Hair Growth Effect of Emulsion Extracted Brevilin A, a JAK3 Inhibitor, from Centipeda minima

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Keywords: network pharmacology, Janus kinase-signal transducer and activator of transcription signaling pathway, Centipeda minima, brevilin A, hair growth

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Janus kinase 3 (JAK3) inhibitors have been used effectively in the treatment of several cases of alopecia universalis and its variants. Our study aims to evaluate whether the emulsion extract of brevilin A from Centipeda minima (CMX) stimulates hair regrowth in a clinical trial, as a JAK3 inhibitor, combined with network pharmacology-based analysis. CMX showed potent inhibition of JAK3 in a concentration-dependent manner. Significant differences in total hair count, terminal hair count, and anagen hair count from the baseline to 24 weeks were observed between the placebo and CMX subjects. The gene set enrichment analysis showed that the targets of CMX are mainly associated with the JAK-STAT signaling pathway, cytokine?cytokine receptor interactions, and the MAPK signaling pathway. This study suggests that the medicinal herbal extract CMX is useful in the treatment of mild to moderate vertex balding that contribute to the visible improvements in hair growth observed in treated patients.

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Hair Growth Effect of Emulsion Extracted Brevilin A, a JAK3 Inhibitor, from *Centipeda minima*

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Abstract: Janus kinase 3 (JAK3) inhibitors have been used effectively in the treatment of several cases of alopecia universalis and its variants. Our study aims to evaluate whether the emulsion extract of brevilin A from *Centipeda minima* (CMX) stimulates hair regrowth in a clinical trial, as a JAK3 inhibitor, combined with network pharmacology-based analysis. CMX showed potent inhibition of JAK3 in a concentration-dependent manner. Significant differences in total hair count, terminal hair count, and anagen hair count from the baseline to 24 weeks were observed between the placebo and CMX subjects. The gene set enrichment analysis showed that the targets of CMX are mainly associated with the JAK-STAT signaling pathway, cytokine–cytokine receptor interactions, and the MAPK signaling pathway. This study suggests that the medicinal herbal extract CMX is useful in the treatment of mild to moderate vertex balding that contribute to the visible improvements in hair growth observed in treated patients.

Keywords: hair growth; brevilin A; *Centipeda minima*; Janus kinase-signal transducer and activator of transcription signaling pathway; network pharmacology

1. Introduction

Alopecia areata, a common autoimmune disease characterized by patchy hair loss, affects all genders, ages, and hair colors [1]. The global prevalence of alopecia areata is approximately 0.1–0.2%, with an estimated lifetime risk of 2%. Besides patchy hair loss, alopecia areata can present with the complete loss of scalp hair (alopecia areata totalis), complete loss of body hair (alopecia areata universalis), hair loss in the occipital scalp (ophiasis), and hair loss over a large scalp area without bald patches (alopecia areata diffusa or alopecia areata incognito) [2,3]. The hair cycle includes three main phases: anagen (active growth phase), catagen (apoptosis-driven phase), and telogen (rest phase). Normally, more than 90% of total scalp hair is in the anagen phase [4]. The hallmark of alopecia areata is the presence of lymphocytes in the bulb region of anagen hair follicles, with the expression of major histocompatibility complex (MHC) class I and II in the follicular epithelium. The abnormal expression of MHC class I leads to the risk of attack by natural killer cells [2].

There is no curative therapy for alopecia areata. To manage this disease, there are two main options: treatment with an immunosuppressive regimen or immune-deviation strategy [2]. Based on the underlying mechanisms of alopecia areata and other autoimmune diseases, therapies associated with the interleukin (IL)-15 pathway, T cell-related mechanisms, or natural killer group 2D (NKG2D) receptor downstream pathways are being developed and tested in early clinical trials [3]. Other future treatment strategies for alopecia areata include recombinant cytotoxic T-lymphocyte-associated protein 4-immunoglobulin targeting, Janus kinase (JAK) inhibition, and stem cell approaches [5]. The infiltration of cluster of differentiation 8 αβ (CD8αβ)+ NKG2D+ T cells into the hair follicle through an IL-15 positive feedback loop with follicular epithelial cells is mediated by the JAK-signal transducer and activator of transcription (STAT) signaling pathway. The upstream-regulated pathways of JAKs are disrupted in patients with alopecia areata [6,7]. Hair regrowth has been observed in patients with alopecia areata treated with tofacitinib (pan-JAK inhibitor), ruxolitinib (JAK1/2 inhibitor), and baricitinib (JAK1/2 inhibitor) [5,8–11].

Brevilin A is a JAK-STAT inhibitor that exerts anticancer activities in several cancer cell lines, such as A549, DU145, MDA-MB-468 [12], and MCF-7 [13] via the suppression of the STAT1 and STAT3 signaling pathways. Therefore, brevilin A could be a potential candidate for the treatment of alopecia areata. In this study, we aim to evaluate whether the emulsion extract of brevilin A from *Centipeda minima* (CMX) stimulates hair regrowth in a clinical trial combined with network pharmacology-based analysis. The initial results showed that a visible improvement in hair regrowth could be observed in patients treated with the medicinal herbal mixture CMX.

Centipeda minima is widely distributed over the areas of China, Korea, and Southeast Asia, also found in Australia and India. It is well-known as a medicinal plant that is used for the treatment of headache, cough, cold, nasal allergy, asthma, diarrhea, and malaria in Chinese medicine [14]. The dried pennywort of *C. minima* is commonly used as herbal tea to cure cold and cough. Recent studies showed that extracts and phytochemicals from *C. minima* have many biological effects like antibacterial [15], antioxidant [14,16], anti-inflammatory [16], neuroprotective [14], anti-melanoma [17,18], acute hepatic injury amelioration [19], allergic rhinitis treatment [20], and anticancer [12,13,21] properties. As the most widespread species of the genus *Centipeda*, *C. minima* is easy to cultivate and has the potential to apply for the development of beverage and natural products.

