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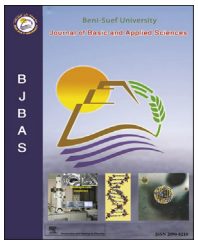

Full Length Article

A comparative in vivo and in vitro evaluation of hair growth potential of extracts and an isolate from petroleum ether extract of *Cuscuta reflexa* Roxb

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ABSTRACT

This study examined the inhibitory effect of Stigmast-5-en-3-O-glucopyranosidetriacetate-5[1]-ol (SGTA), an isolate from petroleum ether extract of *Cuscuta reflexa* and performed comparative study of petroleum ether extract (PTE), ethanolic extract (ETE) and SGTA on hair growth activity in androgenic alopecia rat model. Alopecia induced in albino rats by testosterone administration subcutaneously for 21 days. Finasteride solution was applied topically served as standard. *In vitro* experiment to study the effect of extracts and isolate on activity of 5α-reductase enzyme and comparing with finasteride. *In vivo* experiment showed that rat follicular density and anagen/telogen (A/T) ratio were increased in the PTE, ETE and SGTA treated group when compared to a control group. Skin histological results shown that the PTE, ETE and SGTA treated group had an increase in number and shape of the hair follicles and increase in the follicle anagen/telogen ratio when compared to the finasteride and control group. The result indicated that the ethanolic, petroleum ether extract and isolate of petroleum ether extract of *C. reflexa* found useful in the treatment of androgen-induced alopecia in the experimental animal. In summary, SGTA and extract control the apoptosis of hair cells and retarded the testosterone induce alopecia and therefore be a natural product with much impending for use as a treatment for androgenic alopecia.

1. Introduction

The term androgenetic alopecia frequently used to express the patterned loss of scalp hair in genetically vulnerable men and women. Androgenetic alopecia (AGA) is an androgen-driven condition in genetically prone individuals that affects half of the male population (Otberg et al., 2007). There are various genetic and environmental factors, which are engaged in causing AGA. In androgenetic alopecia miniaturization of

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genetically vulnerable terminal hairs into vellus hairs in the affected region, that is mainly drive by androgen. The key androgen is supposed to be an active metabolite of testosterone called dihydrotestosterone, rather than testosterone itself. The 5α-reductase type-2 isoenzyme is mainly responsible catalyst for the conversion of testosterone into dihydrotestosterone because of this it is main target for treatment of alopecia (Sinclair, 2005).

Presently available therapies for treatment and management of androgenetic alopecia are antiandrogens and biological response modifiers. On the other hand, the low success rate and related adverse effects confines their clinical use (Price, 1999). Nowadays natural products consider in cosmetics and about many kinds of extracts from plant investigated for hair growth activity. Various plants and herbal formulations reported as hair growth promoter as well as used for enhancement of hair quality in traditional Indian system of medicine, but being deficient in sound scientific support and information confines their use (Roy et al., 2005).

C. reflexa Roxb belongs to family Convolvulaceae. It is a parasite, perennial herbs with slender long yellow or golden stems. It is also called as dodder or akashabela or amarabela or swarnalata. It is widely distributed in tropical and temperate regions and common in all over India and Ceylon. It depends on host plants, mostly thorny herbs for growth and nutrition and sometimes entirely covering the bushes and trees (Dorr, 1990; Patel et al., 2012). Conventionally, it is mainly use as a purgative in the management of protracted fever, diaphoretic, and demulcent (Chopra et al., 1992; Kritikar and Basu, 1984). Antisteroidogenic properties of methanolic extract of *C. reflexa* have been reported (Gupta et al., 2003). In our earlier study, we reported the hair growth promotion of the petroleum ether extract of this herb on denuded skin surface of albino (Roy et al., 2006, Pandit et al., 2008). The present study, our investigation was an attempt to compared the efficacy of petroleum ether (PTE), ethanolic extract (ETE) and Stigmast-5-en-3-O-glucopyranosidetriacetate-5[1]-ol (SGTA), an isolate from petroleum ether extract of *C. reflexa* for promoting hair growth in testosterone-induced hair loss and to showed that the ETE and SGTA also inhibits conversion of testosterone to its more potent metabolite, dihydrotestosterone by inhibiting 5α-reductase type 2 enzymes thus inhibiting hair loss as PTE.

