CHAPTER V

DISCUSSIONS

Hair loss is a distressing condition for an increasing number of men and women. Therefore, to develop new therapies for the treatment of hair loss is of great importance. Furthermore, many human diseases are associated with hair loss. There are many causes of hair loss including diseases, nutritional deficiency, aging, hormone imbalance and stress. Many hair promoting agents focus in inducing anagen from telogenic hairs. Despite availability of several 'anti-hair loss' agents, convincing evidence-based medicine is still exception in the market. Till date, just two anti-hair loss drugs (dihydrotestosteron-suppressing 5α-reductase inhibitor, finasteride and the anti-hypertensive potassium channel opener, minoxidil) have been approved by FDA[215]. Conventional anti-hair loss drugs such as finasteride and minoxidil limit their therapeutic use due to the undesirable side-effects and low cure rate[216-217]. Given the limited, transient and somewhat unpredictable efficacy of these approved anti-hair loss medicines, it is important to develop novel pharmacological treatments and agents. These factors lead to the search for novel drugs that revitalize the hair growth with less adverse effects. So screening of medicinal plants and isolation of potential phyto-

compounds as a hair growth promoter was brought into limelight. Attempt being focused to discover effective phyto-compounds from traditional herbal medicines, which prevents hair loss and also regenerate hair follicles[218]. For the purpose, traditionally known medicinal plants were selected, phyto-compounds were isolated, purified and their efficacy as hair follicle regenerator and growth promoter studied.

5.1. Morpho-phenological characterization of the plants selected

5.1.1. *E. alba*

Morphological data (Table 4.1a) revealed that the plant possesses dark-brown stem with average height 40.4 ± 3.36 cm and width 4.06 ± 0.69 cm, covered with whitish hairs. Each plant bears 15-20 leaves, length ranges from 2.56 ± 0.67 - 3.53 ± 0.85 cm and breadth varies between 1.29 ± 0.24 - 1.69 ± 0.25 cm. Leaves are sessile, lanceolate, distantly toothed, narrowed and pointed at both ends. The phenological data (Table 4.1b) revealed that, flowers are white in colour, axilliary, tubular, 6 sepals present in each calyx; flower heads are sub-globose and small, diameter of the flower varies between 0.63±0.16 and 1.02±0.34 cm and flower length varies between 1.0±0.09 and 1.3±0.14 cm. The obtained morpho-phenological data of *E. alba,* is in agreement with the characters described by Kanjilal and Bor[219].

5.1.2. *A. barbadensis*

Data obtained (Table 4.2) on morphological characters of *A. barbadensis* revealed average height to be 56.4 ± 6.35 cm with 10.8 ± 2.77 cm girth. A plant possesses 20-25 leaves with the average length varying within 41.76 ± 3.94 - 50.5 ± 6.08 cm and breadth from 5.12 ± 1.11 to 9.7 ± 0.88 cm. White spots were observed in young leaves, which disappears in the old leaves. Leaves are convexed in the middle with saw like teeth present in the margin. Panda[220] reported similar data for the plants of *A. barbadensis*.

5.2. Genomic study of the plants

5.2.1. DNA isolation of plants

The genomic DNA from *E. alba* and *A. barbadensis* was isolated using Doyle and Doyle[210] protocol with slight modifications. In this study tender leaves were used having high cell density and less polysaccharides[221].The protocol involves repetitive washing with chloroform: isoamyl-alcohol (24:1) to remove the aqueous phase. The ice-cold isopropanol was added and it was kept overnight at room temperature to precipitate the DNA. 1.5M NaCl solution was used in the experiment to remove polysaccharides by increasing their solubility in isopropanol so as to bind and precipitate DNA. Warude *et al.*[222] reported that 1.5 M NaCl was effective in removing polysaccharides. Also it helps to modulate the cation concentration in the extraction buffer[223]. For better yield and purity, RNase was added in the reaction mixture and incubated at 37°C for 1 h. Further to

separate polysaccharides from the DNA, CTAB is used as a detergent in the extraction buffer[224]. Richards *et al.*[225] suggested that the polysaccharides present in the cell may interfere with biological enzymes such as polymerases, restriction endonucleases and also ligases. According to Kawata *et al.*[223], CTAB was included in the extraction buffer as reagent for protein denaturation in the isolation process. Also to remove the magnesium ion, a necessary co-factor for nucleases, EDTA was included in the extraction buffer[223, 226]. Oxidations of polyphenols present in the plant crude extracts reduce the purity of the isolated DNA. For the purpose, β-mercaptoethanol was used as a strong reducing reagent and used to prevent the oxidation of polyphenols[223, 224, 226]. The purity of the isolated and purified DNA from *E. alba* and *A. barbadensis* were 1.81 and 1.83, respectively suggesting high-quality. Moreover, the molecular weights of the isolated DNA were much above 23 kb. The DNA yield was calculated to be 16.5 and 12.3 μg/g in the case of *E. alba* and *A. barbadensis*, respectively.

