

Effect of *Cuscuta reflexa* Roxb on androgen-induced alopecia

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Summary

Background Alopecia is a psychologically distressing condition. Androgenetic alopecia, which affects millions of men and women, is an androgen-driven disorder. Here, *Cuscuta reflexa* Roxb is evaluated for hair growth activity in androgen-induced alopecia.

Methods Petroleum ether extract of *C. reflexa* was studied for its hair growth-promoting activity. Alopecia was induced in albino mice by testosterone administration for 20 days. Its inhibition by simultaneous administration of extract was evaluated using follicular density, anagen/telogen ratio, and microscopic observation of skin sections. To investigate the mechanism of observed activity, *in vitro* experiments were performed to study the effect of extract and its major component on activity of 5 α -reductase enzyme.

Results Petroleum ether extract of *C. reflexa* exhibited promising hair growth-promoting activity as reflected from follicular density, anagen/telogen ratio, and skin sections. Inhibition of 5 α -reductase activity by extract and isolate suggest that the extract reversed androgen-induced alopecia by inhibiting conversion of testosterone to dihydrotestosterone.

Conclusions The petroleum ether extract of *C. reflexa* and its isolate is useful in treatment of androgen-induced alopecia by inhibiting the enzyme 5 α -reductase.

Keywords: 5 α -reductase type 2, androgenic alopecia, *Cuscuta reflexa*, hair growth

Introduction

Alopecia is a psychologically distressing condition. Androgenetic alopecia is the most common form of alopecia which affects millions of men and women; it is an androgen-driven disorder. Androgenetic alopecia is a process wherein continuous miniaturization of sensitive hair follicles takes place. The 5 α -reductase type 2 enzyme plays a central role by intrafollicular conversion of testosterone to dihydrotestosterone, and hair loss is characterized by shortening of the anagen phase and miniaturization of hair follicles which results in thinner and shorter hair. For the treatment of androgen-related disorders like androgenetic alopecia, the synthetic steroidal

drug finasteride has been approved by the US Food and Drug Administration for men.¹ Saw palmetto (*Serenoa repens*), a natural drug, has been investigated for androgen-related disorders like benign prostatic hyperplasia and male pattern baldness, as it has shown the properties of inhibiting 5 α -reductase enzyme.² Natural products have been widely advocated in hair care industry, and the search for natural remedies is being continuously promoted.

Cuscuta reflexa Roxb (Cuscutaceae), known as “amarvela” or “akashbel” in vernacular, is a parasite, with slender long yellow stems. It is distributed in tropical and temperate regions and common throughout India. It grows on different host plants, mostly thorny herbs.³

Traditionally, it is used as a purgative in the treatment of protracted fever, diaphoretic, and demulcent.^{4,5} Methanolic extract is reported to show antisteroidogenic properties.⁶ In our earlier studies, we have shown that the petroleum ether extract of this herb exhibited hair

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growth promotion on denuded skin surface of albino rats.^{7,8} The possible mechanism of hair growth, however, has not been worked out. The present investigation is an attempt to show its efficacy in testosterone-induced hair loss and to show that the drug inhibits conversion of testosterone to its more potent metabolite, dihydrotestosterone.

Materials and methods

Plant material

Stems of *C. reflexa* growing on *Bougainvillia spectabilis* were collected in the month of November 2004 from forests surrounding our university campus. The material was dried in sunlight and reduced to a coarse powder. The identity of the plant was confirmed at the Department of Botany of our university.

Extraction

Coarsely powdered drug was fed in a soxhlet apparatus and extracted with petroleum ether (60–80 °C) until complete extraction. The solvent from the extract was eliminated under reduced pressure (yield 2.5% w/w).

Chromatographic characterization

Cuscuta reflexa is a parasitic plant that draws its nutrients from the host. Phytoconstituents are likely to vary with the host, and it is thus desirable that material is collected from the identified host. For the present study, stems of *C. reflexa* were collected from plants growing on the *B. spectabilis*. The extract was characterized by TLC on precoated silica and developed in toluene/ethyl acetate (97 : 3) as mobile phase.