2. Materials and methods

2.1. Plant material and authentication

Stems of *C. reflexa* growing on *Bougainvillea spectabilis* and *Jasminum multiforum* were collected in the month of Nov–Dec 2010 from forests surrounding our university campus, Sagar and were authenticated by Dr. P.K Tiwari, Department of Botany, Dr. H.S.Gour University (Herbarium no. Bot/Her/2123). The plant material dried in sunlight and reduced to a coarse powder.

2.2. Extraction

Coarsely powdered stems of *C. reflexa* feed in a soxhlet apparatus and extracted with petroleum ether (60–80 °C) and with ethanol (95%) in separate assemblies to obtain petroleum ether extract and ethanolic extract respectively.

2.3. Chromatographic characterization

C. reflexa is a parasitic plant that draws its nutrients from the host. Phytoconstituents expected to show a discrepancy with the host, and it is accordingly desirable that material collected from the recognized host. For the present study, stems of *C. reflexa* collected from plants growing on the *B. spectabilis* and *Jasminum multiforum*. The Petroleum ether extract and ethanolic extract characterized by thin layer chromatography (TLC) on precoated silica developed in toluene/ethyl acetate (97: 3) and Chloroform: Methanol (9:1) as mobile phase respectively. The plates sprayed with anisaldehyde sulfuric acid reagent and heating at 105 °C for 10 min gave eight and four spot respectively.

2.4. Isolation, purification, and characterization of active compound

The isolation of compound done based on solubility. For isolation, the petroleum ether extract suspended in acetone and shaken strongly to dissolve the extract. The insoluble mass centrifuged at 2000 rpm, collected, and suspended into ethyl acetate later than this it separated into ethyl acetate soluble and insoluble fraction. Then, concentrated the ethyl acetate soluble fraction and it yielded yellowish white solid crystalline material after keeping in a refrigerator for about 12 h. It was further purified by crystallization; the isolated compound SGTA melted at 120 °C and gave an R_f value of 0.83. In solvent system toluene: ethyl acetate (97: 3) it gave a single spot. It gave Lieberman burchard test and positive Salwonski test that confirms the sterols moiety. Based on IR, NMR and mass spectroscopic data it confirms the presence of steroidal molecule (Fig. 1).

2.5. In vivo studies on hair growth

2.5.1. Animals

Male Swiss albino rats (6–8 months age, 130–140 g weight) used. The animals were adapted to conventional laboratory conditions with providing standard food and water. The photoperiod kept at 12 h of light and 12 h of darkness and temperature (25 ± 3 °C). All animal experimentation carried out after approval of the protocol by the Institutional Ethical

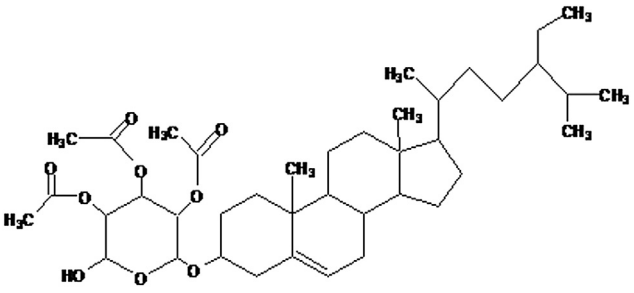

Fig. 1 – Stigmast-5-en-3-O-glucopyranosidetriacetate-5[1]-Ol.

Committee of Dr H.S. Gour University. The guidelines of CPCSEA (India) strictly followed.

2.5.2. Solutions

The vehicle used for dissolving the extracts, the isolate and the standard (Finasteride) was ethanol, propylene glycol and water (8:1:1). Testosterone administered as 1% solution in arachis oil. The concentration of petroleum ether, ethanol extract and Finasteride was 2% in the vehicle solution. The isolate used as 0.5% suspension in the vehicle. Another suspension of mixture of isolate and phosphatidylcholine (PC) (50-50%) in vehicle at concentration of 0.5% were prepared.

