weterings and his associates (Weterings \textit{et al.}, 1981) described a method for culturing epithelial cells derived from the outer root sheath of the human hair follicle, and subsequently Wells in 1982 (Wells, 1982) reported culturing epithelial cells from human telogen hair bulbs. At the same time, Jahoda and Oliver (1981) and Oliver (1966) reported the establishment of \textit{in-vitro} cultures of dermal papilla cells from rat vibrissa follicles. A major limitation of cell culture system is difficulty of establishing whether the cells retain their \textit{in situ} function.

The dermal papilla cells, which have mesenchymal origin, play a very important role in conjunction with epithelial cells. These dermal papilla cells in early subcultures possess the ability to induce hair growth. Hence, plant extracts were screened on characterized HFDPCs. EA01 and EA02 exhibited maximal proliferative activity as compared to other plant extracts.

Whole organ hair culture model has been reported for screening 1, 25 (OH)\textsubscript{2} D\textsubscript{3} (Harmon and Nevins, 1994) and minoxidil (Buhl \textit{et al.}, 1989). It was observed that at relatively low concentrations they potentiated increase in hair shaft and hair follicle, whereas higher concentrations were inhibitory. Whole organ culture was developed and standardized using minoxidil sulphate, an active metabolite of minoxidil. Amongst all the
plant extracts screened EA01 at 2.5 ng/ml exhibited maximal proliferative activity of 115% over and above vehicle control. Further, maximal increase of 113% in hair shaft and 132% in hair follicle was observed in EA01 at 2.5 ng/ml treated hair organ cultures.

Amongst all the animal models reported for screening molecules/extracts for hair growth promotion, pigmented C57/BL6 mice are the most commonly used strain as their truncal pigmentation is entirely dependent on their follicular melanocytes. The truncal 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 (Mori and Uno, 1990; Ahmed et al., 1998; Slominski et al., 1991).

Analysis of hair growth promoters included melanogenesis, increased follicle count in subcutis, and skin thickness. In C57/BL6 mice, melanin synthesis of follicular melanocytes is strictly coupled to the growth stage of hair cycle (anagen), cease during follicle regression (catagen), and is absent throughout the resting stage (telogen) (Slominski et al., 1994). Owing to the strict coupling of follicular melanogenesis and hair follicle cycling in anagen development, C57/BL6 mice have been used widely as a model for screening hair growth promoters (Paus et al., 1989; Slominski and Paus, 1993). The active phase of hair follicle cycling is also accompanied by an increase in size and number of follicles resulting in increase in thickness of subcutis layer between dermis and panniculus carnosus.

Based on the seminal work of Sven Muller-Rover and associates (Muller-Rover et al., 2001), we investigated the follicular growth pattern through sequential histological studies of the back skin of Indian strain of C57/BL6 mice from approximately 23 days to 62 days of age. To the best of our knowledge, this is the first study reporting the pragmatic criteria for recognition of distinct stages of hair cycle in Indian strain of C57/BL6 mice and thus animals could be selected for hair growth promotion studies. The microscopic data obtained tended to support the association of skin pigmentation along
with skin thickness and follicle count in subcutis layer in different phases of hair growth cycle. 62 day old animals were in stable telogen phase and thus selected for screening hair growth promoters.

During the hair cycle, transition of telogen hair follicles to catagen phase takes place via anagen phase of hair growth. In the telogen phase the size of the hair follicle decreases and the localization is restricted to the dermis and upper subcutis layer. The telogen hair follicle enters the anagen phase, where increase in follicle size takes place and lies in deep subcutis. Anagen phase is followed by catagen phase, characterized by shrinkage in follicle size. The changes in skin thickness are in correlation with panniculus carnosus and the border between the dermis and subcutis.

Uno (1991) studied the quantitative evaluation of hair growth potential of minoxidil on macaque monkey and fuzzy rat by determining the percentage transformation of hair follicles from telogen to anagen. The study revealed that the topical application of 5% minoxidil produced approximately 10% conversion of telogen follicles to anagen follicles. Though it is a hypertensive drug, it was postulated that minoxidil readily stimulate the telogen buds and transform to larger anagen hair follicles. Savin and Atton (1993) reported that minoxidil induces proliferation of epithelial cells near the base of hair follicle and may induce the vasodilation of scalp blood vessels, however, the exact mechanism is still not known. Minoxidil is known to work by opening potassium channels ($K_{ATP}$) and is known to stimulate VEGF and prostaiglandin synthesis.

Based on the study of cyclical follicular growth, mice were morphologically preselected for telogen phase of hair cycle. The mice were then used further to validate the model with 1% and 2% minoxidil. The microscopic data obtained from the validation study showed that the topical administration of minoxidil affects the normal cycle by inducing the resting follicles to anagen phase of hair growth in approximately 87% of the treated animals as opposed to 50% efficacy in vehicle treated animals. Our present data confirms the previous reports for large randomized placebo controlled trials on humans conducted by the Upjohn company for androgenetic alopecia where investigators
observed new hair growth in 54% of the minoxidil treated patients at week 32 as compared with 34% of the placebo treated patients (DeVillez, 1990).

