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Research Article
Preparation and Evaluation of Hair Growth Formulations of Indian Ginseng (*Withania somnifera*) for Alopecia

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Abstract

Background and Objective: Hair loss or alopecia or baldness, a dermatological disorder, affects the personality of an individual, psychologically and sociologically. There is a flood of drugs claiming to be useful in the treatment of alopecia but none seems to be developed with a proper rational strategy. The study aims to investigate the hair growth promoting activity of herbal formulations prepared from fruits extract of *Withania somnifera* (family- Solanaceae) collected from two different locations (from Rajasthan, WSR and from Uttarakhand WSU) on healthy male Wistar rats. **Materials and Methods:** The methanolic fruit extracts were sub fractionated into ethyl acetate, butanol and water fractions. All extracts were evaluated for their total phenolic content (TPC), total flavonoid content (TFC) and *in vitro* anti-oxidant activity (by two different methods). Aloe vera based herbal formulations were prepared from ethyl acetate fraction of the plant extracts. The prepared herbal formulations were subjected to primary skin irritation test and *in vivo* hair growth activity in healthy male Wistar rats. All the formulations were observed for hair growth initiation (HGIT) and hair growth completion time (HCIT). The histological study of skin samples was also performed at the end of study to study hair growth at follicular level. **Results:** Ethyl acetate fraction showed high TPC as well as TFC in both WSR and WSU in general. The extracts (particularly ethyl acetate extract) showed significant anti-oxidant activity in DPPH free radical scavenging activity and hydrogen peroxide scavenging assay. Primary skin irritation test showed that the prepared herbal formulations were non-irritating and non-toxic to the skin without any erythema or oedema at the end of 48 h of formulation application on denuded skin of rats. *In vivo* study showed early hair growth initiation and completion time in test group of animals as compared to the control group and the effect was comparable to that of standard group. The histology showed good growth of hair follicle in WSU as compared to WSR, control and standard with visible maximum anagenic population of hair. **Conclusion:** It was concluded that WSU formulation showed good *in vivo* hair growth activity and was well supported by follicular/histological study.

Key words: *Withania somnifera*, total phenolic content, total flavonoid content, anti-oxidant activity, *in vivo* hair growth activity, alopecia

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Hairs form a vital part of the overall personality and contribute immensely to the stylish and elegant looks of a person. Biochemically hairs are composed of protein keratin and are important part of our integumentary system. Hairs are filamentous structures or appendages of skin that originates from hair follicles be cause of coordinated cell proliferation and differentiation process[1-3]. Hairs provide protection against trauma, electromagnetic radiations, insects and parasite penetration. They act as insulator for body heat and as sensory antennae for feeling the surrounding environment[4]. Hair follicles act as production units for hairs. Massive proliferation of these stem cells results in formation of mature hair follicles that exhibit a regular reoccurring cycle of hair regeneration called as hair follicle cycle or hair growth cycle[3]. Hair growth cycle shows three phases: Anagen, catagen and telogen (Fig. 1). Once whole cycle gets completed, anagen phase begins again to start a new hair cycle.

Alopecia generally called as hair loss or baldness is a very common dermatological disorder. Although not being a serious threat to life it can have deep psychological impact on affected person[5]. Medically alopecia is classified in four major types. Androgenic alopecia (AGA), Alopecia areata (formation of circular bald patches on scalp), alopecia total is complete hair loss of scalp and alopecia universal is (entire body hair loss). AGA largely affects male but also affects females to a lesser or mild extent and hence classified into male pattern and female pattern baldness[6-8]. In AGA, as condition of alopecia advances, hair follicles are miniaturized progressively. Terminal hairs normally found on adult scalp were replaced by short, finer and non-pigmented vellus hairs. Shortening of anagen phase and larger telogen phase is characteristic of AGA[9]. Lot of products available in market claim for treatment of alopecia but only few are efficient due to lack of proper diagnosis for actual reason behind alopecia, causing failure of treatment strategy of alopecia by synthetic drugs. In addition, the adverse effects associated with these create more problems. On other hand, herbal formulations being used from ancient times as mentioned in indigenous systems of medicine (Ayurveda, Chinese, Unani etc.) were found and reported to be more efficient and safer and are very popular in cosmetics and dermal products. Various studies have reported the hair growth promotion effect by herbal products[10-12].