5.2.2. Genome size determination

Estimation of DNA content in cell nuclei is an important application in plant sciences which has mostly been done with flow cytometry. It is a well-accepted method for the determination of genome size and estimation of nuclear DNA content because of its accuracy and ease. The instrument can measure a large number of nuclei content from a small amount of tissue and the relative DNA content. The result of the analysis is usually displayed in the form of a histogram of relative fluorescence intensity, representing relative DNA content. The genome

size of an unknown sample can be determined only after comparison with the nuclei of a reference standard[227]. Flow cytometry has two key advantages, first a large number of cells/particles can be evaluated in a very short time, which makes the results statistically strong and representative of the whole population. Even at rates up to 1,00,000 cells/second, approximately 20 parameters from each cell/particle can be collected and analyzed. The second key advantage is the ability to physically separate single cells from mixed populations at rates up to 70,000 cells per second[228]. In the present study, Propidium Iodide (PI) was used as flurochrome for measuring the nuclear DNA content and the genome size of both plants. PI-based flow cytometry produced consistent result based on Feulgen micro-spectrophotometry[229]. For nucei isolation, Otto buffer was used which is phosphate/citric acid buffer, having pH 7.3 and it works well for separating nucleus and DNA. The isolated nuclei can be kept in Otto buffer at room temperature for prolonged time periods without negative influence on staining of DNA[227].

The most important criterion in genome size determination is, the correct choice of reference standard, which has largely been neglected[230]. *P. sativum* was selected as the reference standard for the flow cytometric analysis of the selected plants isolated cell nuclei. *P. sativum* is stable, easy to grow, and high quality nuclei suspensions can be prepared from leaves, which appear to be free from compounds interfering with PI staining[231- 232]. The 2C value of the nuclear genome of *P. sativum* is 9.09 pg[233] and is in the known range of genome sizes of

plants which facilitates calibration of reference standards with higher or lower genome sizes[227]. For the present investigation, the genome size of *E. alba* was shorter as compared to the genome size of *A. barbadensis*.

5.3. Isolation of the phyto-compounds

5.3.1. Isolation from *E. alba*

In the present investigation, phyto-compounds were isolated from *E. alba* using methanol and ethylacetate extract as the solvent. A number of compounds such as wedelactone, dimethyl-wedelactone, demethyle-wedelolactone, strycholactone[234], β-sitosterol, triterpenoids[235], steroidal alkaloids verazine, dehydro-verazine ecliptalbin[236], hydrocarbons like ecliptal, α-formyl-terthienyl[237-238] were isolated by several authors. *E.alba* is a traditionally known medicinal plant containing triterpenes like saponin, eclalbatin, α–amyrin, β-amyrin, ursolin acid, oleanolic acid and wedelic acid[239]. In the present investigation, compound Ea 1 was isolated from methanolic extract of the plant and identified as saponin. Another compound Ea 2 was isolated from the ethylacetate extract of *E. alba* and identified as aliphatic compound. Isolation of saponin compounds from *E. alba* were previously reported by several authors[240-241]. Saponins are widely distributed in the plant kingdom and display several biological properties[242-245]. Some investigations[242-243] reported that saponins isolated from plants depending on their structure, possess antimicrobial activity, principally against plant pathogens, some yeasts pathogens and also humans[246-249].

Various pharmacological uses of saponins as antibacterial, antifungal, antitumor, antiviral, hepatoprotective, anti-inflammatory and anti-ulcer compounds were reported[250-258]. Pharmacological use of plant based aliphatic compounds was also reported by various authors[259-260]. However, isolation of aliphatic compounds from *E. alba* and its biochemical activities were not reported till date. The present investigation reports the isolation of aliphatic compounds from *E. alba* from the first time.

5.3.2. Isolation from *Aloe barbadensis*

A. barbadensis contains various chemical compounds such as anthraquinones (aloe-emodin, aloesaponarin), anthranols (aloesaponol I-IV), anthrones (aloin A and B, aloe emodin), chromones (aloesin, neoaloesin, isoaloesin) [260-262]. In the present investigation, compound Av 3 and Av 4 isolated from *A. barbadensis*, were identified as pyrone. Although pyrone compounds derived from *A. barbadensis* were previously reported[260], however information on pharmacological activities of the compounds is very limited.

