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Original Research Article

Isolation and identification of hair growth potential fraction from active plant extract of *Blumea eriantha* DC grown in Western Ghat of India: In silico study

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ABSTRACT

Background: In *Aayurveda*, *Blumea eriantha* DC has been used in the management of various diseases and is found to exhibit antioxidant and anti-hyperlipidemic, hypoglycemic, anti-diarrhoeal, larvicidal, anti-microbial properties.
Objective: The present study has focused on isolation of the active fraction from *B. eriantha* DC extract and to investigate its effect as a hair growth promoter along with identification of phytoconstituent(s) responsible for hair growth activity and its probable mechanism of action.
Materials and methods: Our work introduces an effective isolation protocol for the active fraction from *B. eriantha* DC extract using chromatographic techniques. Fraction A was isolated by using mobile phase toluene:acetone (9:1). *In-vitro* and *in-vivo* methods were executed for the evaluation of hair growth activity. Moreover, the docked conformations of the isolated phytoconstituent Dimethyl sulfone was compared to Minoxidil for selected proteins namely 2FGF, 2PVC and 4U7P. The PDB identifications 2PVC (DNMT3L recognizes unmethylated histone H3 lysine 4), 4U7P (Crystal structure of DNMT3A-DNMT3L complex and 2FGF (Human Basic Fibroblast Growth Factor) were downloaded from Protein Data Bank.
Results: The study data revealed that *B. eriantha* DC alcoholic extracts exhibited prominent hair growth activity and it was affirmed that Dimethyl sulfone a phyto-constituent isolated from *B. eriantha* DC alcoholic extract contributed for the same.
Conclusion: The findings strongly suggest hair growth promotion potential of the extract of *B. eriantha* DC.

1. Introduction

Hair, often regarded as a vital component and significantly contribute to the general attractiveness of an individual. It is considered as a fundamental element of the overall appeal of the human body [1]. There is no dispute that hair loss is one of the main dermatological complaint globally [2,3]. Alopecia refers to the disappearance of hair development in regions of the human body where hair formerly grow. It may be probably owing to the damage in hair follicles or physical damage. It is typically characterized depending on the reasons and symptoms and named accordingly

Abbreviations: BE, *Blumea eriantha* DC; AR Grade, Analytical grade; CC, Column chromatography; TLC, Thin layer chromatography; GC-MS, Gas chromatography-mass spectrometr; HPLC, High performance liquid chromatography; IAEC, Institutional animal ethical committee; SUK, Shivaji University, Kolhapur; RCSB-PDB, Research collaboratory structural bioinformatics protein data bank; SEm, Standard error mean; S.D, Standard deviation.

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such as alopecia diffuse, premature alopecia, and alopecia totalis. Among them, alopecia totalis is temporary and reported to be fully healed by dermatological therapy in certain instances, although the former two forms of alopecia are challenging to handle. However, premature alopecia is affected by hereditary effects, although it is often stated that it is triggered by abnormal sebiferous, formation of excess dandruff, peripheral circulatory or sex hormone, inadequate blood circulation and excess hair treatment such as brushing and use of cosmetics like shampoo, conditioners etc. Although prevalence of premature alopecia has increased dramatically especially in younger populations at an alarming rate, no effective remedies have been developed yet.

Alternative systems of medicine such as *Aayurveda, Siddha and Unani* have the potential to meet health care needs of ever-increasing population of India [4]. In the current era, use of several medicinal plants has gained wide popularity, as medicines originated from plants/herbs have been considered as safe and greatly devoid of side effects, compared to their synthetic counterparts [5,6].

The plant genus *Blumea* is mainly originated in the tropical and sub-tropical zone of Asia, especially, the Indian Subcontinent and Southeast Asia [7]. The species *B. eriantha* DC (BE) has widespread in India, especially Maharashtra, Karnataka, Madhya Pradesh, dry region of Uttar Pradesh, and Orissa. It is a slim, perennial herb, growing, up to near about one meter in height often dichotomously [8,9]. Singh and Parthasarathy reported that BE extract contain flavonoids, phenolic compounds, carbohydrate and phytosterol etc [10]. In the *Aayurvedic* medicinal system, BE has been used in management of various diseases viz: studies on extracts of BE and their isolate(s) have shown anti-oxidant and anti-hyperlipidemic potential [11], anti-diarrhoeal activity [12], hypoglycemic activity [13], larvicidal activity [14], antimicrobial activity [15] and anti-cancer activity [16]. Moreover, the Bhinge et al (2020) have reported the hair growth potential of prepared iron and silver nanoparticles of BE extract [17]. Also, in India, BE has been claimed for hair growth promotion activity. Thus, it trigerred the search for hair growth promotion activity of BE, subsequently resulting in to an Indian patent on BE by the authors of the present study [5].

Various medications comprising of herb extracts have been widely available in the market to treat alopecia [18]. However, their hair growing outcome is far from satisfactory. Assessing the impact of intervention on resting and growing phases of hairs could be a rational approach for reporting efficacy of alopecia treatments. Because, alopecia results from inhibition of proliferation of hair follicle cells and its conversion to hair in the growing stage. There still exists a desperate need for natural remedies containing natural isolate that may be useful to prevent hair loss and/or regenerate hairs, and has no adverse effects. Owing to long-term usage, the physiological impact cannot be imposed on long-term drug intake/application or cause poor patient compliance. The present study aims to explore the effectiveness of BE extract and isolated fraction recovered from natural source for management of such hair fall and hair related diseases. Characterization of isolated phyto-constituent from the fraction and also explore their probable mechanism with the help of molecular docking study.

2. Experimental methods

2.1. Chemicals

AR grade chemicals were procured for the proposed analysis. MINTOP 2% liquid (Dr. Reddy's Laboratories Ltd, India) containing Minoxidil sample was procured from Shree medicine Shopee, Karad, Maharashtra, India, 415 110 [19]. Alcohol was obtained from Merck Germany.