2.5.3. Treatment

Animals divided into 6 groups of four rats each. The following treatment given to animals of different groups:

Group I: Testosterone solution (s.c.) + Vehicle (Topically)
Group II: Testosterone solution (s.c.) + Finasteride solution (2%) (Topically)
Group III: Testosterone solution (s.c.) + Petroleum ether extract solution (2%) of *C. reflexa* (Topically)
Group IV: Testosterone solution (s.c.) + Ethanolic extract solution (2%) of *C. reflexa* (Topically)
Group V: Testosterone solution (s.c.) + Isolate suspension (0.5%) (Topically)
Group VI: Testosterone solution (s.c.) + Isolate and PC mixture (50-50-%) (0.5% suspension) (Topically)

The method reported by Matia's was followed with minor alteration (Matias et al., 1989, Pandit et al., 2008). Rats in all groups were administered testosterone subcutaneously (0.1 ml) daily. Animals of groups I, II, III, IV, V and VI were given topical application of vehicle, finasteride, petroleum ether extract, ethanolic extract, isolate and mixture of isolate + PC respectively.

Approximately 0.2 ml of the solution or vehicle topically applied on back skin once a day for 20 days. On 21st days, rat from each group selected randomly and sacrificed. The difference in growth of hair in each group noticed by visual observation and recorded by taking photographs. Skin biopsy also undertaken from the balding site of each group of rats, and samples of skin kept in phosphate-buffered formalin for paraffin sectioning. The cyclic phase of hair follicles (anagen and telogen), number of hair follicles were determined, and the anagen/telogen ratio was calculated with the help of ocular micrometer (Table 1).

2.6. In-vitro studies on enzymatic activity

2.6.1. Enzyme preparation

Dihydrotestosterone (DHT) plays an active responsibility in development and progression of Androgenetic Alopecia, prostate cancer, benign prostatic hyperplasia. Therefore, 5α-reductase enzyme is supposed to be in a larger quantity in prostate. Adult male goat prostate (7.5 gm) was homogenate with 30 ml of 20 mM sodium phosphate buffer solution at pH 6.5 containing 250 mmol/L of sucrose and 1 mmol/L of EDTA. The homogenate centrifuged at 5000 rpm for 20 min and supernatant taken as a source of enzyme (Pandit et al., 2008). The fresh suspension used during the reaction. Bradford method used to determine the concentration of enzyme in the suspension (Bradford, 1976).

2.6.2. Preparation of test materials

Testosterone (10 mg/ml), extracts (8.5 mg/ml), isolate (5 mg/ml) and finasteride (10 mg/ml) solution were prepared in ethanol (95%) with gentle heating wherever necessary. The EDTA solution (10 mg/ml) was prepared in distilled water.

2.6.3. Determination of optimum concentration of enzyme

It determined by keeping the concentration of substrate constant and varying the concentration of enzyme. Optimum concentration of enzyme is the point at which it attained highest velocity and highest free enzyme able to interact with substrate or at which enzyme demonstrate maximum activity.

Reaction mixture (1 ml) = Sodium phosphate Buffer (40 mM at pH 6.5) + Testosterone solution (1 mg or 0.1 ml) + Enzyme solution (0.1–0.9 ml).

The reaction mixture incubated at 37 °C for 1 h and adding 2 ml ethyl acetate terminated the reaction. The mixture shaken vigorously for 1 min and the ethyl acetate layer separated. These layers evaporated to dryness, and the residue then dissolved in 2 ml methanol. The methanolic solution followed by used for estimation of testosterone by high performance liquid chromatography (HPLC; Shimadzu, Column C 18).

Table 1 – Hair follicular density and A/T ratio in sections of skin of different groups of animals.

S.No.	Group no.	Treatment	Hair follicular density (no./mm)	Anagen to Telogen
1.	I	Testosterone (s.c)+ vehicle (topical) (Control)	1.5 ± 0.90	1:3.16
2.	II	Testosterone (s.c)+ 2% Finasteride solution (topical)	3.3 ± 0.77*	1.22:1
3.	III	Testosterone (s.c)+ 2% Petroleum Ether Extract of *C. reflexa* solution (topical)	2.5 ± 1.0**	1.6:1
4.	IV	Testosterone (s.c)+ 2% Ethanolic Extract of *C. reflexa* solution (topical)	2.41 ± 0.90**	1.5:1
5.	V	Testosterone (s.c)+ 0.5% suspension of Isolate of *C. reflexa* (topical)	2.91 ± 0.79***	1.63:1
6.	VI	Testosterone (s.c)+ 0.5% suspension of Mixture of Isolate and PC (topical)	3.25 ± 0.62*	1.85:1

Value are mean ± SD, $n = 12$.
$P < 0.001$*, $P < 0.05$**, $P < 0.01$***, significance versus control.
(A/T ratio: Anagen/Telogen ratio; S.C: Subcutaneous; PC: Phosphatidylcholine).