5.4. Chemical characterization and Structure elucidation of compounds

5.4.1. *Eclipta alba*

The methanolic extract of *E. alba* yielded the fraction Ea 1 which appeared brownish in color and sticky. The IR spectrum of the compound possessed the absorption bands at 3410.97, 2925.35–2857.04, 2194, 1414.63, 1258.49 and 1,166.26 cm^{-1}. The absorption at 2925.35−2857.04 suggests the presence of

symmetric and asymmetric –CH_3 stretch and peak at 2194.12 indicates –C≡C– stretch. Absorption peak at 1564.52−1414.63 pointed towards the presence of aromatic ring and COO^- stretching and 1258.49-1166.26 suggested for bridging −O− stretch and also for –CO stretch. The proton NMR spectrum of the compound Ea 1 in Fig.4.12a indicates the presence of aliphatic methyl groups at the range of δ 0.79-2.00. The presence of −CO and −O− stretch points at δs 2.55, 3.34 and 3.6 with a hydroxyl group at δ 3.17. Though, aromatic rings were observed in the carbon NMR spectra at δ 139.3 along with an alkene (C=O) group at δ 114.08. In the range of spectra between δ8-30, the presence of methyl group was shown whereas δ 20-60 indicates the presence of –CH group. On the other hand, presence of alkanes and aldehydes were significant at δs 14.1, 22.7, 28.98, 29.37, 31.95 and 33.83 in the spectra (Fig.4.12b). The proton and carbon NMR spectra of compound Ea 1 support the FTIR spectra of the compound. Among all the saponins isolated from *E. alba*, the present purified saponin was found to be similar with 'Eclalbasaponin'. The molecular formula of Ea 1 was found to be $C_{32}H_{62}O_8$ with mass value 620.3 obtained from the spectral analysis.

Another compound Ea 2 separated from ethylacetate extract of the young leaves of *E. alba* was in powder form and dark green in colour. The isolated fraction showed absorption peaks at 3390.3 for –OH stretching, 2922.2 and 2851.3 signifying —CH_3 stretching (—CH). Absorption at 1738.8 is due to the C=O stretching vibration; also 1458.1 and 1027.6 indicate the presence of COO− group in the compound. The fraction Ea 2 is an aliphatic compound with aromatic rings

present at δs 8.08, 8.2 in proton NMR along with carbonyl group at δ 206.8. In addition to carbonyl group, other functional groups such as CH and CH_2 groups are also present in the compound. The ^{13}C NMR data of Ea 2 presented important signals for vinylic carbons at δs 130 and 128.2, for methyl carbon at 14.2 and for methylene carbons between 34.01 and 22.71. The absence of signals between δ5.32 to 2.82 in proton NMR spectrum and between δs 128 to 34.01 in carbon NMR spectrum ruled out the presence of any carbinol carbon in the molecule. The probable molecular formula of the compounds was found to be $C_{15}H_{28}N_2O_2$ with mass value of 268.22.

5.4.2. *Aloe barbadensis*

The compound Av 3 isolated and purified from young shoots of *A. barbadensis*, was found to be yellowish and sticky. The IR spectra of the compound showed its absorption at 3397.5 and 2940.8cm^{-1}indicating the presence of –OH and asymmetric −CH stretching. Absorption at 1642.5 refers to −C=O group and on the other hand absorption at 1412.5 and 1059.7 cm^{-1} pointed towards symmetric COO^- and −CO stretches. The functional groups present in IR spectra (Fig.4.17) are also supported by the NMR spectra of the compound (Fig.4.18a and 4.18b). The presence of aliphatic groups like $–CH_3$ and $−CH_2$ in δ1.26, 2.04, 2.17 and 2.6 was observed in the proton NMR of the compound Av 3. The spectra indicate –OH at δ 4.97 and ─C=O−Me at both δs 4.23 and 3.46 peaks. The presence of carbonyl group and aromatic rings was showed at δ 207.67 and δ 171.46 whereas methyl groups were indicated at δ 8-30 ppm in the ^{13}C NMR spectra of Av 3. The

compound was identified as pyrone and the molecular formula of the compound was calculated to be $C_{13}H_{18}O_4$ with mass value 238.12 which also indicate compound Av 3 to be a novel compound.

The compound Av 4 exhibited IR absorption bands due to the presence of conjugated esters or lactone group (1642 cm^{-1}) and asymmetric –CH stretching (2941.2 cm^{-1}). Also, other functional groups such as –OH,−CH_3,−COO and CO− were also observed in the IR spectra of Av 4. The proton NMR of Av 4 indicates the presence of aromatic methyl group at δ 2.19 and also δs 3.86 (OMe), 5.47 (C=CH−), 6.15 (C=CH−) and 6.45 (arom H). Accordingly, the spectrum showed the presence of aromatic methyl group along with a methoxyl and four aromatic protons. The carbon spectra of the compound Av 4 showed the presence of aromatic ring at δ 167.2 along with ketone carbonyl group at δ 192.1. However, other than the aromatic methyl group, other groups like CH_2OH and CO were also found to be present in the compound. According to the spectra, the compound Av 4 was found to be similar with aloenin which was also supported by Suga *et al.*[263] and Hirata and Suga[264]. The probable molecular formula of the compound is derived as $C_{19}H_{22}O_{10}$ with mass value 262.26.