Takahashi et al. (2001) reported screening of 1000 different plant products to determine if any of plant extracts could influence hair growth. Proanthocyanidins extracted from grape seeds promoted the proliferation of hair cells by 230% by converting the telogen (non-growing) phase of hair growth into the anagen (growing) phase of hair growth. Thus proanthocyanidins displayed hair-cycle-converting activity, which was similar to that of minoxidil.

Similarly in our study it was observed that the hair follicles periodically are transformed from telogen to anagen phase of hair growth in the animal groups treated with methanol extract of Ecliptic alba and Arnica montana flower and ethyl acetate extract of Eclipta alba. After topical applications with the plant extracts, the secondary germ cells associated with aggregated dermal papillae cells began to proliferate and their continuous growth and differentiation may result in construction of anagen follicles.

In the present study, animals treated with EA02 (at 3.2 mg/15 cm²) showed telogen to anagen transition in ~90% of animals as compared to 0% anagen induction in vehicle treated group (p<0.0001). Similarly animals treated with EA01 (0.4 mg/15 cm²) and ARM02 (6.4 mg/ 15 cm²) also showed telogen to anagen transition in ~90% and ~60% of animals, respectively as compared zero in vehicle treated group (p<0.0001).

Chemotherapy-induced alopecia (CIA) is a frequent and emotionally distressing side effect of cancer chemotherapy (Munstedt et al., 1997; Dorr, 1998) for which there is currently no effective preventive therapy (Chen et al., 1998). CIA is thought to arise when anticancer drugs ablate the proliferating epithelium and block normal maturation of precursor epithelial cells to the final hair strand. The sensitivity of hair follicle cells to anticancer agents is related to their state of proliferation. Many anticancer agents that cause CIA target specific phases of the cell cycle and are therefore selectively toxic to
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Based on the data from *in-vitro* hair organ culture, which correlated with our present *in-vivo* data for hair growth stimulation, our efforts have been directed at the search for agents that are effective against commonly used chemotherapeutic drugs with high propensity to produce alopecia in clinical settings. We started with screening potential plant extracts further to examine the effect on etoposide-induced alopecia.

The murine model allows the study of the effects of chemotherapy on well-defined, homogeneous and mature populations of precisely the type of hair follicles that are severely damaged by chemotherapy, resulting in alopecia and disturbances in hair growth. The murine model also allows as yet unparalleled insights into the basic patterns of follicle response to recovery from chemotherapy, which is further based on the high degree of hair cycle synchrony displayed by the mouse strain. It has been reported that ImuVert, a biological response modifier prepared from the bacterium *Serratia marcescens* protected the animals from alopecia induced by cytosine arabinoside (Hussein et al., 1990). In subsequent studies, similar protection from cytosine arabinoside-induced alopecia was observed with recombinant interleukin 1 β and later with epidermal growth factor and fibroblast growth factor (Jimenez et al., 1991). However, when used under similar conditions none of these agents offered protection from alopecia induced by cytoxan. In the clinical setting, chemotherapy more often involves the use of alkylating agents. Accordingly, we continued our efforts to explore various compounds in this model and ways to prevent alopecia from alkylating agents like etoposide. The results of the present study clearly depict that the murine model used for CIA resembles the clinical situation more closely than any other currently available model.

Development of *in vivo* model for etoposide-induced alopecia included generation of baseline data in C57/Bl6 mice and Swiss albino mice post depilation. Animals at 14-day post depilation that were in active anagen phase were selected for screening extracts reversing chemotherapy-induced alopecia. Etoposide terminates anagen prematurely and causes severe alopecia. EA02 and EA01, which showed potential as hair growth promoter were further selected and screened in chemotherapy induced alopecia model.

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Topical application of EA02 accelerates the anagen phase of hair growth in etoposide induced alopecia unlike the EA01. Further, EA02 accelerates normal pigmentation of regrowing shafts; it retards the occurrence of etoposide-induced alopecia and diminishes the severity of alopecia. This strongly encourages one to explore and develop extracts as drugs for accelerating and improving the clinically and psychologically important regrowth of a normally pigmented hair coat after human CIA.

The novel strategies for the therapeutic management of CIA should more systematically take into account that, like the vast community of patients with abnormal hair loss or gain seen in clinical practice, CIA and its subsequent hair regrowth disorders predominantly reflect defined alterations of normal patterns of hair follicle cycling, rather than of hair shaft production. This implies that real progress in the prevention and treatment of CIA can only be accomplished by dissecting the molecular interactions of alopecia reducing drugs.

It is clear from the above observations that the plant extracts having hair growth promoting activity may not always reverse or prevent chemotherapy-induced alopecia.