2.6.4. *Determination of inhibitory concentrations of extract and isolate*

The reaction mixture (1.5 ml) made by adding testosterone solution (0.1 ml), EDTA solution (0.1 ml), extracts/isolate/finasteride solutions (0.1–0.5 ml) for separate groups, optimum amount of enzyme solution (i.e., 0.6 ml), and sodium phosphate (20 mm) to a final volume of 1.5 ml. Reaction mixture incubated at 37 °C for 60 min and reaction terminated by addition of 3 ml of ethyl acetate. The mixture was vortexed for 1 min; ethyl acetate layer separated and evaporated to dryness. The residue dissolved in methanol and volume made up to 2 ml with methanol. HPLC used to determine the residual testosterone content in methanol. The column was eluted isocratically with a mobile phase of methanol/water (80: 20) at a flow rate of 1.0 ml/min (Purdon and Lehman, 1997).

2.7. *Statistical analysis*

All data presented as MEAN ± SD of at least three samples. Significant difference among groups determined using graph pad instat version 2 for windows.

3. Results

The herb in the present studies selected based on its use in traditional medicines for hair care. Stem of *C. reflexa* evaluated during present investigations. Based on *in-vitro* method results petroleum ether, ethanolic extract and isolate of *C. reflexa* tested in Testosterone induced alopecia model.

3.1. *Extraction*

The yield in the case of petroleum ether extraction was 3.0% w/w whereas 3.6% w/w in the case of ethanolic extraction. This recorded on elimination of solvent under reduced pressure and weighing its residue.

3.2. *In vivo studies on hair growth*

3.2.1. *Morphologic observation*

The animals of groups I showed diffuse alopecia. Loss of hair from the dorsal portion of rats was clearly visible after 20 days of treatment with testosterone (Fig. 2A). In animals of group