2.2. Extraction of plants materials

Plant material of BE was stored from Bhinge-wadi, Atpadi region of Maharashtra, INDIA-415301, in the month of January from their natural territory. The taxonomical recognition of the BE was carried out with Herbaria (RMRC - 502) From Regional Centre of Indian Council of Medical Research, Nehru Nagar, Belgaum, KA-590010 [17]. In order to prevent degradation of the phyto-constituents, plant materials of BE were dried in oven at 40 ± 2 °C for a day and then stored in a air tight jar. About 300 g coarse plant material was defatted with pet-ether for 63–85 °C in an extractor, further it was extracted with an 95% alcohol [5]. The collected alcoholic extract was further concentrated on rotary evaporator and also stored in a vacuum dryer until used [5,19]. The yield of an alcoholic BE extract was calculated to be 15.15% (w/w).

2.3. Qualitative analysis of BE extract

A Phytochemical screening of BE was performed for the qualitative study of different phyto-constituents as per methods previously mentioned by Siddiqui and Ali (1997), Trease (1989), Sithara et al. (2016) [20–22].

2.4. Isolation of fraction A from BE extract

Silica was activated at 110 °C in hot air oven for continuous 1hr and then about 140–150 gms was weighed and filled in a glass column. Hexane solvent was used to build the column. However, small amount of hexane had been permitted to remain at the top of the column around 2 cm. The present air bubbles in the prepared column were removed with gentle tapping.

2.0 gm of BE extract was dissolved in alcohol, prepared sample solution then applied on the bed of silica with the help of pipette. Furthermore, the prepared column was eluted successively with optimized mobile phase i.e Toluene: Acetone (9:1). However, different fractions were isolated and examined by TLC to verify chemical homogeneity, then, same RF value fractions were collected, combined and crystallized for further studies.

2.5. Analysis of isolated fraction A with gas chromatography-mass spectrometry (GC–MS)

The solution of Fraction A was analyzed under GC–MS (Model QP2010 Ultra technology, Make - Shimadzu, Kyoto, Japan), equipped with DB-WAX column having size 60 m (0.25 mm × 0.25 mm, Make - Agilent Technologies, United States of America) [19]. Helium (99.999%) gas was used as carrier and 2 μL of Fraction A solution was injected into the GC/MS system with maintaining flow rate at 1.0 mL min^{-1}. Initial temperature of oven was set at 60 °C then gradually increased to 110 °C at 2 C/min rate, and a final hold at 280 °C for 9 min as per previously reported procedure [23]. Moreover, temperature of interface and ion source was set at 275 °C and 200 °C respectively. A mass spectrum of Fraction A was taken in selected range (*m/z* 45e850). Observed results were confirmed with screening the retention time (RT) and MS with those from SUK, MS 416004, using an automated library search, finally, % of composition was estimated by peak area under curve [19,23].

2.6. Isolation of dimethyl sulfone from fraction A

Column chromatography (CC) and thin layer chromatography (TLC) were applied for the separation and isolation of fraction and phytoconstitutents using silica gel, Silica Gel 60 F, 70–230 mesh ASTM (Merck 7734).

TLC of dimethyl sulfone was analyzed using commercially available Merck Aluminium supported silica gel 60 F254 TLC sheets (Merck 1.05554.0001). The elution of the column with Toluene: Acetone (9:1) afforded colorless semisolid mass of 1, which was purified by Thin Layer Chromatography (Toluene: Acetone; 9:1). A UV spectrum of Dimethyl sulfone was obtained using a JASCO V 630 Ultraviolet–Visible Spectrometer. Moreover, an IR spectrum was recorded in the range of 4000–600 cm^{-1} by using a JASCO FTIR-4600 and the NMR spectra were acquired with a Brucker DNP-NMR (400 and 500 MHz).

2.7. High-performance liquid chromatography (HPLC)

HPLC is an advanced tool used to separate, identify and quantify each individual component in mixture. HPLC system of Cyberlab – chrom - HPLC, V 4.0 (Cyberlabs, United State of America) series was used with injection volume of 10 μL isolate prepared in diluents containing water and acetonitrile (50: 50 v/v). A silica based C18 columns with different stationary phases material were checked for separation of isolated Dimethyl sulfone. The C18 (Shiseido, Japan) DDS5 column with L.D 4.6 mm and length 250 mm (particle size 5 μm) was used for further study. The flow rate of pump was set at 1.0 mL min^{-1}. Mobile phase for the method was optimized as per procedure reported in a previously published report containing buffer solution with pH 3.0 (containing potassium dihydrogen phosphate and triethyl amine): Acetonitrile in the ration of 50:50 [24].

2.8. Hair growth promoting activity

2.8.1. Animals

30 Albino mice (Male 15 and Female 15) weighing between 17 and 26 gm were obtained for evaluating hair growth potential of BE extract. The selected mice were housed in polypropylene cages under standard conditions with 23±3 °C and 35–65% humidity defined by the IAEC Committee. The proposed work was granted by RCP, Kasegaon, Maharashtra, INDIA, 415404 as per guidelines of CPCSEA (Protocol Number - RCP/IAEC/2016-17/P-001). Moreover, mice were quarantined for 7 days prior to the hair growth testing. Activity was continued as per the protocol/guidelines laid by Indian Government.

The efficacy of an extract of BE in mice was studied. The mice in all groups were morphologically preselected in 5 groups (6 mice per group) for their hair growth cycle [19,25]. The selected mice were shaven with an area of 4 cm × 3 cm. The selected mice were shaved from their dorsal portion by a 4 × 3 cm area for this experiment. 0.2 mL of 1.5% w/v BE plant extract, 3% w/v BE plant extract and 3% Fraction A were taken in separate labeled test tube with alcohol (95%). The prepared solutions were applied to denuded area of the respective groups once a day. A Minoxidil 1 % suspension treated group was applied 1% Minoxidil (MINTOP) solution in 95% alcohol [26]. Vehicle control group received saline water. The application of 2% MINTOP suspension was continued for 28 days and simultaneously the treatment hair growth pattern was observed [19,27,28]. Below mentioned parameters were assessed for hair growth promotion and/or hair growth restoration in experimental model.

2.8.2. Skin irritation test

Plant extract should not have any irritating effect on the shaved region and should not cause skin lesions such as itchy eyes, skin rashes, inflammation, stuffy nose etc. [17]. Shaven dorsal region of the selected mice was properly cleaned with surgical spirit, further, BE alcoholic extracts (1.5% and 3%) and 3% Fraction A solution were rubbed on mice shaved region over 1 sq cm and the mice were monitored for 2 days for any skin lesions [19].