5.4.3. Structural similarities of minoxidil with the phyto-compounds Ea 1 and Av 4

The isolated phyto-compounds Ea 1 (eclalbasaponin) from *E. alba* and Av 4 (aloenin) from *A. barbadensis* exhibited good hair regenerating quality in comparison to the synthetic drug minoxidil which is known to activate the hair

follicle cells. The structure of minoxidil compound is shown in Fig.4.18. There are some structural similarities observed between minoxidil with Ea 1 (eclalbasaponin) and Av 4 (aloenin). The presence of two or more aromatic rings was observed in both Ea 1 (eclalbasaponin) and Av 4 (aloenin) as well as in minoxidil. Also, the presence of two or more hydroxyl and methyl groups were noted in Ea 1 (eclalbasaponin) and Av 4 (aloenin), but minoxidil does not possess any ─OH or ─CH_3 group. From the molecular formula, it is observed that minoxidil is a small molecule with nine carbons whereas Ea 1 (eclalbasaponin) and Av 4 (aloenin) are comparatively larger molecules with 32 and 14 carbon molecules, respectively. Molecular weight of minoxidil is also low as compared to the Ea 1 (eclalbasaponin) and Av 4 (aloenin).

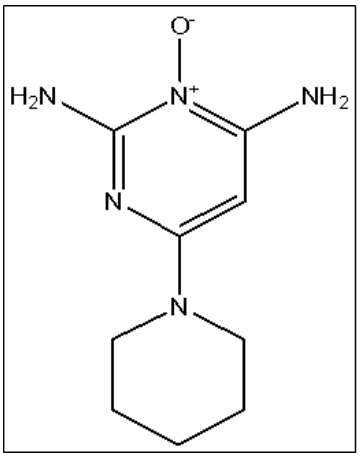

Fig.5.1. Structure of Minoxidil[265]

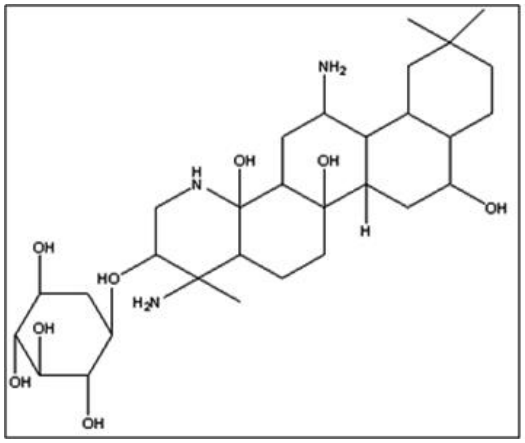

Fig.5.2. Structure of Ea 1

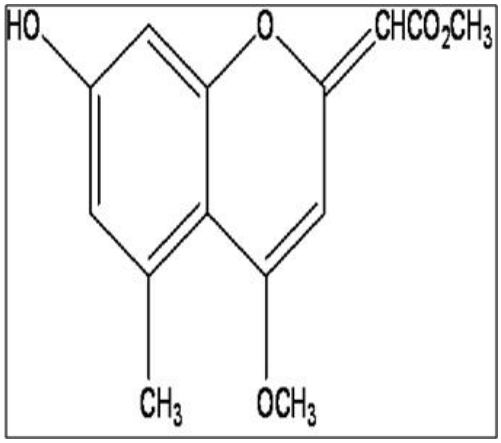

Fig.5.3. Structure of Av 4

5.5. Biochemical assessment of the phyto-compounds

5.5.1. Microbial assay

5. 5.1.1. Antibacterial activity

Antibacterial activity of *E. alba* extracts and its chemical compounds were already reported by various authors. Karthikumar *et al.*[266] determined the antibacterial activity of the various solvent extracts of *E. prostrata* leaves and reported significant activity against a variety of gram positive and gram negative bacteria. Bakht *et al.*[267] reported the inhibitory activity of petroleum ether, dichloromethane, ethyl acetate, butanol, methanol and water extracts of *E. alba* against *B. cereus* by the well diffusion susceptibility assay. In the present investigation, compound Ea 1 (eclalbasaponin) was obtained from the methanolic extract of *E. alba* which showed good antibacterial activity against *B. subtilis*

(MTCC 619) followed by *E. coli* (MTCC 739), *P. aeruginosa* (MTCC 7815), *K. pneumonie* (MTCC 109) and *S. aureus* (MTCC 737). There are several reports on the antimicrobial activity of the plant extracts due to the presence of saponin[268]. There may be several reasons for the differences in the antibacterial activity of saponin towards the different bacterial strains such as: (a) degradation of saponin by some glucosidase enzymes produced by gram-negative bacteria, (b) differences in the cell envelope structure and (c) the variation in the chemical structure of saponins. Variation in the structure of saponin such as glycone side chains in terms of number, chemical composition and specific point of attachment to the steroid or triterpenoid nucleus is critical for the saponins biological effects[269]. The degree of growth inhibition by saponin was much higher towards the gram-positive bacteria used in the test as compared to the gram-negative bacteria. Avato *et al.*[270] reported that the aglycone part of saponin is responsible for their antibacterial activity. Their studies also suggested that the sugar moiety is not important for the antimicrobial efficacy. Other than saponin, the aliphatic compound Ea 2 also showed moderate inhibitory activity against both Gram positive and Gram negative bacterial strains. Antibacterial activity of the aliphatic compound isolated from *E. alba*, has not been studied till date against any microorganism. The purified compound Ea 2 showed its highest antibacterial activity against *B. subtilis* (MTCC 619) followed by *S. aureus* (MTCC 737). But no inhibitory effect was observed against *K. pneumonia* (MTCC 109).