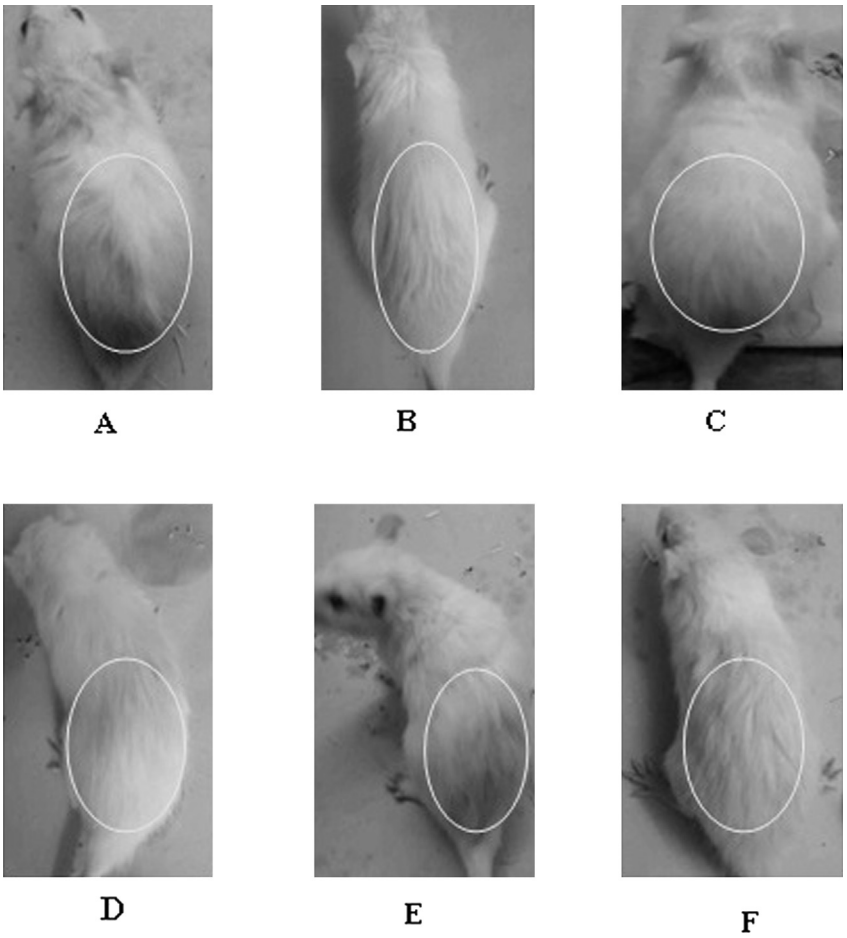

Fig. 2 – Comparison of baldness pattern in each group. (A) Animal treated with testosterone and vehicle showing diffuse alopecia. (B) Animal treated with testosterone and finasteride showing less hair loss. (C) Animal treated with testosterone and petroleum ether extract showing less hair loss. (D) Animal treated with testosterone and ethanolic extract showing less hair loss. (E) Animal treated with testosterone and isolate showing less hair loss. (F) Animal treated with testosterone and mixture of isolate and PC showing less hair loss. (PC: Phosphatidylcholine).

III, IV, V, VI, petroleum ether extract, ethanolic extract, isolate and mixture of isolate and PC respectively administered along with testosterone. The alopecic condition was not visible in this group of animals, showing that the extracts and isolate prevented the action of testosterone and blocked testosterone-induced hair loss (Fig. 2C, D, E, F). The observation was the same in animals of group II who received finasteride along with testosterone (Fig. 2B).

3.2.2. *Histological observation*

Histology of skin sections of group I animals shown that testosterone treatment caused miniaturization of hair follicles. Follicles of group-I showed characteristics of telogen follicles *i.e.* smaller in length, hollow, presence of necrosis, more destroyed follicles, follicle shrinks means diameter decreases and not deeper. Several hair follicles were in the telogen phase (Fig. 3A). The effect of testosterone on miniaturization of hair follicle was inhibited by administration of petroleum ether extract, ethanolic extract, isolate and mixture of isolate and PC in-group III, IV, V and VI animals respectively. The increase in number of hair follicles noted. The number of follicles in anagen phase was considerably increased and fewer follicles in telogen phase observed. Follicles of group-II, group-III, group-IV, group-V, and group-VI showed characteristics of anagen follicles *i.e.* Follicle hair longer in length follicles dense (number increases as compared to group I), less cell necrosis, and present deeper (Fig. 3B, C, D, E, F). In-group V and group VI number of follicle are more than group III and group IV. There was no significant change in epidermis and dermis cells. Again, the number of follicles in hair growth phase increased with duration of treatment. The Hair follicular density and anagen telogen ratio (A/T ratio) was calculated (Table 1).

3.2.3. *Hair follicular density*

The histological study showed that hair density was maximum *i.e.* 3.3 ± 0.77 in case of standard, 3.25 ± 0.62 in case of mixture of Isolate and PC treated, 2.91 ± 0.79 in case of Isolate treated, 2.5 ± 1 in case of petroleum ether extract treated, 2.41 ± 0.90 in case of ethanolic extract treated while it was minimum *i.e.* 1.5 ± 0.90 in testosterone and vehicle treated animals (Table 1).

3.2.4. *Anagen telogen ratio (A/T ratio)*

A/T ratio was maximum *i.e.* 1.85:1 in case of mixture of Isolate and PC treated, 1.63:1 in case of Isolate treated, 1.6:1 in case of petroleum ether extract treated, 1.5:1 in case of ethanolic extract treated, 1.22:1 in case of standard treated while it was minimum *i.e.* 1: 3.16 in testosterone and vehicle treated animals (Table 1).

The prostate gland weight found to be 0.1094 g, 0.0992 g, 0.1044 g, 0.0995 g, 0.1065 g and 0.0981 g of animal treated with Testosterone and vehicle, Finasteride, petroleum ether extract, ethanolic extract, Isolate and mixture of Isolate and PC respectively. Negligible change in prostate weights observed during in vivo studies (Table 2).