Withania somnifera (*Solanaceae* family), vernacular: Ashwagandha or Indian ginseng, was extensively used in ayurvedic formulations since ancient times for its numerous medicinal and health benefits. Leaves, fruits, seeds, stem and roots of WS consists of several phyto constituents responsible for its pharmacological properties. Major secondary plant metabolites present in WS are with aferin A and with anolide D[13]. Some other alkaloids present are with ananine, somniferinine, cuscohygrine, pseudotropine, scopoletin, isopelletierine, anaferine, anahygrin etc. Saponins, steroidal lactones, flavonoids, tannins and amino acids have also been reported[14]. *Withania somnifera* was reported to have anti-inflammatory, aphrodisiac, anti-biotic, anti-fungal,

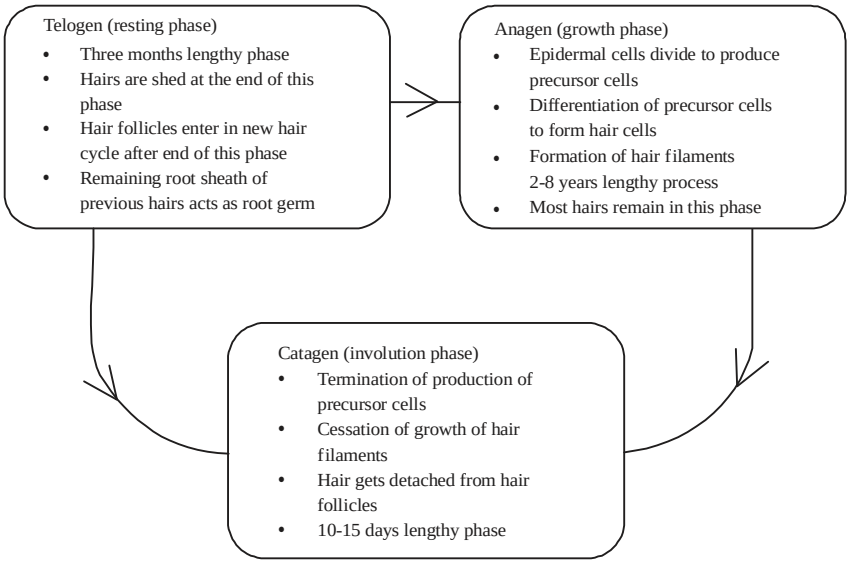

Fig. 1: Different phases of hair growth/hair follicle cycle

immunomodulatory, anti-stress, anti-oxidant, hypolipidermic, diuretic, sedative, hepatoprotective activities and found to be effective in various cardiovascular, inflammatory and nervous disorders[14,15]. It has also been reported to increase lipid peroxidation (due to proteolytic enzyme, chamase) and free radical scavenging activity of enzymes such as superoxide dismutase, catalase and glutathione peroxides[16]. Immunosuppressive effect on human B and T lymphocytes was also reported[17].

In the study aloe vera gel based herbal formulations of WS fruits extract (collected from two different geographical locations) were prepared and evaluated for hair growth potential (on male wistar rats). Besides the hair growth studies, the extracts of WS fruit extracts were subjected to Total phenolic content (TPC), Total flavonoid content (TFC) and *in vitro* anti-oxidant activity (DPPH and hydrogen peroxide scavenging activity).

