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EXPLORATION OF NUTRACEUTICAL POTENTIAL OF HERBAL OIL FORMULATED FROM PARASITIC PLANT

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Abstract

Background: *Cuscuta reflexa* (*C. reflexa*) is a parasitic climber of medicinal importance. The present study was aimed to evaluate the nutraceutical potential of *C. reflexa* stems collected from different hosts and to evaluate the role of the herbal formulation in dandruff, hair fall control as well as hair growth promoter.
Materials and Methods: Hair formulations of *C. reflexa* collected from different host plants were prepared in the form of herbal oils (10% w/v). *C. reflexa* stems were extracted using mustard oil as base oil by using direct boiling technique. Prepared oil was studied as hair tonic. The experimental protocols used were anti-dandruff hair growth activity, as well as hair fall reduction. Herbal hair oils versus mustard oil were evaluated by applying oils on human volunteers with hair fall and dandruff problem whereas promotion of hair growth activity was conducted on rats. The formulated oils were also characterised for proximate analysis, physiochemical composition, as well as antimicrobial activity.
Result: The test oils of *C. reflexa* collected from *Azadiracta indica* and *Zizyphus jujuba* were effective in the promotion of hair growth, dandruff control, as well as reduction in hair fall activity.
Conclusion: All the formulated oils showed potent antimicrobial activity against all selected strains of bacteria and fungi.

Keywords: *Cuscuta reflexa*, host plants, Hair fall, Dandruff, Combing assay, Herbal hair oil, Hair growth activity.

Introduction

Hairs are erupted from ectodermal part of skin. Hairs are protective appendages on the body and considered accessory structure of the integument along with sebaceous glands and sweat glands (Stough et al., 2005).

Throughout history and in most civilisations, scalp hair has been associated with positive signals such as beauty and power. However, baldness has negative impact. Different hair care preparations are the important luxuries for growth of hairs. These are also used for the prevention and treatment of baldness, aggression of hairs, dandruff or other ailments. Hair oil containing herbal constituents are used as hair tonic. Hair care products are categorised into two main categories: hair tonics and hair grooming aids. These are basically the extracts of medicinal plants in an oil base (Takahashi et al., 1998; Banerjee et al., 2009). Each hair grows in three cyclic phases, i.e. anagen [growth], catagen [involution] and telogen [rest]. The anagen phase lasts for 2-6 years. Catagen phase with maximum growth activity lasts between 2-3 weeks. The telogen phase is at which hairs enter into resting state. This phase lasts for 2-3 months. Generally, 50-100 hairs are shed every day (Thorat et al., 2009). Various factors contribute to dandruff and hair fall / loss. Genetic predisposition and hormonal factors predominantly contribute to the hair fall. Diseases, such as typhoid, malaria, jaundice, etc., also cause hair fall. The use of chemotherapeutic agents also causes dandruff as well as hair fall (Yoon et al., 2010). Management of hair fall is extremely complex. Hormone therapy use of a-reductase inhibitors, vasodilators like minoxidil are widely used to reduce the hair fall / loss (Adhiranjan et al., 2003; Roy et al., 2006). The use of some of the herbal oils is also reported to reduce the hair fall / loss. A plethora of herbs have been employed for hair treatments, but lack of sound scientific information limits their usage with confidence (Adhiranjan et al., 2003). A few of these herbs are gooseberry, tamasi, chitraka, marigold, hibiscus, nutmeg, parsley, rosemary, thyme, jujuba, and parasitic plants like *C. reflexa* (Sharma et al., 2010). Hence, the present study was aimed to evaluate the nutraceutical potential of *C. reflexa* stems collected from different hosts and to evaluate their role as herbal formulations in dandruff, hair fall control, as well as hair growth promoter.

Materials and Methods

Collection and authentication of Parasitic Plant Material

Fresh stems of *Cuscuta reflaxa* were collected from different plant hosts like (*Azadiracta indica)* (Indian lilac **A**), *Zizyphus jujuba* (Jujuba **B**), *Morus alba* (Mulbery **C**), *Accacia arabica* (Indian gum **D**) and *Cordia latifolia Roxb* (Sebes tan plum **E**) present in different localities of Punjab province, Pakistan. Samples were washed under the running tap water, air dried under the shade and then homogenised to fine powder and stored in air tight bottle before further physiochemical analysis; whereas for hair oil formulation, fresh stem was used. These stems were identified by comparing them with standard herbarium specimens available in the Dept of Botany, University of Agriculture, Faisalabad.

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Proximate analysis

C. reflexa stems were subjected to proximate analysis including moisture, total ash, protein and mineral contents (Anjum et al., 2006).

Characterization of *C. reflexa*

Different qualitative chemical analyses were performed to identify the chemical constituents of *C. reflexa* (Fulton, 1932; Wall et al., 1954; Balbaa et al., 1988).

Preparation of Herbal Hair Formulation

For making herbal oil, *C. reflexa* stems were cut into small pieces and mustard oil was used as base. The hair oil (10% w/v) was prepared by direct boiling method in which the cut pieces of *C. reflexa* stems were weighed and directly boiled in mustard oil with continuous stirring and heating until the *Cuscuta* stem were completely extracted in the oil base. Oil was separated from the residues and used for further analysis.