Isolation, purification, and characterization of active compound

The isolation of compound was done on the basis of solubility. Petroleum ether extract was suspended in acetone and shaken vigorously to dissolve the material. The insoluble mass was collected after centrifugation at 2000 r.p.m. (179 g) and collected. It was then fractionated into ethyl acetate soluble and insoluble fractions. The ethyl acetate soluble fraction was concentrated and kept in a refrigerator for about 12 h, when it yielded solid crystalline material. It was further purified by crystallization; the isolated compound (CR-1) melted at 68 °C and gave a single spot on the TLC plate (toluene/ethyl acetate, 97: 3) with an R_f value of 0.83, and tested positive for sterols using Lieberman Burchard and Salkowski tests for sterols.

In vivo studies on hair growth

Animals and tissue

The protocol for experimentation was approved by the Institutional Animal Ethics Committee of Dr. Harisingh Gour University, Sagar, Madhya Pradesh, India. Male Swiss albino mice (2–3 months) were housed in cages at room temperature (26 ± 2 °C) and were fed on standard diet with free access to water.

Testosterone test solution

Sterile testosterone solution (1% w/w) was prepared as suspension in aqueous carboxyl cellulose solution.

Finasteride solution

The 2% standard finasteride solution was prepared in vehicle (ethanol/propylene glycol/water, 8 : 1 : 1).

Extract solution

The 2% extract solution was prepared in vehicle (ethanol/propylene glycol/water, 8 : 1 : 1).

Treatment of animals for study

The method reported by Matias was followed with slight modification.⁹ In brief, the mice were divided in four groups of six mice each. Mice in all groups were administered testosterone subcutaneously. Animals of groups II, III, and IV were given topical application of vehicle, finasteride, and petroleum ether extract, respectively. Approximately 0.2 mL of the solution or vehicle was topically applied on back skin once a day for 20 days. After 20 days, mice from each group were selected randomly and sacrificed. The difference in growth of hair in each group was noticed by visual observation and was recorded by taking photographs (Fig. 1). Skin biopsy was also undertaken from the balding site of each group of mice, and samples were kept in phosphate-buffered formalin for paraffin sectioning. Vertical sections (3–4 μ m) were cut parallel to the direction of hair growth and stained with hematoxylin and eosin. The cyclic phase of hair follicles (anagen, telogen) was determined, and the anagen/telogen ratio was calculated with the help of ocular micrometer (Table 1).

In vitro studies on enzymatic activity

Preparation of enzyme solution

Human prostate (about 200 mg) supplied from a local hospital was cut in small pieces and homogenized in 10 mL of medium A (20 mM sodium phosphate, pH 6.5, containing 0.32 M sucrose and 1 mM EDTA). The homogenate was centrifuged at 4000 r.p.m. (716 g) for