MATERIALS AND METHODS

Butylated hydroxy toluene (BHT) and 2,2 diphenyl-1-picrylhydrazyl (DPPH) were purchased from Himedia Laboratory Pvt., Ltd., Mumbai. All other chemicals used in the study were of analytical grade.

Collection of fruits: The fruits of WS were collected from two geographical locations. First place of collection was Udaipur region of Rajasthan situated at an altitude of 598 m from sea level with latitude 24.5854°N and longitude 73.7125°E. Second area of collection was Pauri Garhwal region of Uttarakhand situated in lower Himalayan region at an altitude of 1000-1100 m from sea level with latitude 29.8688°N and longitude 78.8383°E. Fruits collected from Rajasthan and Uttarakh and were identified and authenticated from Department of Botany, H.N.B. Garhwal University, Srinagar (Garhwal) and hence designated as WSR and WSU respectively, for the study purpose.

Extraction of fruits: The collected fruits of WSR and WSU were washed for removal of soil/dust and left for drying for under shade for 20 days. Dried fruits of both plants were then grinded to form a coarse powder. Weighed 200 g of this coarse powder of each plant and dipped in n-hexane for de-fatting and then dried for at room temperature for 5 days. The defatted powder of each plant was then dipped in methanol in a round bottom flask for 4 week and then extracted in rotary vacuum evaporator (Perfit 5600, buchi type, India) at 70°C at 80-120 rpm for 24 h and dried in vacuum to obtain the dried extract.

Dried extracts were further subjected to sub fractionation into ethyl acetate, butanol and water extract fractions. The dried fruit extracts of both plants were sub fractionated using well established pre-reported method[18].

Determination of total phenolic content: The total phenolic content of different extracts of WSR and WSU was determined by Folin-Ciocalteu reagent method using Gallic acid as standard[18]. The total phenolic content was expressed in gallic acid equivalent (GAE) in μg mg^{-1} of extract.

Determination of total flavonoid content: Total flavonoids content of extracts of both plants was calculated using a calibration curve of quercetin as standard and results were expressed as quercetin equivalent (QE) in μg mg^{-1} of extract[18,19].

DPPH free radical scavenging activity: Anti-oxidant activity of the plant extracts (methanolic, ethyl acetate, butanolic and aqueous extract) were determined by DPPH (2, 2diphenyl-1-picrylhydrazyl) free radical scavenging method against the activity of standard, butylated hydroxyl toluene (BHT) using the standard reported method[20].

Hydrogen peroxide (H_2O_2) scavenging activity: Hydrogen peroxide (H_2O_2) scavenging activity of the plant extracts (methanolic, butanolic, ethyl acetate and aqueous extract) were determined by using the standard reported method against the activity of standard, butylated hydroxyl toluene (BHT)[21].

Preparation of formulations: Aloe vera gel was used as base for preparation of formulations of fruit extracts of WSR and WSU, as reported earlier[22]. The gel was extracted from aloe vera leaves from the fresh leaves of aloe vera. Then collected gel was heated to 40°C and a blend of stabilizers (ascorbic acid 0.5% w/w and sodium benzoate 0.5% w/w) were added to it for enhancing its stability. It was then allowed to cool and stored in cool dark place until used for formulation development.

An amount of 20 g of prepared aloe vera gel was taken in a beaker and 0.2 g of dried extract (ethyl acetate fraction) was added to it. Then, both were mixed to form uniform gel (Table 1). The prepared formulations were then subjected to physical evaluation and *in vivo* hair growth studies in healthy male rats.