3.3. *Determination of IC 50*

The optimum amount of enzyme solution to give optimum activity found to be 0.60 ml (213.72 μg enzyme fraction). Varying concentrations of test substances incubated with a

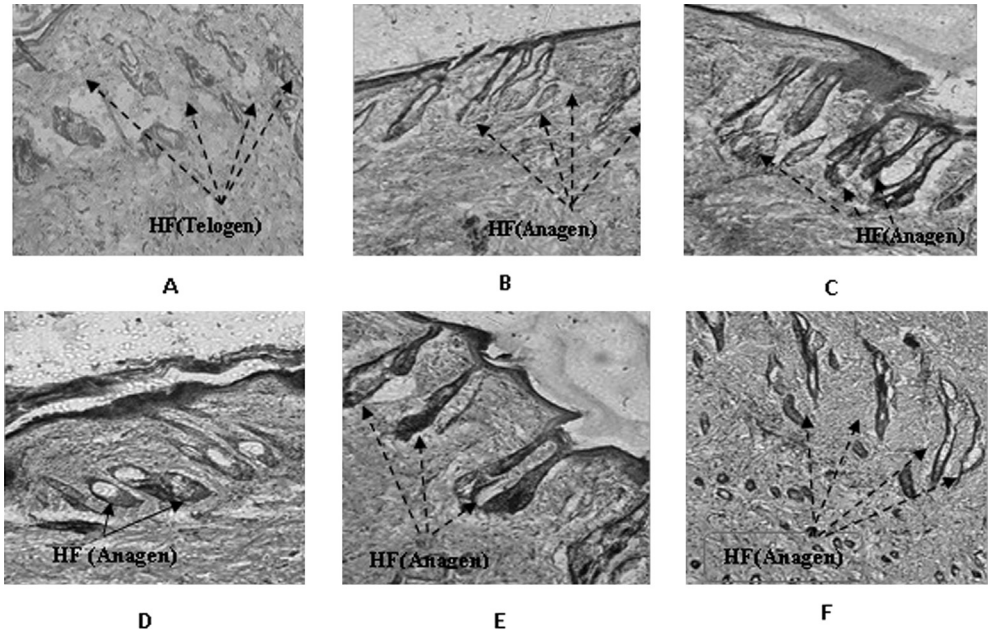

Fig. 3 – Photomicrograph of skin of animal in each group (A) Skin of animal treated with testosterone and Vehicle. (B) Skin of animal treated with testosterone and Finasteride solution. (C) Skin of animal treated with testosterone and petroleum ether extract solution. (D) Skin of animal treated with testosterone and ethanolic extract solution. (E) Skin of animal treated with testosterone and Isolate suspension. (F) Skin of animal treated with testosterone and mixture of isolate and PC. (PC: Phosphatidylcholine; HF: Hair Follicle).

Table 2 – Weight of prostate gland of animals of different groups.

S. No.	Group No.	Treatment	Weight of prostate (g)
1.	I	Testosterone (s.c)+ vehicle (topical)	0.1094 ± 0.002
2.	II	Testosterone (s.c)+ 2% Finasteride solution (topical)	0.0992 ± 0.001
3.	III	Testosterone (s.c)+ 2% Petroleum Ether Extract of *C. reflexa* solution (topical)	0.1044 ± 0.006
4.	IV	Testosterone (s.c)+ 2% Ethanolic Extract of *C. reflexa* solution (topical)	0.0995 ± 0.007
5.	V	Testosterone (s.c)+ 0.5% suspension of Isolate of *C. reflexa* (topical)	0.1065 ± 0.001
6.	VI	Testosterone (s.c)+ 0.5% suspension of Mixture of Isolate and PC (topical)	0.0981 ± 0.003

Values are mean ± SD, $n = 3$.
(S.C: Subcutaneous; PC: Phosphatidylcholine).

constant amount of testosterone and enzyme in reaction mixture, and the residual testosterone content was determined after termination of reaction with ethyl acetate. The residual testosterone content in reaction mixture increased with increasing concentration of ethanolic extract of *C. reflexa*, isolate, and finasteride. The IC_{50} values of extracts, isolate and Finasteride were determined from inhibition curves and stated as 1.06 mg/ml for petroleum ether extract, 1.51 mg/ml for ethanolic extract, 0.76 mg/ml for isolate and 1.81 mg/ml for Finasteride, providing enzyme inhibitory activity of these compounds.

The testosterone induced alopecia model was successful in bringing for the activities of test extracts. The relative efficacy of different material was as follows:

Mixture of Isolate from *C. reflexa* and PC > Isolate from *C. reflexa* > Petroleum ether extract of *C. reflexa* > Ethanolic extract of *C. reflexa*.