Antibacterial activity of *A. barbadensis* extracts and their different chemical compounds were previously studied and reported by various authors[271]. Ahmed *et al.*[272] reported that ethylacetate, hexane, petroleum ether and ethanol extracts of *A. barbadensis* possess antibacterial activity against various gram positive and gram negative bacteria such as *B. subtilis* (MTCC 619), *E.coli* (MTCC 739), *S.aureus* (MTCC 737), *P. aeruginosa* (MTCC 7815), *K. pneumonia* (MTCC 109). In this investigation, the isolated compound Av 3 from *A. barbadensis* showed moderate inhibitory effect against Gram positive and Gram negative bacteria. The highest antibacterial activity of Av 3 was observed against *E. coli* (MTCC 739) followed by *B. subtilis* (MTCC 619). But the compound showed no inhibitory effect against *K. pneumonia* (MTCC 109). The other *A. barbadensis* isolated compound Av 4 (aloenin) showed strong antibacterial activity against both Gram positive and Gram negetive bacteria. This compound showed its maximum inhibitory effect against *E. coli* (MTCC 739) and *B. subtilis* (MTCC 619) followed by *P. aeruginosa* (MTCC 7815), *S. aureus* (MTCC 737) and *K. pneumonia* (MTCC 109). Aloenin was previously isolated by Suga *et al.*[263], but the antibacterial activity of the compound was not studied till date. The present investigation reports the antibacterial activity of aloenin for the first time. Other than aloenin, other pyrone compounds isolated from *A. barbadensis* are aloenin B, aloenin-2´´-p-coumaroyl ester, and 10-O-β-D-gluco-pyranosyl aloenin[273, 274].

In this study, MIC and MBC experiments revealed the minimum concentration at which the isolated compounds acts as bactericidal and bacteriostatic agent against

Gram positive and Gram negative bacteria. Such a bacteriostatic agent restricts the bacterial growth by interfering with protein synthesis, DNA replication, and other cellular metabolism of bacteria. Since complete killing has occurred at higher concentration, it is marked as bactericidal in nature at higher concentration. The degree of growth inhibition by the saponin was much higher towards Gram-positive bacteria as compared to Gram-negative bacteria used. Among all the tested compounds, compound Ea 1 (eclalbasaponin) showed lowest MIC and MBC values against *B. subtilis* followed by Av 4 (aloenin).

The results of antibacterial activity of all the purified phyto-compounds showed that the degree of growth inhibition was much higher in the case of gram positive bacteria as compare to the gram negative bacteria. The reason for the difference in the sensitivity between gram-positive and gram-negative bacteria could be ascribed to the morphological differences between the micro-organisms. Gram-negative bacteria contains an outer phospholipid membrane carrying the structural lipo-polysaccharide components which makes the cell wall impermeable to lipophilic solutes, while porins constitutes a selective barrier to the hydrophilic solutes with an exclusion limit of about 600 Da[275]. The gram-positive bacteria are more susceptible having only an outer peptidoglycan layer which is not an effective permeability barrier[276]. This observation was also supported by Karthikumar *et al.*[266] and Khanna and Kannabiran[277].

5.5.2. Antifungal

Antifungal activity of all the purified phyto-compounds was studied against *F. oxysporium* (MTCC 7392) and *C. albicans* (MTCC 227). Antifungal activity of different extracts of both the plants and their various fractions were studied by various authors. *E. alba* plant extracts were tested against both *F. oxysporium* (MTCC 7392) and *C. albicans* (MTCC 227) by Bakht *et al.*[267]. Compound Ea 1 (eclalbasaponin) isolated from *E. alba*, showed good antifungal activity against both the tested fungal strains. Several reports are available on plant saponins possessing significant antifungal activity[250, 278, 279]. The antifungal activity possessed by both eclalbasaponin and aloenin is reported here for the first time through the present investigation. The mechanism of the antifungal activities of saponins seems to be through the disruption of fungal membranes. This finding is in agreement with that of Keukens *et al.*[280]. According to them, antifungal activity of saponins apparently involves their ability to form complexes with the sterols present in the fungal membranes and causes loss of membrane integrity. Saponin derivatives can also inhibit the growth of *C. albicans* (MTCC 227)[281-282]. Another compound Ea 2 isolated from *E. alba*, showed weak inhibitory effect against both the test fungal species. Antifungal activity of the aliphatic compounds isolated from the *E. alba* were not studied or reported till date.