Physiochemical characterisation of formulated oils

Different physical parameters like density, refractive index, pH and chemical parameters like free fatty acid, iodine value, saponification value and unsaponifiable matter were determined according to standard IUPAC methods as reported by Anjum et al (2013). Colour was estimated by Lovibond tintometer (Tintometer Ltd., Salisbury, United Kingdom) using a 1-in. (2.5 cm) cell Anjum et al (2013).

Antimicrobial activity

Antimicrobial activity of all formulated oils was determined by following the modified method as reported by Anjum et al (2013).
a. Disc diffusion method: The antimicrobial activity of oils was determined using disc diffusion method as reported by Abbas et al. (2012).
b. Determination of minimum inhibitory concentration (MIC): For the determination of MIC, a micro dilution of broth susceptibility assay was used as recommended by the National Committee for Clinical Laboratory Standards (CLSI, 2007).

Haemolytic activity: Haemolytic activity of the oil was studied using Shahid et al. (2013) method.

Evaluation of hair fall activity in human volunteers

Male and female subjects between 18-40 years of age neither suffering from nor any other diseases except hair fall were selected for study for the period of sixty days. After ascertaining the clinical compliance of each subject, the objective and other details of the study were explained to them. Thirty volunteers recruited for the study were divided into six groups of five each to test the efficacy of formulated oils against mustard oil separately. 300 mL of the oil was provided to each of the volunteer and was instructed to apply it in the morning every alternate day on the scalp [6 ml / application] and massage the scalp gently for 10 min for the period of sixty days. On the day of hair wash, they were advised to apply the oil after bath. The volunteers were also advised not to have hair wash with any shampoo four days prior to review in the lab once in a week over study period of sixty days.

a. Evaluation Method

The hairs of all volunteers were gently combed 10 times using a comb in downward direction covering the entire scalp surface on zero days. All the hairs collected in the comb were counted and examined under microscope for the presence of roots. The hairs of all the volunteers were totalled and the number of hairs with roots was calculated and percentage was obtained for each group. The same procedure was repeated on 10, 20, 30, 40, 50 and 60 days after oil usage.

b. Evaluation of dandruff problem in human volunteers

Eighteen patients (six groups having three in each) of both sexes, from the age group of 20-45 years, suffering from mild to moderate dandruff (suffering from nor any other disease except dandruff) were enrolled in the present study. All patients were examined for scalp skin and advised to apply 10 ml of formulated hair oil twice a day for a period of two weeks with gentle massage to the entire scalp and were advised to leave the oil on the scalp for a contact period of minimum 3-4 hours after application. Patients confined themselves to the formulated hair oil as the only treatment for their dandruff and no alternative treatment was allowed during the study period. Thorough scalp examination was done after completion of one week and at the end of the study. The severity of the dandruff symptoms (white scales) was recorded on a score scale from 0 to 3 (0=Nil, 1=Mild, 2=Moderate and 3=Severe).

Hair growth activity of formulated oils *in vivo*

18 Adult albino rats weighing 150-200 ± 5 g (Grouped into six) were obtained from National Institute of Health, Islamabad, Pakistan. Three animals per group were used for the present research work. Polypropylene cages under standard laboratory conditions (Ambient temperature 25±4°C, relative humidity 55±2%, 12:12 h dark and light cycle, standard diet and water *ad libitum*) were used for housing of rats (three rats per cage) at least seven days prior to the experiment. Group one was selected as control. Remaining five groups were given experimental treatment of five formulated oils. All animals were brought to the experimental lab, one hour before the initiation of experiment.

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Primary skin irritation test

4 cm^2 of dorsal portion of all test animals were made free from hair by using branded hair remover and then cleaned with surgical spirit. Fifteen micro litre of hair oil was applied over the respective pre-selected and pre-marked test sites of all the animals. Observations were made for erythema and oedema after 48 hours of applications of all test oils (Banerjee et al., 2009).

Hair growth activity after application of formulated oils

After performing the skin irritation test, shaved skin was cleaned with surgical spirit. Fifteen micro litre of prepared hair oil was applied to shaved area of respective group once a day and control group received mustard oil treatment. This treatment was continued for 28 days.

a. Hair growth initiation and completion time

Hair growth initiation time (minimum time to initiate hair growth on shaved skin region) and hair growth completion time (minimum time taken to completely cover the shaved skin region) with new hair strands on adjoining areas were recorded for each group of animals (Yoon et al., 2010).

b. Hair length determination

Hair length was measured periodically every 7th day during experiment. Ten hairs were plucked from shaved area of all mice after every seven days. Length was measured and average length was determined. The results have been expressed as mean length ± SD of ten hairs (Yoon et al., 2010).