4. Discussion

Androgens are mediators of terminal hair growth all through the body. Androgenetic alopecia (AGA) is a dihydrotestosterone (DHT)-mediated process, characterized by continuous miniaturization of androgen reactive hair follicles and accompanied by perifollicular fibrosis of follicular units in histological assessment (Yoo et al., 2006). Alopecia induced in rat by administration of testosterone. Dihydrotestosterone, which is a more potent androgen than testosterone, results in miniaturization of hair follicle and change in cyclic phase of hair growth cycle, which leads to androgenic alopecia. In balding men, DHT binds to androgen receptors in vulnerable hair follicles and, by an unknown mechanism, activates genes liable for follicular miniaturization. Both plucked follicles and skin from balding scalp revealed to contain increased levels of DHT compared to follicles and skin from non-balding scalp. For the conversion of testosterone to dihydrotestosterone, 5α-reductase type 2 enzyme is responsible (Kaufman, 2002). Finasteride, a synthetic anti-androgenic drug, marketed for hair growth, and the mechanism involved is inhibition of 5α-reductase activity.

Alopecia in the AGA rat occurs because androgens induce a decline in the rate of hair growth and markedly prolongs the duration the resting phase or telogen. That is the reason for more telogen follicle in control group I. The alopecia, induced in the rat by testosterone, counteracted when extracts and isolate administered simultaneously to the rat. The alopecia not observed in groups treated with extracts or isolate or finasteride along with testosterone and anagen follicles are more than telogen follicles because of retention of late anagenic follicles as well as increase in follicular length and prevention of their miniaturization may therefore attributed due to 5α-reductase inhibitory activity. Besides visual observation, quantitative data (e.g., A/T ratio and follicular density) also suggested inhibition of androgenic activity of the extract. Result of *in-vitro* studies showed that these extracts and isolate also inhibit 5α-reductase enzyme activity as finasteride.

Phospholipids i.e. phosphatidylcholine (PC) play a major role in drug delivery technology. There are abundant advantages of phospholipids in addition to solubilizing property while taking into consideration them for a carrier system (Upadhyay et al., 2012). Among the treated group, maximum activity shown by group VI, in this group PC increases absorption of isolate and other group exhibited comparable or better hair growth than finasteride treated group. The predominance of hair follicle in anagenic growth phase indicates reversal of androgen induced hair loss in extracts, isolate and finasteride treated group.

The prostate is affluent in enzyme 5α-reductase type 2, and prostate homogenate demonstrates conversion of testosterone to dihydrotestosterone in reaction mixtures (Steers, 2001). The *in-vitro* study demonstrated 5α-reductase inhibitory activity and reduction of conversion of testosterone to DHT. Increased testosterone level in reaction mixture was because of inhibition of 5α-reductase because it is not converted to its metabolite dihydrotestosterone. Addition of ethanolic extract, petroleum ether extract and isolate of *C. reflexa* as well as finasteride in reaction mixture showed increased levels of unchanged testosterone levels in reaction mixture, suggesting inhibition of enzyme action by these test materials.

In testosterone-induced alopecia, plant extracts have safe topical effect. As negligible change in prostate weights, observed during this study. This suggests that dose given by topical applications were not sufficient to inhibit 5α-reductase in prostate and only inhibition caused in skin 5α-reductase. A hair growth promoter should have high anagen to telogen ratio, high follicular density. Therefore, it can said that the petroleum ether extract of *C. reflexa* Roxb, isolate from petroleum ether extract of *C. reflexa* and ethanolic extract of *Cuscuta reflexa* plant can be used as hair growth promoter in

androgenetic alopecia. These may be act by inhibiting 5α-reductase enzyme.

5. Conclusion

It can concluded that *C. reflexa* petroleum ether extract, ethanolic extract and its isolate from petroleum ether extract i.e. Stigmast-5-en-3-O-glucopyranosidetriacetate-5^1-ol can be used as a hair growth-promoting agent in androgenetic alopecia, which is caused by an increase in androgen levels in the skin. It also revealed that isolate has maximum efficacy than extracts and petroleum ether extract have better effect than ethanolic extract. Other androgen-dependent conditions like benign prostatic hyperplasia, prostatic cancer and acne were also due to dihydrotestosterone (Pérez-Ordeals et al., 2005). The observed 5α-reductase inhibitory activity of the extract and its isolate makes them potential candidates worthy of further investigation in management of these conditions.

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