The topical route was chosen for the potential applicability to the clinical settings. For evaluating the safety of EA02, acute toxicity was performed in accordance with OECD guidelines. Hematological, biochemical and all pathological findings were within normal limits.

Based on the above observations recorded from various studies herein, one should offer a new potential approach to both hair growth stimulation and chemotherapy induced alopecia. Our efforts have been directed at the search for agents that are effective against commonly used chemotherapeutic drugs with high propensity to produce alopecia in clinical settings.
The most potent extract, EA02 was subjected to acute dermal toxicity, in accordance with the OECD guideline for testing of chemicals (Proposal for a new draft guideline 434: Acute dermal toxicity – fixed dose procedure: first edition -2004). Acute dermal toxicity study of EA02 was carried out in adult female Swiss albino mice to assess its effects on morphological, gross behaviour, body weight changes as well as to find out histopathological, biochemical and hematological changes. EA02 reflected innocuous nature of this Eclipta alba preformulation on hepatic, renal and hematological system even at a limit dose of 2 g/kg.

The results of bioactivity assays were used for elucidation of the mechanism of action for anagen induction for EA01 and EA02 as hair growth stimulators. In the present study, presence and absence of surrogate markers were studied for plant extracts along with their antioxidant activities.

In both mice and human, hair cycle is regulated by interplay of stimulatory and inhibitory growth factors (Stenn and Paus, 2001). Various members of fibroblast growth factor (FGF) family are expressed in the skin and are involved in the dynamics of dermal function including physiological processes as wound healing and hair growth. Expression of these FGF varies throughout hair growth cycle; FGF7 and FGF10 are expressed in dermal fibroblasts and papilla cells and stimulate proliferation of keratinocytes, which is characteristic of anagen phase of hair growth (Oro and Higgins, 2003). In resting hair follicles, BMP4 mRNA predominates over noggin in epithelium and mesenchyme. Anagen development is accompanied by down regulation of BMP4 and increased noggin mRNA in hair follicle (Bitgood and McMahon, 1995). Shh expression and target gene induction occurs only in the anagen phase of hair growth. At the start of catagen when apoptosis occurs, expression of Shh ceases and its expression is undetectable in the quiescent telogen hairs (Botchkarev et al., 2001). Under physiological conditions, expression of FGF 7 and Shh is restricted to anagen phase of hair growth and BMP4 to telogen phase of hair growth.
The follicle counts in subcutis and skin thickness data is complemented by our immunohistochemical analysis for fibroblast growth factor-7 (FGF-7) and sonic hedgehog protein (Shh), which may serve as surrogate markers for anagen phase of hair growth. This is as opposed to bone morphogenetic protein 4 (BMP4), a marker for telogen phase of hair growth. The ethyl acetate enriched fraction EA01 containing two coumestans was screened for hair growth promoting activities. EA01 showed hair growth promoting activity at a concentration as low as 0.4 mg/15 cm². EA01 also showed induction of FGF7 and Shh antigen as surrogate markers. The mechanism of action for EA01 and EA02 as hair growth promoters was elucidated immunohistochemically for telogen to anagen switch in C57/BL6 mice. It was observed that the mechanism was modulated to great extent by increases in expression of FGF-7, Shh protein and decrease in BMP4 protein.

The plant extracts were analyzed to compare the extracts using antioxidant activity and SOD mimetic activity. The results of both assays were in agreement that the sub-fraction EA01 displayed highest antioxidant capacity, followed by EA02.

SOD mimetics like tempol, lamin and analogs of lamin like proziphen-N-proxiphen-N ("Prox-N") was variously reported for hair growth promoting activities. Topical SOD can be used alone or in combination with other hair growth stimulants or addittaments, which are available to enhance hair growth stimulation, like the hydroxyl radical scavengers, antiandrogens. SOD mimetics like orgotein and M40403 are also reported in other therapeutic areas.

Both ABTS and DPPH assays measure the total antioxidant activity (TAA) of the extracts on organic medium (all the extracts are prepared in methanol and ethyl acetate), which is neither an indication of total antioxidant activity of the plant / plant part, nor is it an indication of the antioxidant activity of the plant extract in-vivo. As most of the plants were extracted with methanol, many other antioxidants, which were insoluble in methanol and ethyl acetate, might not have been extracted from the plant. The method of heating during extraction procedure as well as consumption of the plant might also have
effects on the amount of useful antioxidants obtained from \textit{in-vivo}, thus the results of these tests were limited.

In conclusion the results suggest that the plant extracts, EA01 and EA02 can be the potential candidates for treatment of different kinds of alopecia. The plant extracts are much safer when compared to minoxidil and finasteride, which has numerous toxicities associated with. In a nutshell with the concerns in mind, to arrive at a drug, which can be much safer to the humankind, we suggest the plant extracts, EA01 (ethyl acetate extract of \textit{Eclipta alba}) and EA02 (methanolic extract of \textit{Eclipta alba}) as the potential candidates.