***In vivo* hair growth activity:** *In vivo* activity was carried out in male Wistar rats weighing from 180-270 g. Animals were

Table 1: Composition of hair growth formulations of *Withania somnifera* Rajasthan (WSR), *Withania somnifera* Uttarakhand (WSU), standard (S), control (C)

Formulations	Composition
Withania somnifera Rajasthan (WSR)	Aloe vera gel (20 g)+herbal extract (0.2 g)
Withania somnifera Uttarakhand (WSU)	Aloe vera gel (20 g)+herbal extract (0.2 g)
Standard (S)	10% w/v minoxidil solution
Control (C)	Aloe vera gel only

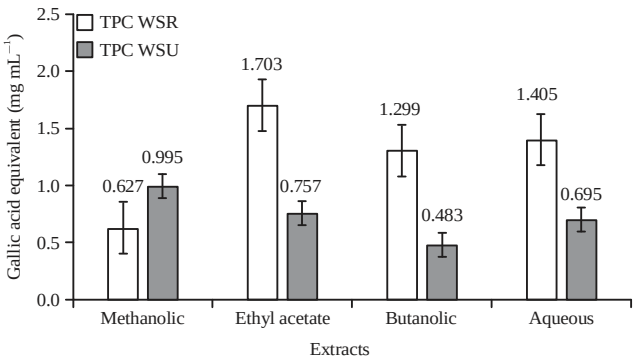

Fig. 2: Total phenolic content (TPC) of different extracts of *W. somnifera* Rajasthan (WSR) and *W. somnifera* Uttarakhand (WSU)

kept in standard environmental conditions, fed with standard diet and allowed free access to drinking water. Animal study protocols were duly approved by Institutional Animal Ethical Committee of Department of Pharmaceutical Sciences, Kumaun University Bhimtal campus (KUDOPS/57; 22/10/2016). Minoxidil 10% w/v solution (Mintop, Dr. Reddy's Lab. India) was used as standard and the heat stabilized plain aloe vera gel was used as control in study. Preliminary primary skin irritation test was conducted in three healthy male rats. The prepared hair gel was applied over the denuded test sites (on one side of spine) and then the test sites were observed after 48 h for any toxic side effects, erythema or oedema and compared with the results of standard and control.

In the major *in vivo* study, four groups of healthy male rats containing three animals in each group were used. Hairs from an area of 4 cm^2 on the dorsal side were removed by application of marketed hair removal cream and denuded skin was wiped with surgical spirit this serves as the test area. One group on which only control (aloe vera) was applied served as the control group. On one other group standard (10% minoxidil solution) was applied. Out of remaining two groups one was treated with herbal extract formulation of WSR and the other one was treated with herbal extract formulation of WSU. Every formulation was applied to the denuded skin twice a day for 30 days on animals of test groups. The hair growth pattern was observed visually in the test animals, recorded and evaluated on basis of two parameters: hair growth initiation time (minimum time for initiation of hair growth on denuded skin) and hair growth completion time (minimum time taken to completely cover the denuded skin with new hairs)[22].

After 30 days of study a rat was selected randomly from each group. Skin biopsy was taken from the area under test of the selected rat. Skin specimens of the rats were then embedded in paraffin and sections were cut out using rotary microtome. These sections were then stained with haematoxylin and eosin dyes and histological slides were prepared. These histological slides were then observed under stereo microscope for studying the follicular development.

Statistical analysis: Results were expressed as mean values±standard deviations. A probability value less than 0.05 ($p<0.05$) was considered to be a significant value.

RESULTS

To evaluate the possible use of WS for treatment of alopecia the extraction of fruits was done and subjected to various assays for development of an effective herbal formulation for alopecia treatment.

Determination of total phenolic content: The TPC of both WSR and WSU extracts was determined by using Folin-Ciocalteu method. Phenolic contents of different extracts were calculated using standard curve of gallic acid and expressed as gallic acid equivalents (GAE) in μg mg^{-1} of dry weight of extract. In WSR total phenolic content within different extracts were found to decrease in following order; ethyl acetate>aqueous>butanolic>methanolic. While in case of WSU the pattern of presence of TPC was as followed, methanolic>ethyl acetate>aqueous>butanolic. Results indicated that phenolic components are important phyto constituents in both WSR and WSU (Fig. 2).