2.8.3. Hair growth initiation and completion by qualitative analysis

The hair growth was confirmed with visual observation of two well known parameters namely, hair growth initiation (less period in days to begin detectable hair growth) and hair growth completion period (less period in days taken to fully cover the shaved skin area with new hair) [2,29]. The qualitative analysis for the determination initiation and completion period of hair were counted for BE extract, Fraction A, Minoxidil (standard) treated animals, which was significantly compared with control or vehicle treated animals.

2.8.4. Hair length and weight

Twenty hairs were pulled from the earlier denude region of selected mice on 20th, 25th and 30th day of study [19]. The measured lengths of hairs were noted and the mean length in cm was taken for the final calculation. The final results were represented as the mean length ± S.D. of plucked 20 hairs [19,27,30].

BE extract (1.5 and 3%), 3% Fraction A, 1% Minoxidil and vehicle treated mice group were euthanicated on the 28th day of drug application [19]. Moreover, the excised mice skin was set on frame consisting of a metal coin with a radius of 0.9 cm [19]. After weighing skin with hair and without hair, difference in weight was calculated as a hair weight [19,31,32].

2.8.5. Histological studies

Histological studies were performed in accordance with the previously reported procedure [30] with minor modification. The mice from each group were euthanicated on the 31st day after the treatment. The dorsal skin biopsies of the mice from each group were taken and it was fixed in buffer formalin (10% neutral) solution. The sample applied mice tissues were fixed in paraffin wax and uniformly sliced (4 μm) and were stained with haematoxylin and eosin [17]. Digital photomicrographs were captured from respected area at fixed magnification of 100x [17]. However, captured photo of mice tissues were cropped with width at 700 pixels. From the prepared slides, anagen and telogen ratio in % of hair follicles and the total count of hair follicles/mm [33] were calculated. Also, the thickness of the skin using UTHSCSA image tool 300 from epidermis to panniculus carnosus was estimated [19,34].

Optical microscope (Nikon) has been utilized for the quantification of size of follicles [31]. Lastly, numbers of hair follicles were manually counted by the blinded viewer at fixed size area from the dermis and subcutis layer.

2.9. Molecular docking studies

Autodock software, version 4.2 was used for the ligand receptor interaction, whereas, dimethyl sulfone and minoxidil were considered as co-crystallized ligand against selected receptors. Autodock 4.2 software estimated free energies with bound and unbound state of ligand–receptor complexes. Selected protein structures were retrieved from the RCSB-PDB Protein Data Bank. The PDB id are, 2PVC (DNMT3L recognizes unmethylated histone H3 lysine 4), 4U7P (Crystal structure of DNMT3A-DNMT3L complex and 2FGF (Human Basic Fibroblast Growth Factor). Firstly, the structures of Dimethyl sulfone and minoxidil were taken from ACD-Chem-sketch software and then they were converted to molecular 3D form. To identify the putative binding site, we performed blind docking and local docking protocol using the autodock 4.2 [35]. A grid box set covering the entire surface of the protein receptors was generated for identifying the binding pocket. The number of van der Waals Hydrogen-bond interacting residues were obtained from

PyMOL 1.7.4 and Discovery Studio Visualizer [36]. The amino acids with the binding pockets in receptor and selected ligands were predicted at Q-site finder server [37].

2.10. Statistical analysis

The obtained values were presented as mean ± standard error of mean (SEM) of the respective clusters [19]. The difference between subgroups (no of follicles, size of follicles, hair weight, hair length, hair initiation and completion histopathological differences, etc) was assessed using student t test. A value of $P < 0.0001$, 0.05, 0.01, or 0.001 was considered statistically significant [16,19].

3. Results

3.1. Phytochemical screening of BE extract

Anthraquinone; cardiac and saponin glycosides; alkaloids; tannins; carbohydrates and flavonoids were observed in the BE extract after the qualitative analysis [16].

3.2. Isolation of dimethyl sulfone

In recent years, the analysis of organic compounds from plants and the chemical process that are caused by them have been in remarkable focus [38]. The hyphenated tool namely GC−MS is an excellent isolation and identification technique for qualitative determination of phytoconstituents (volatile and semi-volatile) [39]. In the present study total 14 bioactive compounds were confirmed in the Fraction A with their vital chemical properties. The dominating component noted in the Fraction A was Dimethyl sulfone (81.33% RT at 8.275 min with area 7062946). The results are showed in Table 1 and Fig. 1. Therefore, the elution of the column with Toluene: Acetone (9:1) afforded colorless semisolid mass of 1, which was purified by Thin Layer Chromatography (Toluene: Acetone; 9:1) yield: 0.6 g (0.008% yield), Rf 0.46.

Fraction A was re-crystallized and to obatin pure compound Dimethyl sulfone as a white crystalline power from toluene:acetone (9:1) eluent. The UV λ_{max} (MeOH) of Dimethyl sulfone was found to be 265 nm. Moreover, the results of IR spectra confirmed characteristic absorption bands for functional groups namely S=O (1159 cm^{-1}), $-CH_3$ (2923 cm^{-1}) and $-C\text{-}H_{bend}$ (694 cm^{-1}). The ^1H NMR spectrum of Dimethyl sulfone exhibited evidence for six proton singlet at 2.46 ppm.

Single sharp peak at retention time of 4.501 min at flow rate of 1 mL min^{-1} indicate that isolated component is Dimethyl sulfone based on previously reported data (Fig. 1) [24]. The isolated compound Dimethyl sulfone separated by column chromatography were analyzed by HPLC, under the optimum analytical conditions, and the chromatograms are presented in this Fig. 1. As shown in the figure, the HPLC analysis of the Dimethyl sulfone revealed that the purities of compound were 98.90% (compared with standard Dimethyl sulfone).

3.3. Hair growth promoting activity

3.3.1. Skin irritation test

Studies of skin irritation revealed no signs of atypical effects on the denuded region of the selected mice. Moreover, no erythema (redness of the skin), edema (swelling) and scaling/drying of skin was observed at the denuded area on the treated mice. BE extract and Fraction A were found to be safe with zero irritation potential.