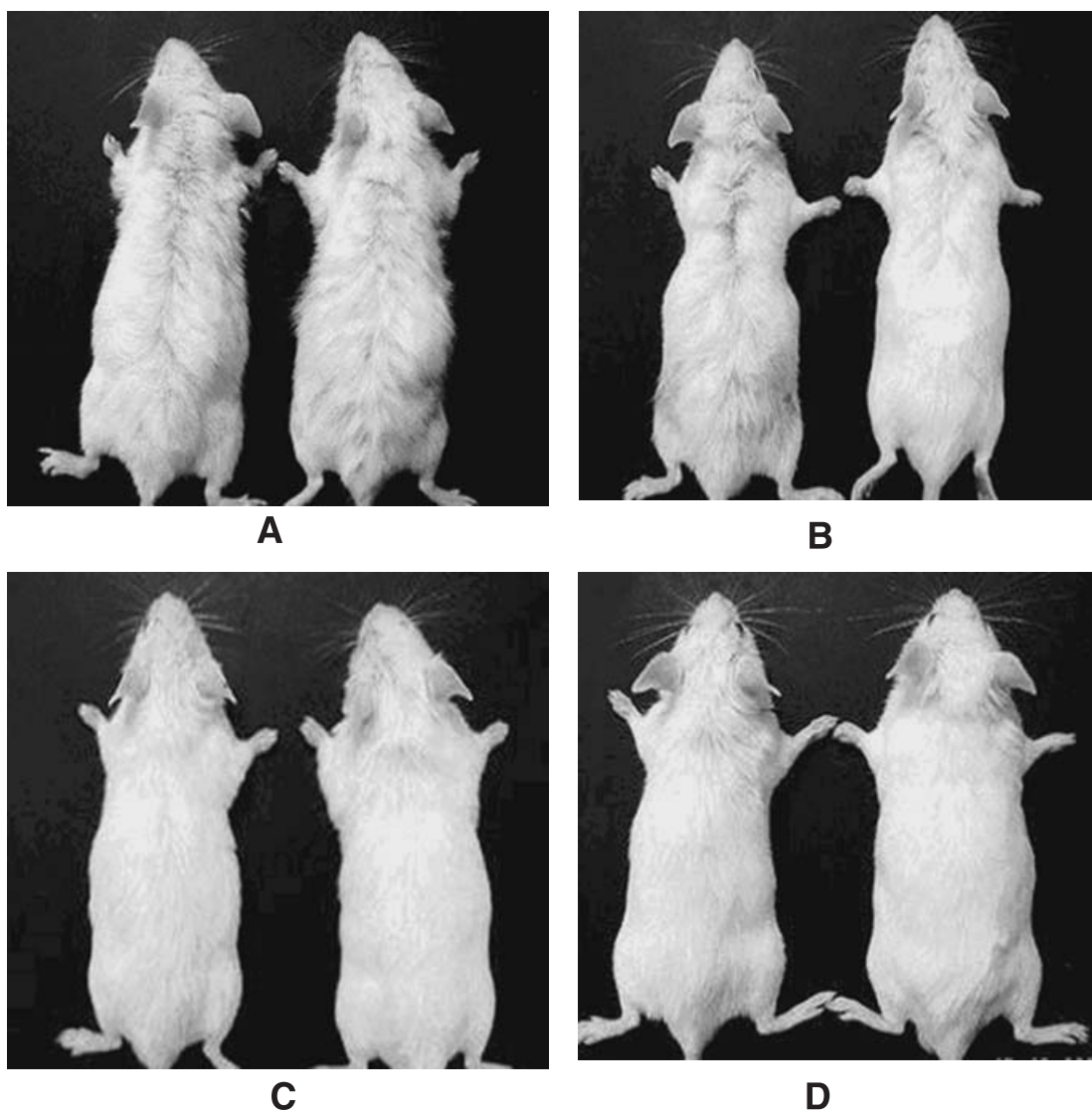


Figure 1 Comparison of baldness pattern in each group. (A) Animal treated with testosterone showing diffuse alopecia. (B) Animal treated with testosterone and vehicle showing hair loss. (C) Animal treated with testosterone and finasteride showing less hair loss. (D) Animal treated with testosterone and petroleum ether extract showing less hair loss.

Table 1 Follicular density and anagen/telogen ratio in sections of skin of different groups of animals.

Sample no.	Group no.	Treatment	Follicular density (no./mm), mean \pm SD ($n = 12$)	Anagen/telogen ratio
1.	I	Testosterone (s.c.)	1.33 \pm 0.77	0.1 : 1
2.	II	Testosterone (s.c.) + vehicle (topical)	2.33 \pm 0.77	0.25 : 1
3.	III	Testosterone (s.c.) + 2% Finasteride solution (topical)	2.83 \pm 1.02	0.91 : 1
4.	IV	Testosterone (s.c.) + 2% Extract solution (topical)	2.5 \pm 1.00	1.6 : 1

15 min. The supernatant was used as a source of enzyme. The concentration of enzyme in the supernatant was determined by Bradford Method of Protein estimation.¹⁰

Preparation of test materials

Testosterone (1 mM), extract (1 mg/ml), isolate (50 µg/ml) and finasteride (10 µg/ml) solution were prepared in ethanol (95%) with gentle heating wherever necessary. The EDTA solution (10 mg/ml) was made in distilled water.