Determination of total flavonoid content: The TFC of extracts of both plants was determined by aluminium chloride colorimetric method and calculated by using standard curve of quercetin. It was expressed as quercetin equivalents (QE) in μg mg^{-1} of plant extract. In WSR flavonoids concentration in

Table 2: DPPH scavenging activity (%) of different extracts of *Withania somnifera* Rajasthan (WSR) and *Withania somnifera* Uttarakhand (WSU)

	DPPH scavenging activity (%)									
	BHT		Methanolic extract		Ethyl acetate extract		Butanolic extract		Aqueous extract	
Concentration (μg mL^{-1})	WSR	WSU	WSR	WSU	WSR	WSU	WSR	WSU	WSR	WSU
50	87.02	94.54	96.31	80.70	66.67	2.75	92.02	79.06	88.81	76.17
100	96.90	98.60	95.83	89.38	82.98	54.42	92.38	83.44	93.10	80.70
150	97.50	98.75	95.48	91.91	88.33	71.34	93.10	82.26	93.10	81.59
200	97.62	98.91	92.98	94.28	89.52	77.28	93.45	83.59	91.90	82.18
250	97.86	98.85	96.79	93.91	91.79	80.48	93.93	83.07	93.81	83.44
500	98.81	99.27	98.10	96.81	92.26	89.24	94.80	83.15	94.76	83.44

Table 3: H_2O_2 scavenging activity (%) of different extracts of *Withania somnifera* Rajasthan (WSR) and *Withania somnifera* Uttarakhand (WSU)

	H_2O_2 scavenging activity (%)									
	BHT		Methanolic extract		Ethyl acetate extract		Butanolic extract		Aqueous extract	
Concentration (μg mL^{-1})	WSR	WSU	WSR	WSU	WSR	WSU	WSR	WSU	WSR	WSU
5	25.07	20.87	18.03	13.25	40.78	49.07	45.45	82.88	46.90	81.25
10	32.22	30.62	18.55	17.78	44.20	72.48	45.05	89.53	47.07	88.93
20	36.70	40.05	18.50	18.50	49.40	81.03	46.05	91.40	47.75	91.52
25	41.88	71.53	19.57	19.42	43.27	81.92	46.58	90.00	47.97	92.73
50	51.12	78.30	22.53	19.57	49.73	90.40	47.33	93.73	48.92	94.38

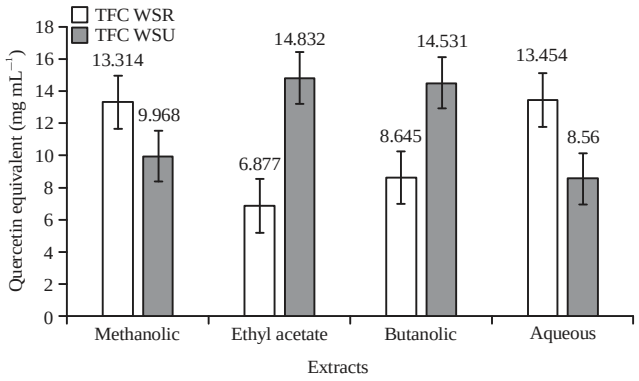

Fig. 3: Total flavonoid content (TFC) of different extracts of *W. somnifera* Rajasthan (WSR) and *W. somnifera* Uttarakhand (WSU)

different extracts was found to be decreased in following pattern, aqueous>methanolic>butanolic>ethyl acetate. The WSU showed following pattern of flavonoid concentration of different extracts, ethyl acetate>butanolic>methanolic> aqueous (Fig. 3).

DPPH free radical scavenging assay: DPPH radical scavenging activity of standard (BHT) and of both WSR and WSU extracts were determined at concentrations ranging from 50-500 μg mL^{-1}. Extracts of both the plants showed significant anti-oxidant activity especially at higher concentrations i.e., 250 and 500 μg mg^{-1}. Different extracts of WSR showed anti-oxidant activity in the following order; methanolic>butanolic>aqueous>ethyl acetate at concentration of 500 μg mg^{-1} (Fig. 4, 5). In case of WSU order of activity of extracts was found to be in following order, methanolic>ethyl acetate>aqueous>butanolic at 500 μg mg^{-1} concentration (Table 2).