Results

Proximate analysis of Cuscuta stems

Proximate analysis and qualitative chemical tests were performed to identify the presence of active constituents responsible for control of hair fall, dandruff as well as increasing hair growth activity (Table 1). Moisture contents of all *Cuscuta* stems collected from different plants were in the range of 13.82-22.19%. Maximum moisture was found in stem collected from Indian gum (*A. arabica*), whereas minimum value was detected in stem collected from mulberry (*Morus alba*). Total protein was in the range of 15.37-24.10% of which 5.11-9.20 % was water soluble. Maximum level of protein was detected in *Cuscuta* stem collected from jujuba and Indian gum (*Z. jujuba* and *A. arabica*). Ash contents were detected in the range of 5.93-10.34%. Maximum ash (10.34%) was noticed in stem collected from Jojoba tree (*Z. jujuba*) which was followed by Indian lilac (9.16%), Indian gum (7.11%), Sebes tan plum (6.59%) and Mulbery (5.93%). Mineral analysis of *Cuscuta* stems was performed by AAS and results are reported in Table 1. It is clear from this table that all stems of *Cuscuta* contained considerable amount of (mg/Kg) Ca (453.08-321.09), Cu (2.79-337), P (129.02-138.93), Fe (43.17-55.19) and Zn (10.27-15.72). Maximum level of calcium, copper and zinc (mg/kg) (453.08, 3.37 and 15.72) was detected in *Cuscuta* stem collected from sebes tan plum, whereas iron (55.19) and phosphorus (138.93) were found maximum in stem collected from jujuba tree.

Table 1: Proximate analysis of *Cuscuta reflexa* collected from different host plants

Parameters	***A.indica***	***Z. jojoba***	***M. alba***	***A. arabica***	***C. latifolia***
Moisture contents	15.02^{d}±1.5	18.11^{b}±0.9	13.82^{e}±0.9	22.19^{a}±1.6	17.15^{c}±0.9
Total protein	21.03^{b}±1.4	24.10^{a}±1.3	15.37^{e}±0.6	17.03^{d}±1.1	18.51^{c}±1.0
Water soluble protein	6.13^{c}±0.7	9.20^{a}±0.7	5.11^{d}±0.5	6.02^{c}±0.5	8.35^{b}±0.7
Ash contents	9.16^{b}±0.5	10.34^{a}±0.7	5.93^{d}±0.2	7.11^{c}±0.6	6.59^{c}±0.5
Mineral Contents(mg/Kg)					
Calcium	321.09^{d}±5.01	378.18^{c}±6.12	421.17^{b}±6.12	377.24^{c}±5.62	453.08^{a}±9.19
Copper	3.02^{b}±0.21	3.11^{b}±0.1	2.83^{c}±0.03	2.79^{c}±0.03	3.37^{a}±0.28
Iron	51.23^{b}±2.11	55.19^{a}±1.45	43.17^{e}±0.21	47.21^{d}±0.98	49.01^{c}±2.18
Phosphorus	129.02^{c}±3.14	138.93^{a}±5.01	126.55^{d}±4.81	132.72^{b}±8.13	129.63^{c}±7.39
Zinc	13.24^{b}±0.9	15.21^{a}±0.25	10.27^{d}±0.37	11.36^{c}±0.29	15.72^{a}±1.91

Values (mean ± SD) are average of three samples of each *Cuscuta reflexa* analysed individually in triplicate (n = 1x3 x 3), ($P < 0.05$). Superscript letters within the same row indicate significant ($P< 0.05$) differences of means within the nutritive values of *Cuscuta reflexa* collected from different host plants

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Qualitative analysis of Cuscuta stems

Different chemical tests were performed for the identification of important chemical constituents in the stems which might be important in dandruff, hair fall control and hair growth activities. Chemical constituents present in *C. reflexa* collected from different hosts are depicted in Table 2. This table indicates that *Cuscuta* stems contain glycosides, alkaloids, proteins, flavonoids, phytosterol and saponins.

Table 2: Qualitative chemical tests for *Cuscuta reflexa* stem collected from different host plants

Mobile phase	Test	Cuscuta extract				
		A	B	C	D	E
Carbohydrates, Glycosides	Fehling test, Molisch test	+	+	+	-	-
Alkaloids	Mayer,s test	+	+	-	-	-
Proteins	Biurate test	+	+	+	-	-
Flavonoids	Acid-Mg test	+	+	+	-	-
Phytosterols	Salkowiski test	+	+	-	+	-
Saponins	Boiling test	+	+	-	+	+

A=Neem (*Azadiracta indica)* B=Jojoba (*Zizyphus jujuba)* C= Mulbery (*Morus alba*) D= Indian gum (*Accacia Arabica*) and E= Lasora (*Cordia latifolia*)

Physiochemical characterisation of formulated hair oils

The prepared formulations are yellow to reddish-brown in colour with pH (6.8-7.5) in accordance with human skin neutral to slightly acidic. Specific gravity was found in the range of 0.9434-0.9527. Refractive index was noted in the range of 1.421-1.461. Iodine values were in the range of 87.29-102.25 mg /100g. The saponification values of oils were detected from 197.29 to 234.17 mgKOH/g. In all formulated oils, unsaponifiable (unsap) matter was detected in the range of 0.201-0.511%. Maximum level of unsaponifiable matter was found in oil A (0.511%) followed by oils B (0.406%), D (0.315), C (0.212%) and E (0.201%) (Table 3).