Determination of optimum concentration of enzyme

It was determined by keeping the concentration of substrate constant and varying the concentration of enzyme. Testosterone solution (1 mM) was prepared in ethanol. Reaction mixture (1 mL) was prepared by adding testosterone solution (0.1 mL), enzyme solution (0.1–0.9 mL), and sodium phosphate buffer (20 mM). The reaction mixture was incubated at 37 °C for 1 h. The reaction was terminated by addition of 2 mL of ethyl acetate. The reaction mixture was then shaken vigorously for 1 min and the ethyl acetate layer was separated. It was evaporated to dryness, and the residue dissolved in 2 mL of methanol. Testosterone content in methanolic solution was estimated by high-performance liquid chromatography (HPLC; Shimadzu, Column C 18).

Determination of inhibitory concentrations of extract and isolate (CR-1)

The reaction mixture (1.5 mL) was made by adding 0.1 mL of testosterone solution, 0.1 mL of EDTA solution, 0.1–0.5 mL of extract/isolate/finasteride solutions for separate groups, optimum amount of enzyme solution (i.e., 0.5 mL), and sodium phosphate (20 mM), to a final volume of 1.5 mL.

Reaction mixture was incubated at 37 °C for 60 min, and reaction was terminated by addition of 3 mL of ethyl acetate. The mixture was vortexed for 1 min; ethyl acetate layer was separated and evaporated to dryness. The residue was dissolved in methanol and volume made up to 2 mL with methanol. The residual testosterone content in methanol was determined by HPLC. The column was eluted isocratically with a mobile phase of methanol/water (80 : 20) at a flow rate of 1.0 mL/min.¹¹

Results

In vivo hair growth studies

Qualitative study

The animals of groups I and II showed diffuse alopecia. Loss of hair from the dorsal portion of mice was clearly visible

after 20 days of treatment with testosterone (Fig. 1A,B). In animals of group III, the petroleum ether extract of *C. reflexa* was administered along with testosterone. The alopecic condition was not visible in this group of animals, showing that the extract prevented the action of testosterone and blocked testosterone-induced hair loss. The observation was the same in animals of group IV who received finasteride along with testosterone.

Quantitative study

Microscopic examination of skin sections of group I animals revealed that testosterone treatment caused miniaturization of hair follicles. The follicles had bulbous appearance and were short. Several hair follicles were in the telogen phase (Fig. 2A,B).

The effect of testosterone on miniaturization of hair follicle was blocked by administration of petroleum ether extract in group III animals. The increase in length as well as number of hair follicles was noted. The number of follicles in anagen phase was considerably increased and fewer follicles in telogen phase were observed. Again, the number of follicles in hair growth phase increased with duration of treatment. The follicular density (i.e., ratio of follicles in anagen to telogen phase) was calculated (Table 1). Anagen/telogen ratio was significantly affected by drug extract, which was observed 1.6 : 1 in the extract-treated group as against 0.1 : 1 observed for testosterone-treated control and 0.91 : 1 for finasteride-treated animals. The follicular density (i.e., number of hairs/mm) was calculated. The follicular density observed in the petroleum ether extract-treated group was 2.5 ± 1 , whereas it was 1.33 ± 0.77 in testosterone-treated control and 2.83 ± 1.02 in finasteride-treated standard group (Table 1).

As evident from the above data, the activity of petroleum ether extract of *C. reflexa* is comparable with finasteride control. The predominance of hair follicle in anagenic growth phase indicates reversal of androgen-induced hair loss in petroleum ether extract- and finasteride-treated group.

Determination of IC_{50}

The optimum concentration of the enzyme was found at 0.5 mL (239.9 µg). Varying concentrations of test substances were incubated with a 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 petroleum ether extract of *C. reflexa*, isolate CR-1, and finasteride. The IC_{50} values calculated for petroleum ether extract, isolate CR-1, and