In the case of compounds Av 3 isolated from *A. barbadensis*, exhibited very less or no antifungal activity against both *F. oxysporium* (MTCC 7392) and *C. albicans* (MTCC 227). However, the other compound Av 4 (aloenin) isolated

from the same plant, showed good antifungal activity against both the fungal strains. The antifungal activities of the various extracts as well as chemical compounds from *A. barbadensis* extracts were studied against both the fungal species by various workers[283-285]. All the phyto-compounds showed better inhibitory effect against *C. albicans* (MTCC 227) than *F. oxysporium* (MTCC 7392) and Av 4 (aloenin) showed highest antifungal activity among all the phyto-compounds.

5.5.3. Antioxidant assay of the isolated compounds

The importance of reactive oxygen species (ROS) and free radicals has attracted increasing attention over the past couple of decade. ROS which include free radicals such as superoxide anion radicals (O_2-), hydroxyl radicals (OH−) and non-free radical species such as H_2O_2 and singlet oxygen ($^1O_2$) are various forms of activated oxygen. ROS is continuously produced during normal physiological and biochemical events and they can easily initiate the peroxidation of membrane lipids, leading to the accumulation of lipid peroxides. Under pathological conditions, ROS is overproduced and results in oxidative stress making the disease condition more inferior. There are a lot of antioxidants that are introduced to minimize the adverse effect of ROS[286]. Antioxidants have been reported to prevent oxidative damage by scavenging free radicals and ROS, which may increase the occurrence of disease such as cancer and aging. DPPH is a relatively stable and free radical. The phyto-compounds reduce DPPH to hydrazine by reacting with the hydrogen donors. DPPH can convert the unpaired reactive

oxygen species and the resulting solution becomes discoloured stoichiometrically depending on the number of electrons take part[287]. The DPPH free radical scavenging activity of the purified phyto-compounds was evaluated using methanolic solution of the chemical compound. A freshly prepared DPPH solution exhibits a deep purple colour with a maximum absorption at 517 nm. The color disappears as and when an antioxidant is introduced into the medium. Antioxidant molecules can quench DPPH free radicals and convert them to a colourless product, resulting in a decrease in absorbance. The effect of antioxidants on DPPH radical scavenging activity was assumed to be due to their hydrogen-donating ability. The reduction capability of DPPH radical is determined by the decrease in the absorbance at 517 nm induced by the antioxidants. Usually the phyto-compounds and their purified components are able to reduce the radical DPPH to the yellow-coloured diphenyl-picryl-hydrazine[288]. The free radical scavenging activity of DPPH is directly proportional to the concentration of the tested compound. The concentration of the sample compound at which the inhibition percentage reaches 50% is known as IC_{50} value. This IC_{50} value is negatively related to the antioxidant activity as it expresses the amount of antioxidant needed to inhibit the radical concentration by 50%. The lower the IC_{50} value, the higher is the antioxidant activity of the tested sample. Among all four phyto-compounds isolated from *E. alba* and *A. barbadensis* (Ea 1, Ea 2, Av 3 and Av 4), the compound Ea 1 (eclalbasaponin) showed the highest free radical scavenging activity. Saponins have been reported

to have potent antioxidant activity[289]. They could act as radical chain terminator, transforming the reactive free radical species into more stable non-reactive products. The antioxidants break the free radical chain by donating a hydrogen atom[290]. From Fig.4.25 it was observed that the compound Ea 1 (eclalbasaponin) has highest radical scavenging activity, followed by the compound Av 4 (aloenin). On the other hand, Ea 2 showed moderate antioxidant activity with 51.23% and Av 3 showed 15.67% scavenging activity. The DPPH scavenging activity of all the four compounds was studied with respect to the standard quarcetin and gallic acid. The compound Av 3 with the highest IC_{50} value exhibited the lowest antioxidant activity and the compound Ea 1 (eclalbasaponin) exhibited the lowest IC_{50} value among all the isolated phyto-compounds.

5.5.4. Cytotoxicity assay

In the present investigation, all the phyto-compounds isolated from *E. alba* and *A. barbadensis*, exhibited no inhibitory effect against the macrophage cell line (RAW 264.7) suggesting their non-toxic activity as compared to the positive control canamycin upto a concentration of 100mg/ml. The cytotoxic effect of the low molecular weight fractions isolated from the plant *A. barbadensis* was already reported by Avila *et al.*[291], but the crude extract of the plant failed to possess cytotoxic effect[292]. Also, the cytotoxic effect of *E. alba* extracts and their constituent compounds were studied by different authors[293]. The LD_{50} values of the isolated phyto-compounds were found to be much higher as compared to the standard drug kanamycin which also suggested the non-toxic nature of the phyto-

compounds. For the first time, cytotoxic study of *E. alba*-derived saponin and aldehyde are presented through the present investigation. Also, *A. barbadensis* isolated pyrone compounds were studied against the RAW 264.7 for the first time.