Table 3: Physiochemical characterization of oils formulated by using *Cuscuta reflexa* stem collected from different host plants

Parameter	*A. indica*	*Z. jujuba*	*M. alba*	*A. arabica*	*C. latifolia*
Color (red units)	2.01^{c}±0.06	2.63^{a}±0.07	1.98^{d}±0.06	1.99^{d}±0.06	2.52^{b}±0.09
Color (yellow units)	1.89^{b}±0.03	1.99^{a}±0.01	1.45^{d}±0.01	1.73^{c}±0.00	1.81^{b}±0.01
Refractive Index (40 °C)	1.424^{c}±0.01	1.437^{b}±0.01	1.461^{a}±0.00	1.435^{b}±0.03	1.421^{c}±0.01
Density (20 °C kg/ m3)	0.9534^{a}±0.04	0.9434^{b}±0.05	0.9444^{b}±0.05	0.9527^{a}±0.03	0.9454^{b}±0.05
pH	6.9^{c}±0.81	7.1^{b}±0.92	7.5^{a}±1.01	7.0^{b}±0.59	6.8^{c}±0.71
Free fatty acid	1.78^{b}±0.06	1.24^{d}±0.04	2.13^{a}±0.11	1.74^{b}±0.05	1.35^{c}±0.05
Iodine value	100.09^{b}±2.18	99.58^{b}±2.72	87.29^{c}±3.01	100.14^{b}±3.11	102.25^{a}±2.98
Saponification value	208.05^{c}±4.12	234.17^{a}±6.14	217.19^{b}±6.92	205.11^{d}±5.99	197.29^{e}±5.17
Unsaponifiable matter (%)	0.511^{a}±0.01	0.406^{b}±0.03	0.212^{d}±0.01	0.315^{c}±0.02	0.201^{d}±0.01

Values (mean ± SD) are average of three samples of each formulated oil, analysed individually in triplicate (n = 1x3 x 3), (P < 0.05); superscript letters within the same row indicate significant (P< 0.05) differences of means within the nutritive values of *Cuscuta reflexa* stem collected from different host plants

Antimicrobial activity

The antimicrobial activity of formulated oils was evaluated against a set of microbes like Gram positive and Gram negative bacteria and selected fungal strains (Tables 4a and 4b). All the formulated oils exhibited different degrees of antimicrobial activity against all micro-organisms tested. Results obtained from disc diffusion method, followed by measurement of minimum inhibitory concentration (MIC), showed the following sensitivity order:

Staphylococcus aureus > *Bacillus subtilis* > *Pasturella multocida*> *Escherichia coli*

The results indicated that *Staphylococcus aureus* and *Bacillus subtilis* were the most sensitive bacteria among selected bacterial strains tested by formulated oils. A significantly higher antimicrobial activity was recorded for the formulated oil A and B, whereas oils C, D and E showed comparatively less activity. A similar pattern was also observed for MIC values (Tables 4a and 4b). A comparatively higher activity of oils A, B and E was exhibited against Gram positive bacteria than Gram negative strains. The antibacterial activity of all formulated oils was comparable with the standard drug, Amoxicillin. The oil A and B exhibited highly significant antifungal activity as compared to standard drug, but oils C, D and E showed less antifungal activity than standard drug (Flumequinene). Sensitivity order of selected fungal strains was *Candida albicans* > *Pityrosporum ovale* > *Fusarium solani* > *Microsporum canis* > *Aspergillus flavus*

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Haemolytic activity

The formulated oils were screened using a rapid assay against human and bovine erythrocytes. The results are summarised in Table 5. No toxicity was observed by formulated oils except oil C as shown in Table 5.

Table 4a: Antimicrobial activity and minimum inhibitory concentration of formulated oils against selected bacterial and fungal strains

Selected Organism	Oil-A		Oil-B		Oil-C	
	DD[B]	MIC[C]	DD[B]	MIC[C]	DD[B]	MIC[C]
Bacterial strains						
Staphylococcus aureus	26.2^{a}_{a}±2.0	122.2^{d}_{d}±2.6	24.5^{b}_{a}±2.1	156.1^{c}_{b}±2.1	19.0^{c}_{a}±1.1	178.3^{a}_{d}±2.9
Bacillus subtilis	24.5^{a}_{b}±1.1	129.5^{d}_{c}±2.3	24.1^{a}_{a}±1.5	151.6^{c}_{c}±1.9	19.8^{b}_{a}±1.4	187.7^{a}_{c}±0.9
Pasturella multocida	21.9^{a}_{c}±1.2	140.5^{e}_{b}±2.4	19.8^{b}_{b}±1.0	152.4^{d}_{c}±2.0	15.2^{e}_{b}±1.3	202.1^{a}_{b}±2.1
Escherichia coli	21.5^{a}_{c}±0.9	158.2^{d}_{a}±2.1	19.5^{b}_{b}±1.5	170.1^{c}_{a}±1.9	11.5^{d}_{c}±0.9	229.8^{a}_{a}±2.7
Fungal strains						
Candida albicans,	16.3^{c}_{d}±1.4	199.2^{e}_{a}±3.0	18.3^{a}_{b}±2.5	232.8^{b}_{a}±2.6	10.1^{d}_{b}±1.4	271.2^{a}_{b}±2.7
Pityrosporum ovale	17.4^{b}_{c}±1.5	182.2^{d}_{b}±3.5	18.6^{a}_{b}±1.9	202.1^{c}_{c}±2.6	8.9^{c}_{c}±1.1	297.3^{a}_{a}±3.5
Aspergillus flavus	23.2^{a}_{a}±1.1	151.3^{d}_{d}±2.1	20.6^{b}_{a}±2.4	173.4^{b}_{e}±1.3	8.6^{e}_{c}±0.6	222.5^{a}_{c}±3.0
Fusarium solani	17.9^{a}_{c}±1.7	175.4^{d}_{c}±1.5	18.1^{a}_{b}±1.2	214.2^{b}_{b}±2.4	07.1^{b}_{d}±1.6	187.3^{c}_{d}±3.4
Microsporum canis	21.3^{a}_{b}±0.9	151.6^{d}_{d}±1.5	21.0^{a}_{a}±0.9	198.3^{a}_{d}±1.5	11.6^{d}_{a}±1.3	185.2^{b}_{d}±3.1