3.3.2. Hair growth initiation and completion

Growth of the hair was confirmed from the shaved part on 13.4 days in control vehicle applied animal group; for standard and Fraction A treated group took 6.6 and 7.2 days, and in the mice treated with BE extract, the growth of hair was initiated on 7.2 days and 6.8 days, for 1.5 and 3% BE alcoholic extracts respectively, as depicted in Fig. 2.

The observations recorded for hair growth completion (days required to achieve hair growth similar to hair present on unapplied skin) from the shaved part of animal model. It was noted as 28.2 and 19.4 days for control (vehicle) and standard drug treated animal group respectively. The hair growth completion in case of 1.5 and 3% BE alcoholic extracts treated animal groups were observed to be 21.4 days and 18.2 days respectively. Whereas 21.8 days were observed in Fraction A treated animal group. The final outcomes are depicted in Fig. 2. The results are indicating the mean values ± SEM. As shown in Fig. 2, the whole denuded region of the selected mice after the application of BE extract, vehicle, standard, was observed to be covered.

3.3.3. Measuring of hair length and hair weight

The outcomes observed in Fig. 3, selected mice treated with BE extract produced greater results on the hair growth as compared to standard Minoxidil and control group. 1.5% BE extract, 3% BE extract and Fraction A treated group showed length of hair values as 9.86 mm, 11.08 mm and 10.28 mm respectively, While standard Minoxidil and control treated group exhibited 9.90 and 7.45 mm length of hair respectively (Table 2).

For the group treated with 1.5 and 3% BE crude extracts, hair weight was recorded to be 3.2133 ± 0.0185 and 3.7333 ± 0.0440

Table 1
Major volatile compounds from BE extract by GC-MS.

Sr. no.	Compound(s)	RT	Molecular weight	Molecular formula	Concentration (%)
1.	Dimethyl sulfone	8.240	94.13 g mol^{-1}	$C_2H_6O_2S$	81.33
2.	4,5-Dichloro-1,3-dioxolan-2-one	8.673	156.4 g mol^{-1}	$C_3H_2Cl_2O_3$	1.13
3.	Benzoic acid	10.460	122 g mol^{-1}	$C_7H_6O_2$	0.21
4.	3,5-Diisopropoxy-1,1,1,7,7,7-hexamethyl-3,5-bis(trimethylsiloxy)tetrasiloxane	11.190	546 g mol^{-1}	$C_{18}H_{50}O_7Si_6$	0.13
5.	Biphenyl	13.395	154.21 g mol^{-1}	$C_{12}H_{10}$	12.50
6.	Tricyclo[3.1.0.0(2,4)]hexane, 3,3,6,6-tetraethyl-,*trans*–	14.065	192 g mol^{-1}	$C_{14}H_{24}$	0.56
7.	2-Buten-1-ol, 2-ethyl-4-(2,2,3-trimethyl-3-cyclopenten-1-yl)-	15.505	208 g mol^{-1}	$C_{14}H_{24}O$	0.52
8.	Neophytadiene	18.645	278 g mol^{-1}	$C_{20}H_{38}$	0.28
9.	n-Hexadecanoic acid	19.933	256.4 g mol^{-1}	$CH_3(CH_2)_{14}COOH$	1.29
10.	Propanoic acid, 2-methyl-, 2-[3-[(acetyloxy)methyl]oxiranyl]-5-methylphenyl ester	20.750	292 g mol^{-1}	$C_{16}H_{20}O_5$	0.27
11.	Oleic Acid	22.590	282 g mol^{-1}	$C_{18}H_{34}O_2$	0.52
12.	Bis(2-ethylhexyl) phthalate	28.915	390 g mol^{-1}	$C_{24}H_{38}O_4$	0.29
13.	Naphthalene, decahydro-4a-methyl-1-methylene-7-(1-methylethylidene)-, (4aR-trans)-	31.310	204 g mol^{-1}	$C_{15}H_{24}$	0.41
14.	Naphthalene, decahydro-4a-methyl-1-methylene-7-(1-methylethylidene)-, (4aR-trans)-	33.075	204 g mol^{-1}	$C_{15}H_{24}$	0.57