5.6. Acute dermal irritation study of the phyto-compounds

Acute dermal irritation study was performed in the present investigation to analyze the *in-vivo* effects of the phyto-compounds to ascertain whether the compounds may potentially be used for topical applications without damaging the skin. In the present study, transdermal patches and the placebo patches in rabbits showed no dermal responses including erythema or edema, as compared to the negative control animal models. It is also evident from the results of acute dermal toxicity study that none of the animals in each group showed any clinical signs (like making noise immediately after treatment, struggling etc) and any overt signs of toxicity from the first day until the end of the experiment. To date, toxicological effects of eclalbasaponin and aloenin were not studied. Because of the importance of dermal applications of the compound, all the phyto-compounds were tested for acute dermal irritancy in rabbits. Previously, Roy et al.[155] and Datta et al.[186] studied the dermal irritancy of various *E. alba* extracts on animal models to determine their toxic effect on mammalian skin. Although, acute dermal irritancy tests for various other plant isolated compounds including *A. barbadensis* which are known for their effects on hair growth promotion, were not reported[20, 72, 98, 100, 109]. The body weights of the phyto-compound treated groups did not differ significantly from the control group. Hence, it can be inferred that

all the phyto-compounds have no tendency to cause drastic tissue destruction and they do not interfere in the absorption of nutrients. Food and water consumption among the treated and the control groups of animals were almost similar without any significant difference. In this investigation, none of the phyto-compounds applied to albino rats caused erythema, redness, drying, scaling or any other side effects. As all the isolated compounds were non-toxic to the mammalian skin, they can be used in cosmetics or other related products.

5.7. Inducing alopecia in wistar albino rats

Many workers studied the hair growth promoting effect of synthetic drugs or phyto-compounds on alopecia induced animal models. Fuzzy rats, macaq monkeys, C3H/HeS1C rats, C3H/He rats, C57/BL6 rats, swiss albino mice, sprague-dawley rats, albino mice were widely used animal models for hair growth related experiments[102,184,192,195,293]. Wistar albino rats are one of the most extensively used animal models for the hair growth promoting experiments[72, 86, 184]. The disadvantages associated with the rat model include a high follicle density and the fact that the rodent hair cycle progresses in a wave pattern[271], unlike that of the mosaic pattern observed in humans[294]. However, the periodic intervals of hair cycles, particularly the duration of the anagen phase are much more consistent in rat models as compared to others[293]. Although the rats do not undergo a 'vellus-to-terminal' hair or suffer from androgenic alopecia, it is an excellent model system for studying the hair cycle for several reasons. The first two cycles of the rat hair follicle are synchronized whereas in humans, when

biopsies are taken, the neighbouring hair follicle cycles are independent of each other. Secondly, the rat hair cycle is short, nearly 3 weeks but the human scalp hairs have a time cycle of several years and even vellus hairs take months. The short synchronized hair cycle thus allows hair follicles to be harvested and thus the growth of the follicle at specific time points in the cycle can be examined. In addition, the stages of the hair cycle have been well characterized in the rats. Anagen is sub-divided morphologically into six and catagen into eight different stages[23]. However, there is no evidence to suggest that the rat hair follicle cycle differs structurally in any way from the human hair follicle cycle, except for the specialized vibrissae follicles in which the follicle does not retract and only the bulb appears to be remodelled during catagen[3]. For these advantages, the wistar albino rat was selected as the animal model for studying induction of alopecia and then regeneration of hair follicles by treating with the phyto-compounds.

Alopecia can be induced by applying testosterone in the rat model[110]. Other than testosterone, there are many other drugs available in the market for the various other diseases which may also cause alopecia as a side effect in the case of patients taking these drugs. Among them chemotherapy drugs, hypertension, psychotropic drugs, antithyroid drugs, retinoids, antineoplastic agents are known to cause alopecia in patients as side effect[295-296]. The association of hair loss and anticoagulant like heparin, heparinoid, warfarin etc are already known for more than 30 years as reviewed on complications of the anticoagulants treatments[297]. Earlier reports established a high incidence (42-78%) of association of the oral

anticoagulants on alopecia. Anticoagulants such as heparin and heparinoid drugs primarily affect scalp hair. Hair loss is generally observed after 3 months from the initiation of the treatment and varies from 7 days to 90 days from the last dose. Although the unfractionated heparin has been the primary anticoagulant linked to drug-induced alopecia; but low molecular weight heparin and warfarin implicated as the causative agents[298-300]. The incidence of alopecia has been observed in patients using warfarin. The regeneration of hair after discontinuation of the drug is observed in some cases but, in most of the cases the regeneration takes too long time if continuation of warfarin is necessary in case no other oral substitution of anti-coagulants is available in the market having no side-effects[301]. Warfarin typically induces mild and diffused alopecia in 3-20 weeks after the initiation of the therapy. In most of the patients, alopecia as the side effect following the application of warfarin goes usually unnoticed. However, 20% patients experience hair loss associated with the use of the drug exhibiting clinically evident alopecia. The scalp hair is the primary target of this disease though eyebrows, then auxiliary and pubic hairs are affected. Telogen effluvium is often observed in the case of treatment with warfarin, which may convert the hair follicles to a permanent resting phase[302]. In the present investigation, the use of warfarin at 1.7 mg/kg to induce alopecia without causing any side-effect in the animal model has been standardized.