Hydrogen peroxide (H_2O_2) scavenging activity: The H_2O_2 scavenging activity analysis was carried out for all extracts of both plants at concentration ranging from 5-50 μg mg^{-1} and BHT was used as standard in same concentration range. Extracts of both WSR and WSU showed significant H_2O_2 scavenging activity. In case of WSR study showed the following pattern of percent H_2O_2 scavenging activity, ethyl acetate>aqueous>butanolic>methanolic at concentration of 50 μg mg^{-1}. The order of H_2O_2 scavenging activity for WSU was found to be; aqueous>butanolic>ethyl acetate> methanolic at 50 μg mg^{-1} concentration (Table 3).

Physical evaluation of formulations: Due to presence of high TPC, high TFC and good antioxidant activity in general, ethyl acetate extracts of both WSR and WSU were selected for development of herbal formulation. The selection was based on evaluation of TPC, TFC and anti-oxidant activities. Herbal formulations were prepared using aloe vera gel as base. The prepared formulations showed light Green texture with characteristic odour.

***In vivo* hair growth activity:** Primary skin irritation test conducted before main *in vivo* test showed that the prepared

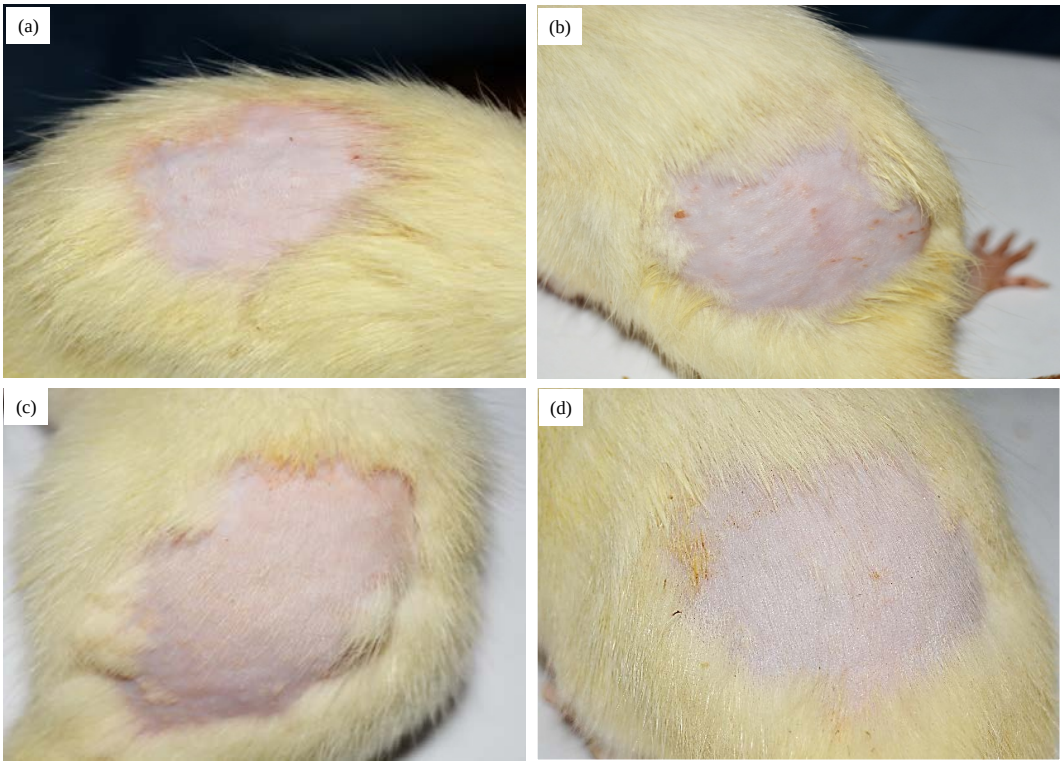

Fig. 4(a-d): *In vivo* hair growth activity showing hair growth initiation time in rats treated with (a) Control (11th day), (b) Standard (9th day), (c) *Withania somnifera* Rajasthan (WSR) (10th day) and (d) *Withania somnifera* Uttarakhand (WSU) (10th day)