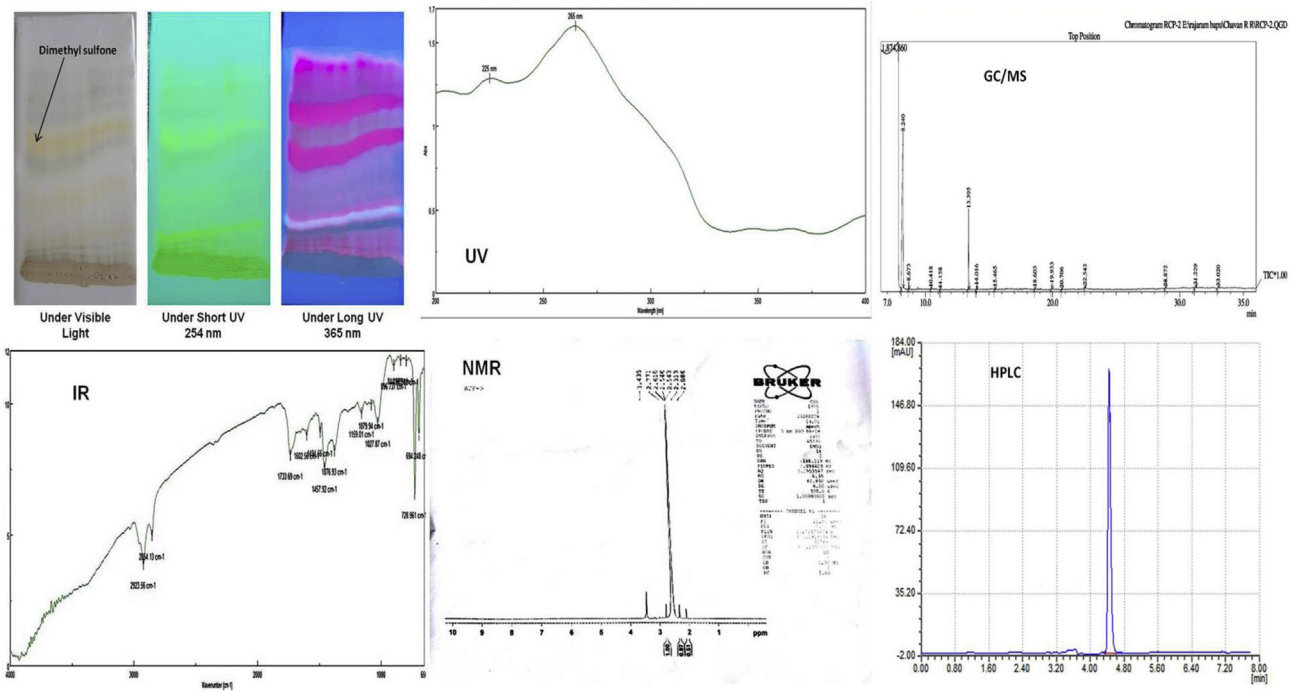

Fig. 1. GC-MS Chromatogram and TLC of Fraction A, UV, IR and NMR and HPLC graph of Dimethyl sulfone.

respectively. The weight of hair was found to be 3.3866 ± 0.1197 and 3.2466 ± 0.0517 for Fraction A and 1% Minoxidil treated mice, respectively (Table 2). However, the vehicle-treated animals noted a far lower hair weight than the other groups. Fig. 2 demonstrates the hair growth pattern. Even, the effect of BE crude extract on growth of the hair was significantly high compared to standard and vehicle group, as depicted in Fig. 3.

3.3.4. *Histological studies*

Fig. 3 depicted the biopsies of the skin, showed noticeable difference in hair follicles (anagen and telogen cyclic phase) of euthanicated mice from BE crude extract and vehicle groups. Conversion of hair follicle in % from telogen phase to anagen phase was noted to be 56.92 ± 1.3% in the clustered applied with of 3% BE extract, whereas in the group treated with 1.5% of BE extract it was

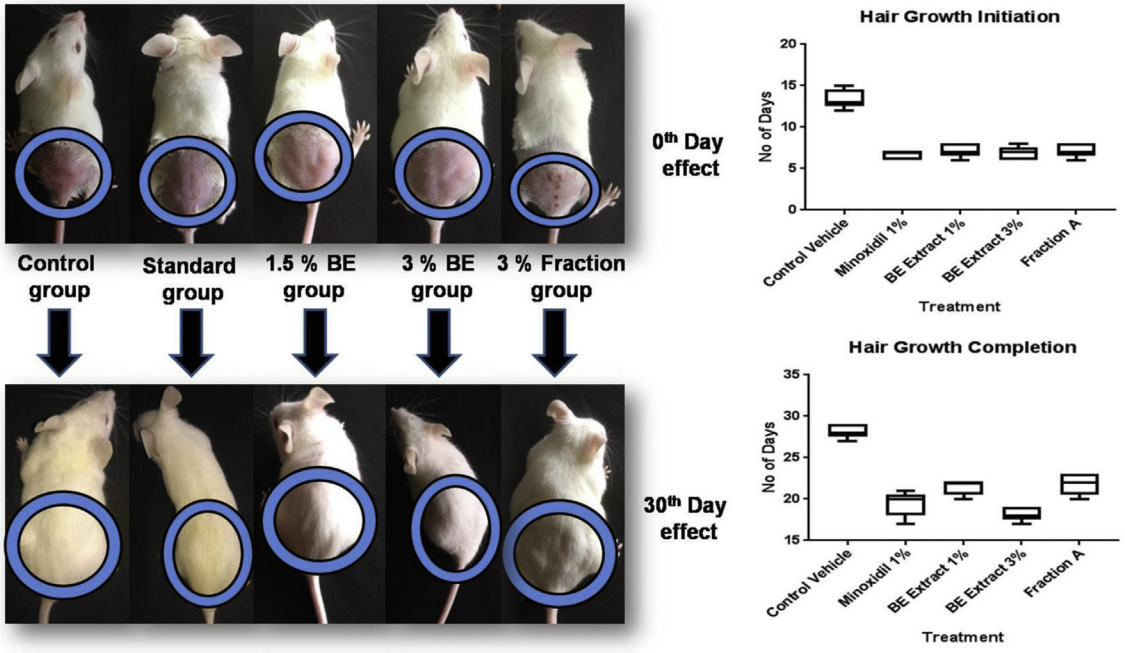

Fig. 2. Hair growth pattern after 30th day effect.