Values (mean ± SD) are average of three samples of each formulated oil, analyzed individually in triplicate (n = 1x3 x 3), (P < 0.05); superscript letters within the same row indicate significant (P< 0.05) differences of means within the antimicrobial activity of formulated oils of Cuscuta collected from different host plants. Subscript letters within the same column indicate significant (P< 0.05) differences of means of antimicrobial activity of formulated oil against different microbial strains.

[A] Amoxiclin (30 µg/disk) for bacterial and Flumequine (30 µg/disk) for fungal strains.

[B] DD, Diameter of inhibition zone (mm) including disc diameter of 6 mm.

[C] MIC, minimum inhibitory concentration (µg/mL).

Table 4b: Antimicrobial activity and minimum inhibitory concentration of formulated oils against selected bacterial and fungal strains

Selected Organism	Oil-D		Oil-E		Antibiotic[A]	
	DD[B]	MIC[C]	DD[B]	MIC[C]	DD[B]	MIC[C]
Bacterial strains						
Staphylococcus aureus	19.7^{c}_{a}±2.2	166.4^{b}_{d}±2.2	24.0^{b}_{a}±1.3	153.7^{c}_{c}±2.5	29.3±1.2	109.4±3.0
Bacillus subtilis	19.6^{b}_{a}±1.5	172.6^{b}_{c}±2.1	17.4^{c}_{c}±0.9	152.6^{c}_{c}±2.7	32.5±0.9	90.1 ± 2.8
Pasturella multocida	17.2^{d}_{b}±1.1	191.2^{b}_{a}±2.0	18.2^{c}_{b}±1.1	177.5^{c}_{b}±2.2	30.6±0.7	73.8± 2.3
Escherichia coli	14.3^{c}_{c}±1.3	189.6^{b}_{b}±1.7	13.6^{c}_{d}±1.5	185.3^{b}_{a}±1.9	30.1±2.1	101.2±3.2
Fungal strains						
Candida albicans,	17.3^{b}_{a}±0.9	225.1^{c}_{c}±3.4	18.3^{a}_{a}±2.2	213.4^{d}_{a}±1.6	25.6±0.9	121.8±3.1
Pityrosporum ovale	17.1^{b}_{a}±1.1	231.7^{b}_{b}±4.1	18.1^{a}_{a}±2.1	201.3^{c}_{b}±2.0	27.1±1.0	124.7±3.7
Aspergillus flavus	15.7^{c}_{b}±2.1	171.4^{b}_{d}±2.4	13.6^{d}_{b}±2.7	159.6^{c}_{c}±1.2	26.4±0.6	135.2±2.1
Fusarium solani	17.5^{a}_{a}±1.2	239.6^{a}_{a}±2.4	18.5^{a}_{a}±3.6	147.5^{e}_{d}±3.1	32.1±2.1	100.2±1.7
Microsporum canis	17.5^{b}_{a}±1.1	153.1^{d}_{e}±2.8	14.3^{c}_{b}±0.7	161.9^{c}_{c}±2.5	24.9±0.8	106.5±3.1

Values (mean ± SD) are average of three samples of each formulated oil, analysed individually in triplicate (n = 1x3 x 3), (P < 0.05); superscript letters within the same row indicate significant (P< 0.05) differences of means within the antimicrobial activity of formulated oils of *Cuscuta reflexa* stem collected from different host plants. Subscript letters within the same column indicate significant (P< 0.05) differences of means of antimicrobial activity of formulated oil against different microbial strains.

[A] Amoxiclin (30 µg/disk) for bacterial and Flumequine (30 µg/disk) for fungal strains.

[B] DD, Diameter of inhibition zone (mm) including disc diameter of 6 mm.

[C] MIC, minimum inhibitory concentration (µg/mL).

Table 5: Haemolytic activity of formulated oils against human and bovine erythrocytes

Samples	Human erythrocyte	Bovine Erythrocyte
Oil-A	ND	ND
Oil-B	ND	ND
Oil-C	3.7±0.3	9.1±0.5
Oil-D	ND	ND
Oil-E	ND	ND
Phosphate buffer saline	ND	ND
Triton-X-100	98.32	97.18

Values (mean ± SD) are average of three samples of each formulated oil, analysed individually in triplicate (n = 1x3 x 3), (P < 0.05)
ND: Not detected.