herbal formulations were non-irritating and non-toxic to the skin as no signs of erythema or oedema were observed after the end of 48 h of formulation application on denuded skin of rats. *In vivo* study showed that hair growth was initiated in control group of animals receiving aloe vera gel only at the 11th day of the study whereas in case of herbal formulations, the hair growth initiation occurs at the 10th day of the study (Fig. 4). In group receiving standard 10% Minoxidil solution hair growth initiates at 9th day of the study. HGCT in case of standard and control groups was 22th day and 24th day respectively. The HGCT for WSU and WSR both was on 22th day. Histological study of the skin specimens showed that in comparison to the control, standard and WSR the developmental rate of follicles in the group treated with WSU was greater (Fig. 5).

DISCUSSION

The TPC and TFC provide a gross but close prediction of a plant's biological activity. It was observed that ethyl acetate fraction showed maximum TPC in WSR and second maximum TPC in WSU. Various previous studies on TPC and TFC of WS well supported the results of the present study. Fernando *et al.*[23] determined the TPC of *W. somniferadunal* from three different growth stages of the plant and reported that leaves, flowers, fruits, roots and stem all possess the phenolic components and leaves have the highest phenolic content. Alam *et al.*[24] also reported high concentration of phenolic components from all parts of the WS.

Ethyl acetate fraction of WSU showed maximum TFC. However, as a paradox ethyl acetate fraction showed least TFC in WSR. The abundance of flavonoids in both plants was also well supported by previous studies. Sharma *et al.*[25] carried out phytochemical evaluation of WS collected from the north west Himalaya and reported for the presence of higher concentration of flavonoids in leaf extract of plants collected from roadsides in comparison to plant collected from forests. Singh *et al.*[26] reported the presence of both free and bound flavonoids in leaves, roots, stem and fruits in *W. somniferadunal* and tested them for their anti-microbial activities against selected pathogens.

Methanolic fractions of both plants showed highest DPPH Scavenging activity. Study also indicated that anti-oxidant activity of extracts increases with increase in concentration