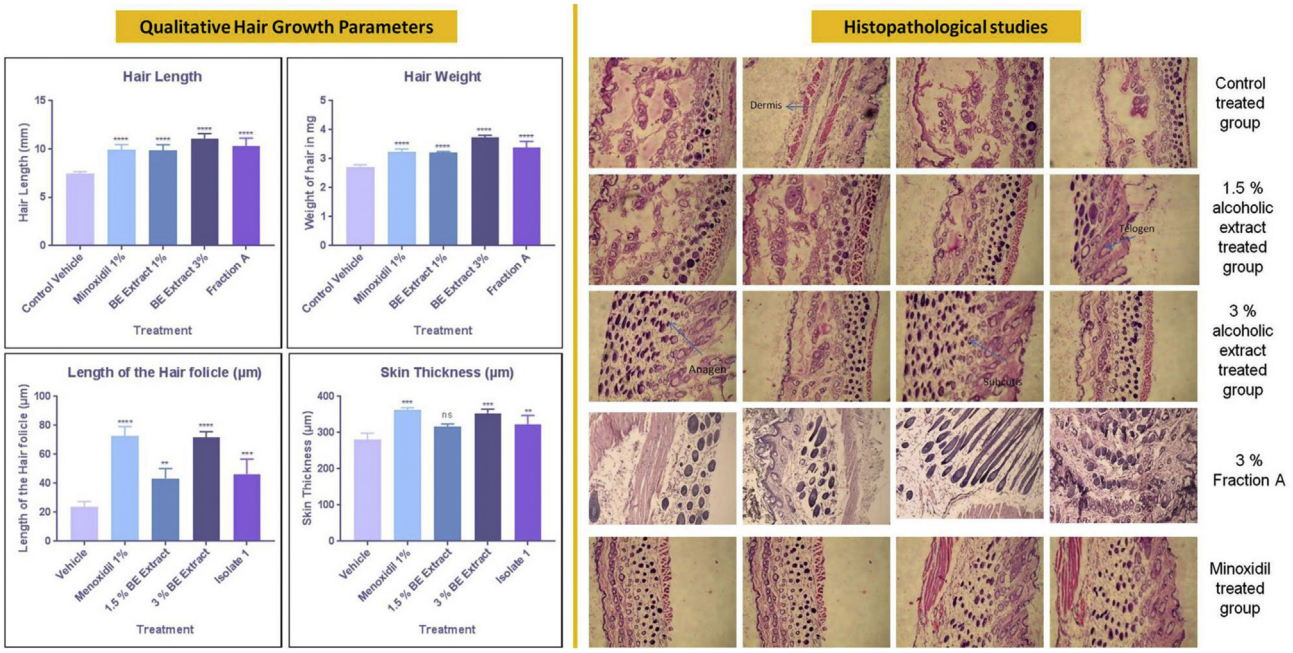

Fig. 3. Effect of BE ethanolic extract on qualitative hair growth parameter **(A)**. Histopathological studies, the number of hair follicle in subcutis layer of animal treated with 3% BE alcoholic extract, 1.5% BE alcoholic extract, 1% Minoxidil solution and control vehicle **(B)** (The results are shown as the mean values ± SEM, **P < 0.0, ***P < 0.001, ****P < 0.0001, when compared to respective control values by Student's *t*-test).

noted to be 53.26 ± 1.3% [17]. The selected mice treated with 1% Minoxidil and Fraction A showed 53.69 ± 1.1 and 54.96 ± 1.2% anagen hair follicle induction, respectively (Table 3). The mice applied with vehicle alone noted very less anagen induction. The all outcomes are showed as the average values ± SEM. ****$p < 0.0001$, when compared with the vehicle values by the t-test [19].

Thickness of the mice skin was quantified using UTHSCSA image tool 300 after 28 days of the treatment [17,19]. The outcomes are noted as the average values ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns − not significant, compared to the vehicle values using the t-test [19]. For the group treated with 1.5 and 3% BE crude extracts, skin thickness of the mice was observed to be 316.66 ± 4.2557 and 353.66 ± 6.3595 µm respectively. Skin thickness was observed to be 323.33 ± 13.9562 and 362.33 ± 3.8441 µm for animal treated with Fraction A solution and 1% Minoxidil, respectively (Table 2). However, the vehicle-treated animals noted a far lower skin thickness than other selected groups (Fig. 2).

3% alcoholic crude extract applied in mice showed a notable differences on the length of hair follicle than those of the groups treated with 1.5% alcoholic extract, vehicle and 2% Minoxidil groups (Fig. 3) [19,31]. The animal applied with 3% and 1.5% BE alcoholic extracts attained an average length of 71.66 ± 2.3333 and 43.33 ± 3.9299 µm, respectively, whereas length of the hair follicle was noted to be 72.6666 ± 3.7564 and 46.3333 ± 5.9254 µm in the Minoxidil and Fraction A solution treated mice, respectively (Table 2).

3.4. Molecular docking study

The ligand−protein interaction geometrics assessment presented in Table 4 revealed the docking scores for Dimethyl sulfone and Minoxidil with interacting 2FGF, 2PVC and 4U7P residues with H-bond, hydrophobic and Van-der Waals interacting residues. It was confirmed that the H-bond and hydrophobic interaction of Dimethyl sulfone and Minoxidil with selected three proteins further stabilized the enzyme−inhibitor interaction. The least binding energy docked conformation of Dimethyl sulfone was found to be −2.74, −3.50 and −3.22 kcal mol^{-1} with the targeted proteins such as 2FGF, 2PVC and 4U7P, respectively. Moreover, Dimethyl sulfone showed binding energy with above listed proteins in the range of −2.74 to −3.50 kcal mol^{-1} (Table 4). The hydrogen bonding interactions of Dimethyl sulfone with target proteins are shown in Fig. 4. Furthermore, the analysis of docked conformation of Minoxidil shows hydrogen bonding and van deer Waals interactions with target proteins (Table 4 and Fig. 4). The least binding energy docked conformation of Minoxidil was found to −4.91, −5.49 and −4.59 kcal mol^{-1} with the targeted proteins namely 2FGF, 2PVC and 4U7P, respectively. Minoxidil exhibited binding energy in the range of −4.59 to −5.49 kcal mol^{-1}.

The H−bond interaction of Dimethyl sulfone with above mentioned receptors further stabilized the enzyme−inhibitor interaction due to Arg107, Arg97, Arg604, Phe608, Pro507,

Table 2
Effect of BE alcoholic extract on hair length (mm), hair weight (mg), Skin Thickness (µm) and Length of the Hair follicle (µm) after 28 Days.

Treatment on mice	Hair Growth Length (mm) (Mean ± SEM)	Hair Growth Weight (mg) (Mean ± SEM)	Skin Thickness (µm) (Mean ± SEM)	Length of the Hair follicle (µm) (Mean ± SEM)
Control Vehicle treated	7.45 ± 0.0878	2.7233 ± 0.0392	281.66 ± 9.8206	23.66 ± 2.1858
Minoxidil 1% suspension treated	9.90 ± 0.2564***	3.2466 ± 0.0517***	362.33 ± 3.8441**	72.66 ± 3.7564***
BE alcoholic extract (1.5%) treated	9.86 ± 0.2616***	3.2133 ± 0.0185***	316.66 ± 4.2557^{ns}	43.33 ± 3.9299^{ns}
BE alcoholic extract (3%) treated	11.08 ± 0.2285***	3.7333 ± 0.0440***	353.66 ± 6.3595*	71.66 ± 2.3333***
Fraction A	10.28 ± 0.3809***	3.3866 ± 0.1197***	323.33 ± 13.95628^{ns}	46.33 ± 5.9254*

The outcomes are exposed as the mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns − not significant when compared to vehicle readings by Student's t-test.