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Efficacy of formulated hair oil in hair fall control

All the volunteers had reported that they used to comb the hair nearly 2-3 times a day and had been obtaining similar number of hairs in the range of 390-434 during each combing. The number of hairs obtained from the test oil users had reduced to 291-361 after 10 days of oil usage. The number of hair fall reduction (15-82 hairs per each combing) was maximum on the 60th day of experiment. Maximum hair fall reduction (96.38%) was noticed by using oil A indicative of the fact that nutritive value of *C. reflexa* stem collected from Indian lilac is more suitable for hair oil formulation, whereas in the case of mustard oil (Control oil) users, hair fall reduction was negligible (14.38%). Other test oils B (92.40%), C (78.97%), D (89.67%) and E (94.89%) also showed significant hair fall reduction as depicted in Table 6a. Microscopic examinations have revealed that 84.64-96.86% of hairs obtained from each group of volunteers on day zero have roots. On 10d of test oil usage of A, B, C, D and E, reduction in number of hairs with roots was 33.42, 40.80, 33.96, 17.26 and 53.74% respectively and that reached its maximum level of reduction (100, 100, 94.88, 99.40 and 98.62 % respectively) on 60d of oil usage. In case of control oil users, the hairs with hair roots obtained before and after usage were almost same, such as 14.44% and 18.89% on 10d and 60d (Table 6b).

Table 6a: Hair fall reduction in volunteers (5 in each) after use of the formulated or mustard oil by combing assay

Sr No.	Formulated hair oils	No of hair fall after use of oils by combing assay (%)						
		Before use	10d	20d	30d	40d	50d	60d
1	Control	421±5.01	401±6.01	321±5.00	378±4.23	382±3.27	352±2.92	361±2.63
2	A	414±6.11	310±4.25	154±2.76	101±4.44	72±1.01	13±0.02	15±0.22
3	B	434±4.99	298±4.00	167±3.01	156±5.32	91±2.95	58±1.11	33±0.13
4	C	390±3.85	302±3.22	289±3.11	231±3.56	167±2.57	111±2.58	82±1.42
5	D	397±5.36	361±3.71	300±2.89	199±2.14	132±1.77	82±0.94	41±0.82
6	E	411±5.26	291±5.10	215±2.79	178±2.41	100±1.01	56±0.15	21±0.41

Note: Control=Mustard oil, Oils of *Cuscuta reflexa* stem collected from *A. indica* (A), *Z. Jujuba* (B), *M. alba* (C), *A. arabica* (D) and *C. latifolia* (E)

Table 6b: Hair fall reduction (With hair root) in volunteers (5 in each) after use of the formulated or mustard oil by combing assay

Sr No.	Formulated hair oils	No. of hairs with hair roots obtained after use of oils by combing assay (%)						
		Before use	10d	20d	30d	40d	50d	60d
1	Control	381±4.11	326±3.13	292 ±2.67	214±1.36	235± 2.33	287± 3.01	309 ±4.01
2	A	401±2.80	267±2.11	124 ±2.01	36 ±0.44	21 ±0.22	02 ±0.00	0
3	B	402±4.36	238±2.35	115±2.83	93 ±0.93	32 ±0.23	0	0
4	C	371±2.79	245±2.33	211 ±2.69	178 ±2.99	132 ± 1.11	67 ±1.03	19± 1.02
5	D	336±3.15	278 ±3.51	226 ±1.72	79 ±2.35	58 ±0.92	11±0.23	2 ±0.00
6	E	361±4.01	167 ±2.14	123 ±1.22	98 ±1.26	45 ±0.27	15 ±0.21	5 ±0.00

Note: Control=Mustard oil, Oils of *Cuscuta reflexa* stem collected from *A. indica* (A), *Z. Jujuba* (B), *M. alba* (C), *A. arabica* (D) and *C. latifolia* (E)

Efficacy of formulated hair in dandruff control

For the evaluation of antidandruff activity of formulated hair oil, eighteen patients suffering from dandruff were enrolled for present study. Highly significant reduction in the score (2.5891 and 2.5997 to 1.00 and 1.012) of white scale formation was observed in patients of groups A and B treated with oils A and B, whereas in group D and E, reduction in score was from 2.7139 and 2.6983 to 1.020 and 1.017, having treatment of oils D and E. Group C having treatment of oil C showed comparatively less reduction in dandruff (2.726 to 1.589) at the end of two weeks. Control group showed no response against dandruff (2.5492-2.3891), indicating that mustard oil could not be used as antidandruff. These score results are indicated in Table 6c.

Table 6c: Improvement in the mean score for scalp white scales with antidandruff hair oils (Formulated oils)

Sr No.	Formulated hair oils	Score for scalp white scales in human volunteers	
		Before use	2nd week
1	Control	2.5492±0.01	2.3891±0.03
2	A	2.5891±0.01	1.000±0.00
3	B	2.5997±0.00	1.012±0.01
4	C	2.726±0.03	1.589±0.01
5	D	2.7139±0.05	1.020±0.00
6	E	2.6983±0.03	1.017±0.00

Note: Control=Mustard oil, Oils of *Cuscuta reflexa* stem collected from *A. indica* (A), *Z. Jujuba* (B), *M. alba* (C), *A. arabica* (D) and *C. latifolia* (E)

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Efficacy of formulated hair oils in hair growth activity

Hair growth activity was observed in six groups of mice (five in each) after applying the test oils for the period of 28 days. Firstly, skin irritation test was applied on test animals for the period of 48hrs. All the animals were subjected to testify for oedema and arythma. It was found that all test oils were safe and produced no irritation to test animals.