5.8. Qualitative study of hair growth in wistar albino rats

Hair growth initiation and completion time was significantly reduced upon

treatment with the phyto-compounds isolated from *E. alba* and *A. barbadensis*. However, all the compounds were not equally effective in regeneration of hair follicles. Among them, only two compounds Ea 1 (eclalbasaponin) and Av 4 (aloenin) showed effectiveness in regeneration of hair follicles and hair growth in the first week. In case of the positive control group (minoxidil treated), hair growth was observed in the alopecia-affected areas in the second week. Similar study done by Roy et al.[155] reported that petroleum ether extract of *E. alba* completed the hair growth in Wistar albino rats on 19th day whereas for minoxidil treatment, the complete hair growth was observe on 19th day. Although similar kind of study is not reported in case of *A. barbadensis* extracts/isolated compounds. After 15 days of topical application, Ea 1 (eclalbasaponin) treated Wistar rats showed increment in length and weight of hair as compared to Av 4 (aloenin) and minoxidil treated Wistar rats. There was no treatment-related mortality in any of the groups of Wistar rats following patch administration in male and female rats for 15 days. There has been no gross toxicity, as revealed by no-loss of body weight of the treated rat models which established non-toxicity of the phyto-compounds as well as no side-effects in the treated rat models.

5.9. Haematology and biochemical analysis of serum

Both the phyto-compounds Ea 1 (eclalbasaponin) and Av 4 (aloenin) isolated from *E. alba* and *A. barbadensis*, were topically applied on wistar rats and their effects on haematological and biochemical parameters were assessed. The tested compounds did not show any significant changes on hematology and serum of the

treated wistar rats after 14 days of the treatment. The observed haematological values were within the normal range, hence it can be suggested that any serious haemato-toxicological implications would not persist during the topical application of these purified phyto-compounds. Among the haematological parameters studied (Table 4.11), the percentage of lymphocyte count of the treated groups was found to be lower as compared to the positive control. However, white blood cell (WBC) count was higher in the case of the phyto-compound treated wistar rats against both positive and negative control groups. All the other parameters including percentage of hemoglobin was similar for all the treated and control group animals. No adverse effects were observed in the levels of glucose (GLUC), cholesterol (Chol), creatinine (CRE), urea (UREA) and uric acid (UA) in serum after treating with the phyto-compounds in comparison to the negative control, thus confirming lack of nephrotoxic potential of the transdermal patches. Transaminases, that is, Serum Glutamic Pyruvic Transaminase (ALT/SGPT) and Serum Glutamic Oxaloacetic transaminase (SGOT) are good indices of liver damage. The treatments of the phyto-compounds did not affect the levels of transaminase indicating that the tested compounds possessed no negative impact on the liver of the rats. No similar studied has been conducted on the haematological and serum biochemical parameters of phyto-compounds isolated from *E. alba* and *A. barbadensis*. Also, haematological and serum biochemical effects of the isolated compounds (aloenin and eclalbasaponin) have not been reported till date.

5.10. Histological analysis

Histology study of the skin tissue sections made out of the control and treated Wistar rats showed varying results. The negative control group did not show any hair re-growth during the entire study period although both minoxidil and phyto-compound (eclalbasaponin and aloenin) treated Wistar rats showed regeneration of hair follicles as well as promotion of hair growth. The hair follicle regeneration was initiated on the 4^{th} day of topical application with Ea 1 (eclalbasaponin) treated Wistar rats and in the case of Av 4 (aloenin) treated group, it was observed after 6^{th} day of treatment. Histology of Ea 1 (eclalbasaponin) treated skin showed more number of hair follicles as compared to the Av 4 (aloenin) treated skin. The microscopic observations showed that the hair follicular density increased in the case of standard and the phyto-compound-treated groups in dose dependent manner (Table 4.13). The follicle density was found to be higher in the case of the Ea 1 (eclalbasaponin) treated Wistar rats skin; however, it was observed to be nearly similar in case of the Wistar rats treated with Av 4 (aloenin) and minoxidil. Previously, various researchers[155, 186] performed the histology of the animal model skin tissues after treating them with different *E. alba* extracts and the follicular density was counted by microscopic observation. However, no histological study was done for *A. barbadensis* extracts against alopecic animal models. Histology study for aloenin and eclalbasaponin compound treated alopecic animal model have not been reported till date.