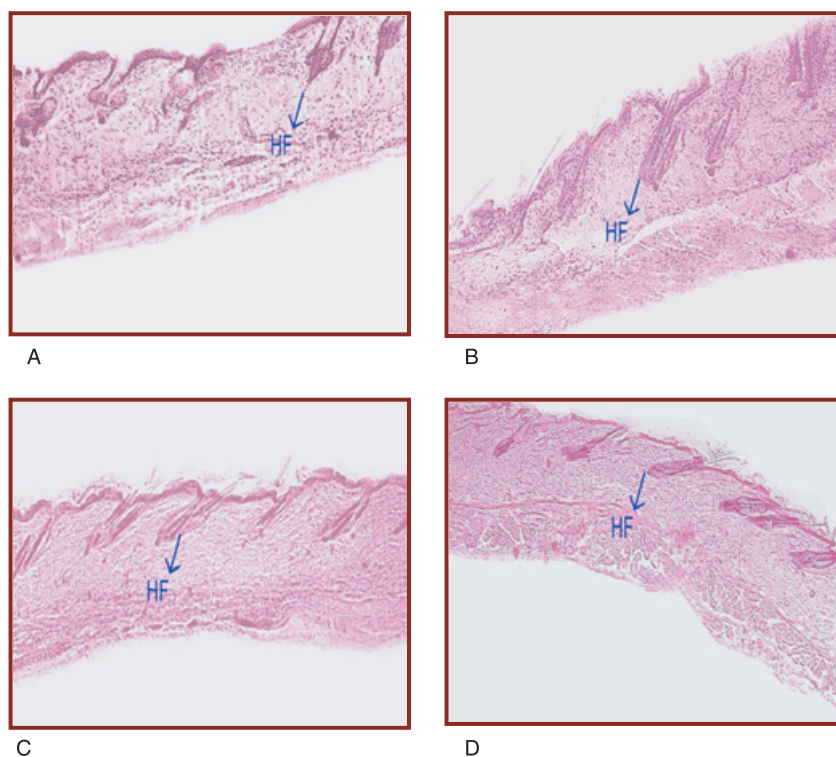


Figure 2 (A) Skin of animal treated with testosterone. (B) Skin of animal treated with testosterone and vehicle. (C) Skin of animal treated with testosterone and finasteride solution. (D) Skin of animal treated with testosterone and extract solution.

finasteride were 1.78 mg, 9.19 μ g, and 0.77 μ g, respectively, providing enzyme inhibitory activity of these compounds.

Discussion

In the present investigation, alopecia was induced in mice by administration of testosterone. Conversion of testosterone to dihydrotestosterone, which is a more potent androgen, results in miniaturization of hair follicle and change in cyclic phase of hair growth cycle, which leads to androgenic alopecia. The enzyme 5α -reductase type 2 is the key enzyme responsible for conversion of testosterone to dihydrotestosterone.¹² Finasteride, a synthetic anti-androgenic drug, is marketed for hair growth, and the mechanism involved is inhibition of 5α -reductase activity.

In our earlier studies,^{7,8} we have shown hair growth-promoting activity in petroleum ether extract of *C. reflexa*. The mode of action of the extract, however, was not worked out. The investigations under this report were taken with a view to ponder on this aspect.

The alopecia, induced in the mice by testosterone, was counteracted when petroleum ether was administered simultaneously to the mice. The alopecia was not

observed in groups that were treated with petroleum ether extract or finasteride along with testosterone. Besides visual observation, quantitative data (e.g., A/T ratio and follicular density) also suggest inhibition of androgenic activity of the extract. The prostate is rich in enzyme 5α -reductase type 2, and prostate homogenate demonstrates conversion of testosterone to dihydrotestosterone in reaction mixtures.¹³ Increased testosterone levels in reaction mixture is a result of inhibition of 5α -reductase because it is not converted to its metabolite dihydrotestosterone. Addition of petroleum ether extract of *C. reflexa*, the isolate CR-1, as well as finasteride in reaction mixture shows increased levels of unchanged testosterone levels in the reaction mixture, suggesting inhibition of enzyme action by these test materials. Furthermore, the inhibition of conversion by these materials in a dose-dependent manner clearly reflects that activity is blocked and, therefore, more testosterone remains unchanged in the reaction mixture. Dihydrotestosterone has also been implicated in androgen-dependent conditions like benign prostatic hyperplasia, prostatic cancer, and acne.¹ The observed 5α -reductase inhibitory activity of the petroleum ether extract and its isolate CR-1 makes them potential candidates worthy of further investigation in management of these conditions.

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