These animals were then subjected to hair growth activity test. The topical application of herbal hair oils on denuded skins of experimental animals showed excellent hair growth initiation, as well as completion which were comparable to control. However, exact mechanism of hair growth stimulation was still unknown and further studies are conditioned in order to evaluate exact mechanism behind the hair growth stimulation. It was also observed that in hair oil treated group, the texture of hair was smooth as compared to control. Hair growth activity was initiated in the first week (sixth day) in test animals (Groups A and B) treated with oils A and B. This activity was approached to its optimum on the seventeenth and eighteenth day; whereas in test animals in groups C and D treated with oils C and D, growth activity was started on eight day of test period approaching to its extreme on the twenty-first and seventeenth day. Group E treated with oil E showed initiation of hair growth on the tenth day and reached its completion on the twentieth day. In control group animals, hair growth initiation in denuded skin area was observed on the eleventh day that took twenty six days of completion as shown in Table 7a.

Hair growth activity was also observed by measuring the average length (mm) of hairs (ten hairs from each animal in each group) at different time intervals after beginning the treatment of formulated hair oils. Significant increase in length was observed in animals treated with formulated hair oils which were comparable to control group. On the seventh day, average lengths of hairs were (mm) 2.1, 2.5, 1.6, 1.9 and 1.9 of groups A, B, C, D and E treated with oils A, B, C, D and E respectively. Length of hairs exceeded its maximum (13.15, 12.77 and 10.49) on the twenty-first day in test animals of groups A, B and E; whereas in case of animals of groups C and D, maximum length of hairs 10.12 and 12.31 was obtained on the twenty-eight day as reported in Table 7b.

Table 7a: Hair growth observation (Minimum time to initiate/complete hair growth)

Formulated hair oils	No. of test animals	Minimum time to initiate growth (days)	Minimum time to complete growth (day)
Control	5	11±0.11	26±0.82
A	5	6±0.14	17±1.11
B	5	6±0.01	18±2.11
C	5	8±0.02	21±0.91
D	5	8±0.21	17±0.32
E	5	10±0.93	20±1.11

Note: Control=Mustard oil, Oils of *Cuscuta reflexa* stem collected from *A. indica* (A), *Z. Jujuba* (B), *M. alba* (C), *A. arabica* (D) and *C. latifolia* (E)

Table 7b: Length of hair at different time intervals after beginning the treatment of formulated hair oils (mm)

Sr No.	Formulated hair oils	Length of hair at different time intervals (mm)			
		7 day	14 day	21 day	28 day
1	Control	1.2±0.001	3.4±0.002	3.5±0.01	3.5±0.01
2	A	2.1±0.003	10.12±0.1	13.1±0.11	13.05±0.17
3	B	2.5±0.001	10.04±0.41	12.77±0.2	12.71±0.11
4	C	1.6±0.001	8.5±0.13	10.11±0.1	10.12±0.07
5	D	1.9±0.002	8.7±0.02	12.04±0.3	12.31±0.09
6	E	1.9±0.001	9.3±0.11	10.49±0.13	10.18±0.07

Note: Control=Mustard oil, Oils of *Cuscuta reflexa* stem collected from *A. indica* (A), *Z. Jujuba* (B), *M. alba* (C), *A. arabica* (D) and *C. latifolia* (E)

Discussion

The variation in moisture contents of *Cuscuta* stems might be attributable to variation in genetic makeup. Protein contents indicate that *Cuscuta* stem is a potential rich source of protein and could be used in any dietary tonic or hair formulations. Maximum level of ash indicates the maximum level of mineral contents present in stem which is essential in many growth activities of plant. These minerals are different salts of Ca, Cu, P, Fe and Zn. These salts are important in *Cuscuta* growth, especially in enzyme activities. Variation in mineral level of *Cuscuta* stems is dependent on the mineral contents of the host plant present in different environment of soil and air. Gupta and Sharma (2006) and Hussain et al. (2009) also reported the presence of minerals in *C. reflaxa.*

Table 2 indicates the nutritive value of *Cuscuta* stem. As *C. reflexa* is a parasite and dependent on its host for its survival, it acquires all nutritive requirements from host body; so, its nutritive value is comparable to host. Gupta and Sharma (2006) also reported the similar nutritive value of *Cuscuta* stem. Wong et al (2006) also reported the antioxidant activity of the *Cuscuta* stem. *C. reflaxa* is one of the commonly used herbal constituents and is a functional attribute that is used in medicinal tonics. It is often added as nutrient in porridge and alcoholic beverages to improve vision and impotence, and also used to prevent abortion as well as aging in clinical treatment. It possesses anticancer and immune stimulatory activities due to its dietary factors. It also shows anti-inflammatory, antipyretic, antiviral, and pesticidal activities (Mahmud et al., 2009). Kumaar et al (2011) examined many phytochemical constituents with effective pharmacological study in *C. reflexa*. In Pakistan, medicinal plants have contributed immensely to healthcare just because they are recognised in traditional medical system and are identified as indigenous medicinal plants which are cheap and easy to access. These established a good support to the use of this plant as medicine (Suffredini et al., 2004).