Table 3
Effect of BE alcoholic extract on quantitative hair growth.

Treatment on mice	After 28 Day (% Hair follicle)		
	Telogen	Anagen	Ratio
Control Vehicle treated	81.14 ± 1.3	18.86 ± 0.97	4.3000
Minoxidil 1% suspension treated	46.31 ± 1.2****	53.69 ± 1.1****	0.8627
BE alcoholic extract (1.5%) treated	46.74 ± 1.4****	53.26 ± 1.3****	0.8775
BE alcoholic extract (3%) treated	43.08 ± 1.3****	56.92 ± 1.3****	0.7571
Fraction A	45.04 ± 1.1****	54.96 ± 1.2****	0.8196

The outcomes are exposed as the mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns − not significant when compared to vehicle readings by Student's t-test.

Arg790, and Ser894 amino acids (Fig. 4). Whereas, the hydrogen bond interactions between Minoxidil and selected proteins further stabilized the enzyme−inhibitor interaction due to Pro132, Tyr115, ILE327, Ser325, Arg331, Tyr235 and Lys219 amino acids (Fig. 4).

4. Discussion

Limited modern therapeutics are prescribed for hair disorder like alopecia. Among these therapeutcs, Minoxidil formulation at a concentration of 1−5% has been used topically for the treatment of hair loss [39]. Previous reports have indicated that Minoxidil is responsible for causing cell propogation in epithelial cells close to the root of hair follicles and provokes vasodilation of blood vessels located at the scalp region [40]. However, its use is not recommended at greater concentrations especially in females, owing to its tendency to produce hyotensive effects or hypertrichosis. Another synthetic moiety namely Finasteride has been earlier used as antiprostatic agent, thereafter, it has been used as hair growth promoter and it was observed that daily application of Finasteride for one year, will help in management of hair loss problems [41]. However, it also possesses certain side effects such as difficulty in orgasm, swelling of body parts, impotence, abnormal ejaculation, etc [42].

The use of herbal remedies has gained wide popularity owing to their medicinal benefits over their allopathic counterparts. Phyto-constituents have been known to be safe and relatively free from severe side effect(s), and therefore their use in the treatment of alopecia may prove to be more useful than Minoxidil, Finasteride and other therapeutics. Therefore, it is a need of the hour to put in efforts to explore newer phyto-constituents which will prove to be beneficial to prevent premature loss of hair and also initiate hair regrowth with minimal side effects. The Indian traditional system of medicine Ayurveda have provided multiple treatments for preventing damage to the hair, avoidance of hair loss and management of several other hair related problems. Numerous hair formulations mainly consisting mixture of phyto-herb extracts have been used/reported. However, from the critical evaluation viewpoint, comprehensive qualitative and quantitative studies focusing on the problems of hair growth and/or its regeneration are scarce. In the present study, an attempt was made to isolate the active constituent responsible for the hair growth activity and its probable mechanism with the help of docking studies.

The preclinical study data indicate that, the topical application of 1.5% BE extract solution, 3% BE extract solution, 3% isolated fraction A suspension significantly minimize period for hair-growth initiation and completion compared to vehicle cluster and significantly comparable with standard drugs. Hair length outcomes confirmed that the topical application of BE alcoholic extract (1.5% and 3%) and 3% isolate fraction solution significantly improved the hair length compared to control group. These outcomes confirmed the effectiveness of 3% BE extract in promoting hair growth. Compared to control group, the groups treated with BE alcoholic extract (1.5% and 3%) and 3% isolate fraction suspension clustered showed significant hair growth activity. Also the control group experienced less number of the hair follicles in the anagen stage. Particularly, in the cluster treated with BE crude extract and isolate solution, most of the hair follicles were found in the anagen phase [31]. Also, hair growth activity of BE crude extract was markedly high as compared to the vehicle treated mice. Although the Minoxidil treated group of mice noted the telogen bulb were converted into the bigger anagen follicle [30]. Also, Minoxidil may help to stimulate the vasodilation of scalp blood vessels and the proliferation of epithelial cells near the base of hair follicles [19,40]. The study data suggestes that, the telogen stage of the hair follicle were accelerated into anagen stage in selected mice groups, and to a greater extent in the BE alcoholic extract treated [19] and isolate fraction solution groups. Moreover, we have successfully isolated the major fraction from BE extract responsible for the hair growth activity. The isolated fraction contains almost 81% of dimethyl sulfone. Shanmugam et al., 2009 have proved that dimethyl sulfone has been effectively used for the treatment of alopecia [43]. The effect of the BE alcoholic extract can be attributed to the presence of Dimethyl sulfone, which is the major constituent of the isolated fraction.

Table 4
Hydrogen bonding, van deer waals interactions of Dimethyl sulfone and minoxidil with different target proteins.

Protein	Binding energy Kcal/mol	Atoms involved in the H-bonding	Distance atom pair (1−2) Å	Bond angle in between (1-2-3) Å	Vdw + Hbond + Desolv energy (kcal/mol)
Dimethyl sulfone					
2FGF	−2.84	Arg107-N …..O-Drug	2.0	117.1	−2.74
		Arg107-N …..O-Drug	2.7	111.4	
		Arg97-CH….O-Drug	1.8	156.1	
2PVC	−3.55	Arg604-N …..O-Drug	1.8	153.6	−3.50
		Phe608-CH….O-Drug	2.1	146.9	
		Pro507-CH….O-Drug	2.3	139.3	
4U7P	−3.34	Arg790-NH….O-Drug	2.5	137.5	−3.22
		Arg790-NH...OH-Drug	2.6	135.4	
		Ser894-NH …...O-Drug	2.4	138.4	
Minoxidil					
2FGF	−4.57	Pro132-O …..HO-Min	2.4	122.8	−4.91
		Tyr115-O …..HN-Min	2.8	112.6	
2PVC	−5.16	ILE327-O …..HN-Min	2.4	124.2	−5.49
		Ser325-O …..HO-Min	1.9	149.8	
		Arg331-NH …..O-Min	2.8	102.2	
4U7P	−4.34	Tyr235-O….HC-Min	2.0	151.1	−4.59
		Lys219-CH….N-Min	2.5	166.9	