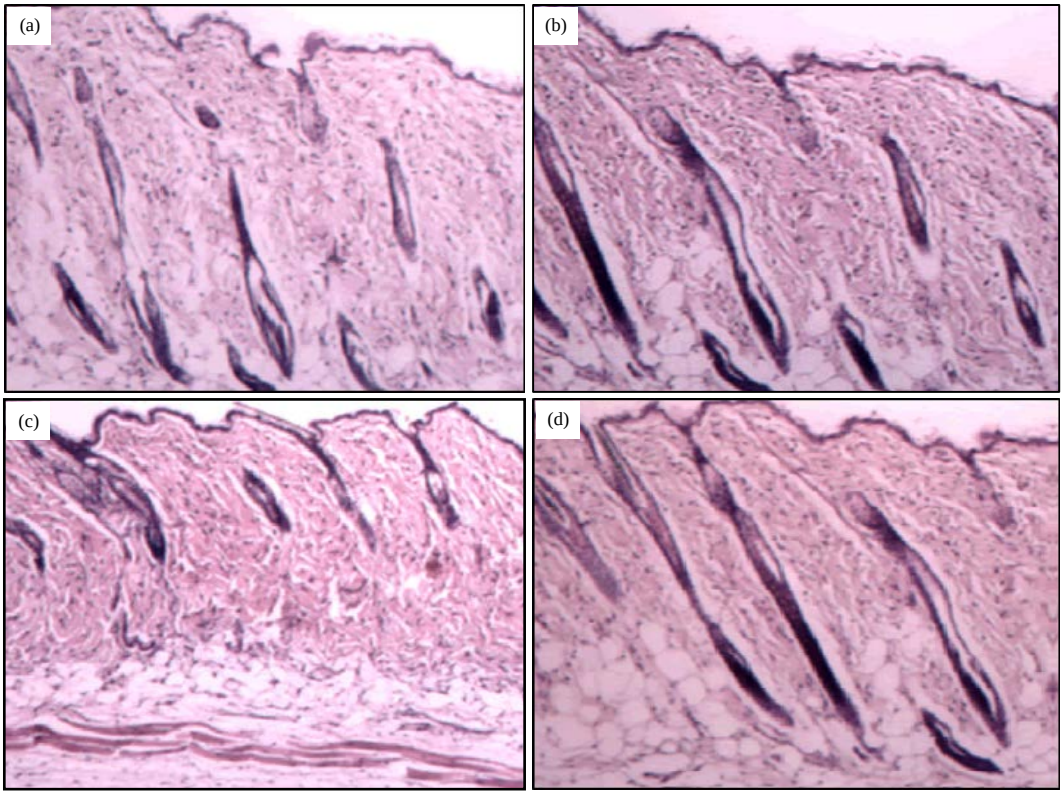

Fig. 5(a-d): Histological images of *in vivo* hair growth study showing follicular developmental stages in test animals treated with different formulations, (a) Control, (b) Standard, (c) *Withania somnifera* Rajasthan (WSR) and (d) *Withania somnifera* Uttarakhand (WSU)

upto certain concentration at least. Study also revealed that WSU showed higher H_2O_2 scavenging activity in comparison to that of WSR. Previous studies also supported the results, Alam *et al.*[27] reported that methanolic extracts of leaves, fruits and roots of *Withania somnifera* have significant anti-oxidant activities especially leaves, having highest anti-oxidant property.

Results of the *in vivo* study indicates that in group of rats treated with herbal formulations of WSR and WSU a reduction in HGIT was observed and they also showed a better hair growth pattern in comparison to control and standard. The early initiations of hair growth by other aloe vera based herbal formulations have also been reported with plants like Trigonella[22,28]. Aloe vera contributed as spreading agent for the herbal drug. Though it has also been reported to contribute in hair growth, the effect has been nullified by its use in control group during the hair growth study. Histology study also revealed that population of hair follicles in anagen phase was higher in comparison to the telogen phase in the test animals treated with WSU herbal formulations. The increase in anagenic hair population was a further confirmation of significant hair growth activity of the prepared herbal formulations.

CONCLUSION

The study showed that extracts of fruits of Withania WSU and WSR have significant antioxidant activity as well as the potential to stimulate the hair growth with more advanced hair follicular development in the animals treated with the herbal formulation of extract. The activity of Withania from high altitude region (WSU) was found to be better. Therefore, the study concluded that formulation of fruit extract of *Withania somnifera* (WSU) can have a therapeutic application as a natural and safe herbal remedy for treatment of alopecia.

SIGNIFICANCE STATEMENT

Alopecia, hair loss or baldness is a very common dermatological disorder which shows deep psychological

impact on affected person. No precise medicine without any side effect is available in the global market. The study explored the fruits of *Withania somnifera* (also called Ashwagandha or Indian ginseng) for hair growth activity by development of topical herbal formulation. The study is significant and novel due to first time reporting of hair growth properties of fruits of Withania (collected from two different regions) through the prepared herbal formulation. The study also helps to correlate the antioxidant activities, flavone/phenol content with that of hair growth properties. The results of the study if validated through human trials, may lead to the development of safe herbal formulation for alopecia.

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