The prepared formulations are yellow to reddish-brown in colour. This change in colour might be due to the presence of browning substances that are very polar due to active radicals like phospholipids. These substances result from Maillard type non-enzymatic reaction and caramilization. Phospholipids were reported to cause darkening of the oils as a result of phospholipid degradation during heat treatment. Increase in

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darkening substances may be attributable to the increase in contents of other lipids like glyceroglycolipids (Anjum et al., 2006). Specific gravity was found in the range of 0.9434-0.9527 which showed that it is less dense than water, but denser than other edible oils. This might be due to the occurrence of polymerisation that makes the oil denser. Refractive index was noted in the range of 1.421-1.461, indicating that saturated fatty acids in the oils might undergo polymerisation during formulation of hair oil. Iodine values (87.29-102.25 mg /100g) indicate that all formulated oils contained significant amount of saturated fatty acid which is evident from free fatty acid values that were found in the range of 1.24-2.13 mgKOH/g. The saponification values of oils were in the range of 197.29-234.17 mgKOH/g. These high saponification values are an indication of the fact that the oil may be suitable for soap making and shampoos. Unsaponifiable (unsap) matter was detected in the range of 0.201-0.511%, indicating the presence of minor constituents like tocopherols, tocotrienols and phytosterols in the oils.

The antimicrobial and haemolytic activities of formulated oils were evaluated against selected bacterial and fungal strains. All the formulated oils showed potent antimicrobial activity against all formulated oils. Antimicrobial activities of *C. reflexa* stem were also reported previously (Pal et al., 2006; Faiyyaz et al., 2011; Mehjabeen et al., 2011; Mateen et al., 2011), but formulated oils were not evaluated for antimicrobial activity before. Our findings were similar to the results by Harsh (1998). It can be inferred that the oils A and B have stronger and broader spectrum of antimicrobial activity. Antimicrobial activity of *Cuscuta* stem was also reported by earlier scientists (Pal et al., 2006; Uddin et al., 2007; Khan et al., 2010; Kumar et al., 2011; Ashwan et al., 2012). Formulated oils were found safe and showed no haemolytic activity except oil C whereas other scientists showed haemolytic activity of C. stem as reported by Lotufo et al (2005) who reported the haemolytic activity of *C. reflexa* against mouse erythrocytes. Previous studies have also demonstrated the cytotoxic activity of *C. reflexa* (Jose et al., 2001; Khan et al., 2010).

These observations confirm the folk uses of these medicinal plants and justify the ethno botanical approach in the search for novel bioactive compounds. The present status of medicinal plants provides opportunity to benefit from the emerging marks as developing countries posses most biodiversity of medicinal plants. It is concluded that in accordance with the chemical literature findings on resistant strains of organism, plant biodiversity may lead to unexpected research findings (Mahmud et al., 2009).

Formulated hair oils were subjected for hair treatment. All formulated oils showed considerable improvement in hair fall reduction, dandruff control as well as hair growth activities indicative of the fact that oils formulated from C. stem contained significant nutritious components essential for hair growth. Similarly, Roy et al. (2008) also reported the hair oil formulation from *Cuscuta* stem having hair growth promotion capabilities. *Cuscuta* stem is an excellent hair tonic due to its nutritive value as it contains different antioxidants, phytosterols and saponins as reported by Jadhave et al (2009). Similar findings have been reported by Roy et al (2006) and Patni et al (2006).

Hence, along with the mechanism of action and compounds showing bioactive properties in formulated hair oils, further research work is required to isolate and develop compounds exhibiting properties of dandruff and hair fall control, as well as promoting hair growth activities.

There were no clinically significant adverse reactions, either reported or observed, during the entire study period and overall compliance to the treatment of formulated oils was excellent.

Dandruff is the mildest manifestation of seborrheic dermatitis and is caused by *P. ovale* combined with multiple host factors. Androgenic influence may be responsible, when the level of sebaceous activity is at its peak. Dandruff is commonly aggravated by changes in humidity, trauma (scratching), season and emotional stress. *Pityrosporum* organisms are linked to T-cell depression, increased sebum levels and an activation of the alternative complement pathway.

The aim of treatment was to reduce the level of the *Pityrosporum ovale* as well as other micro-organisms on the scalp, and the goals of therapy were to reduce morbidity and prevent complications. A variety of topical compounds with antipityrosporal activity are useful in treating dandruff. Imidazoles, selenium sulphide, zinc pyrithione, coal tar and salicylic acid are the common drugs used for the treatment of dandruff, either alone or in combination. But, with the available therapies, the problem of complete symptomatic control and clinical cure are not addressed and dandruff usually recurs on the stoppage of the treatment.

This study has been observed as a significant reduction in the mean scores of white scales. The excellent antidandruff action of formulated hair oil might have been due to the synergistic antifungal, anti-inflammatory and local immune stimulatory actions of its ingredients.

The principal ingredients of Indian lilac, Jujuba, Mulbery, Indian gum and Sebes tan plum are glycosides, flavonoids, proteins, phytosterol and triterpenoid glucosides as depicted in table 2. Formulated hair oil has potent antioxidant efficacy attributable to the presence of flavonoids as reported by Wong et al (2006).

Conclusion

Present investigation has clearly shown that formulated oils from stems of *C. reflexa* collected from different host plants exhibited antimicrobial, antidandruff, anti hair fall, as well as hair growth promotion activity that might be due to the presence of a number of nutrients like different proteins, fatty acids, flavonoids, and saponins. Oils A and B revealed more significant response towards hair problems, as well as hair growth promotion activities. .

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