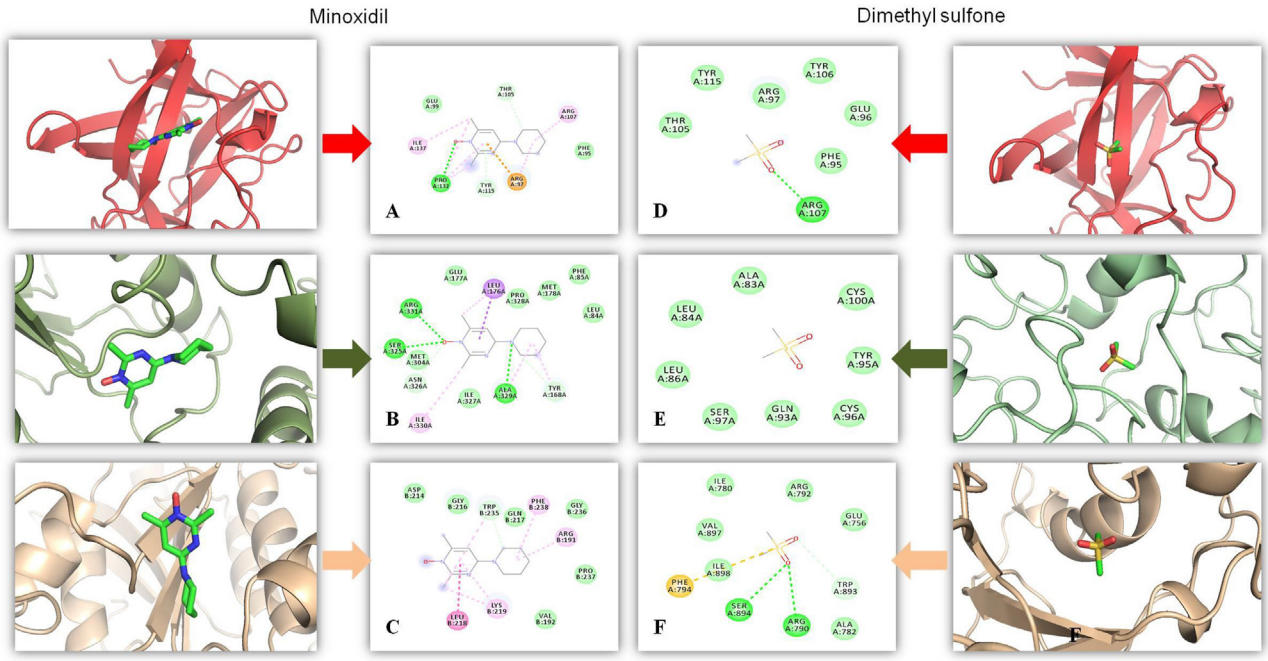

Fig. 4. 2D & 3D binding mode of the Minoxidil and Dimethyl sulfone on 2FGF, 2PVC and 4U7P. Where, in 2D images, the hydrogen bond and Pi-sulfur are represented in fluorescent green and orange yellow in color respectively. And, In 3D image, the Helix, Turn/C Alpha and Sheet are represented in yellow, green and blue in color respectively.

In order to check the mechanism of significant hair growth activity of BE crude extract, GC–MS analysis was used to find the possible phytoconstituents which responsible to get the targeted effect. Molecular docking studies of Fourteen expected structures were confirmed after quantifying the data of GC–MS of BE plant extract. Amongst the fourteen compounds, Dimethyl sulfone was the principal phyto-constituent selected for the further studies based on the review of literature which suggested that this compound is particularly used for the skin and hair care products [44] and therefore it was isolated from the BE plant extract. Further, we continued the docking studies of Dimethyl sulfone and Minoxidil to find their mechanism of action and to compare the docking score of the selected compounds with that of Minoxidil.

In this approach we have studied the ligand–protein interaction along with their docked confirmation on selected protein namely 2FGF, 2PVC and 4U7P. The said proteins belong to the class of DNMT1, DNMT3a, DNMT3b, DNMT3L and TETs, which explored the stability of the selected structure to the genetic material and expression by epigenetic modification of DNA, which proved the noteworthy role in hair growth [45–47]. The results revealed that the docking score of Dimethyl sulfone was almost comparables result with Minoxidil.

These findings suggeste that compared to the other groups, the conversion of hair follicles from telogen stage to the anagen stage was at a greater extent in 3% BE crude extract group. Also, this effect may be due to the presence of Dimethyl sulfone in the BE alcoholic extract as confirmed from the experimental result and the docking studies. The entire work depicted as flow chart diagram is represented in Supplemental 2.

5. Conclusion

The effect of 3% BE extract for hair growth was noted to be more effective compared to 1.5% BE extract, Fraction A and vehicle applied mice. Whereas, quantitative outcome of 3% BE plant extract significantly promoted hair growth by inducing hair follicles from telogen to anagen stage which signify that the mice applied with alcoholic crude extract of BE plant at a concentration of 3% demonstrated effectiveness compared to vehicle and standard treated animal groups. Also, the % of anagen induction with 3% BE plant extract and Minoxidil exhibited almost significant results. Thus, it can be expected that 3% BE alcoholic extract will have similar hair growth pattern as shown by Minoxidil. The hair growth effect may be attributed to the presence of Dimethyl sulfone isolated from the Fraction A and the presence of Dimethyl sulfone in Fraction A as confirmed using GC/MS, IR, NMR and Mass spectra results. The predicted mechanism was also confirmed with docking studies which revealed significant docking scores of Dimethyl sulfone.

Declaration

The authors have filed Indian patent entitled “Composition for Hair Disorder and Process of preparation thereof” No. CBR – 11453, Application No - 201621016966 A, dated 17/05/2017.

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None.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

SDB, NRJ, DSR carried out the design, conduct of the study and wrote the manuscript. SDB and DSR conducted the preparation of extracts. SDB, DSR, NRJ carried out the design, conduct of the study and wrote the manuscript. SDB participated in the data analysis. DSR carried out the data collection. SDB, MAB, RC, BVK carried out

the design, conduct of the study and wrote the manuscript. All authors read an approved the final manuscript.

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Appendix A. Supplementary data

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