The major components of *C. minima* have been identified and quantified by high-performance liquid chromatography-quadrupole-time of flight-mass spectrometry (HPLC-Q-TOF-MS) and HPLC-diode array detector, which identified 12 common compounds including phenolic and polyphenolic acids, flavones and their glycosides, and sesquiterpene lactone [22]. Additionally, various pharmacological activities, including antibacterial, antioxidant, and anti-inflammatory properties, of aqueous extracts and isolated compounds have been evaluated [15,16].

To evaluate the effects and underlying mechanisms of CMX on hair loss, we proposed a novel framework that integrates an in vitro investigation, a clinical study, and a network pharmacology-based analysis. We evaluated the inhibitory effects of CMX and brevilin A, the active compound of CMX, on JAK3. Then, we tested the efficacy of CMX on total hair, terminal hair, and anagen hair counts in patient with mild to moderate vertex balding. We conducted a network pharmacology-based analysis to investigate the underlying mechanisms of brevilin A. As natural products exert therapeutic effects via the activation of multiple targets simultaneously [23], network pharmacological analyses are well-suited to investigate the systems-level mechanisms of CMX. Our comprehensive strategy is summarized in Figure 1.

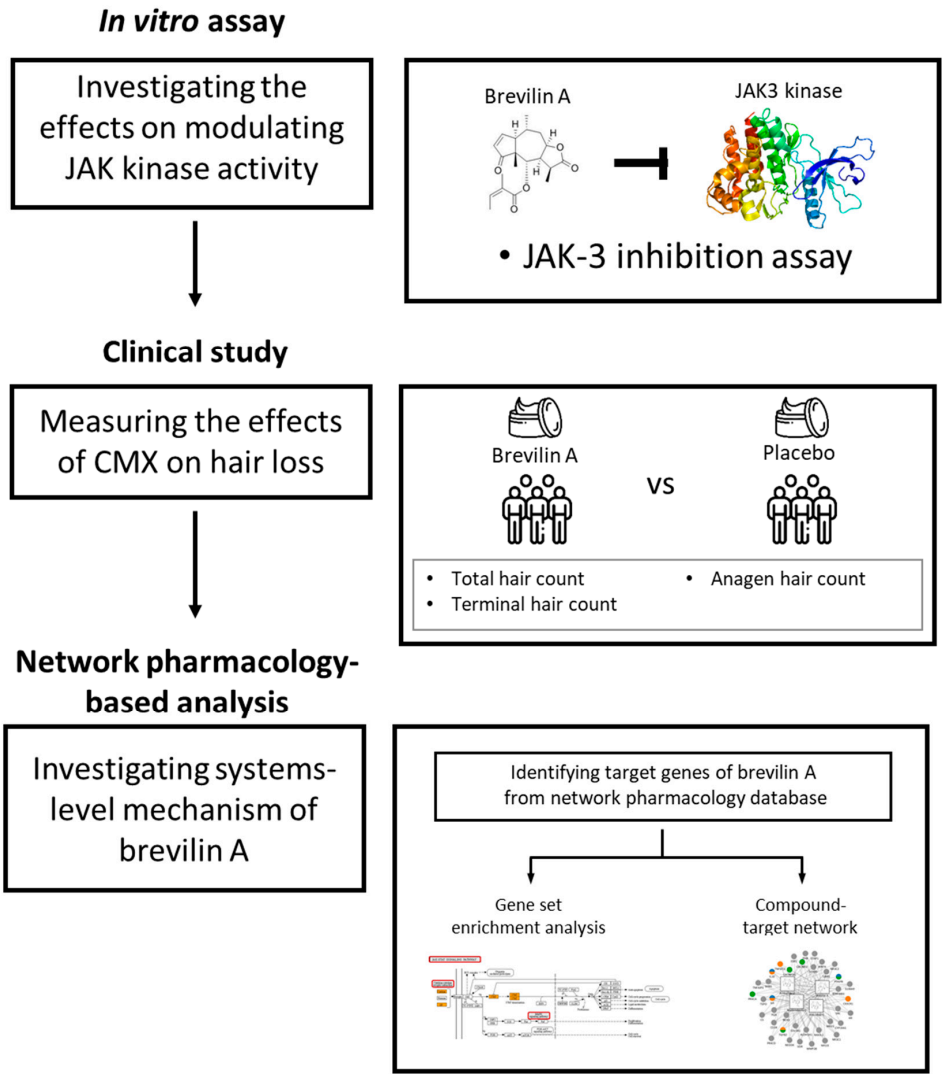

Figure 1. Overview of the study process combined with the clinical study and network pharmacology-based analysis. CMX, emulsion extract of brevilin A from *Centipeda minima*; JAK, Janus kinase.

2. Materials and Methods

2.1. Plant Materials and Preparation of CMX

C. minima was purchased in December 2019 from Natural-herb (Goesan, Korea). The material was identified by one of the authors (J.P.). A voucher specimen of the material (CM-2019-001) was deposited in the herbarium at Kyungsung University. CMX (ANACELL^{TM}) was prepared by D. Nature Co., Ltd. (Seongnam, Korea) by the efficient separation of brevilin A from *C. minima* by inducing phase separation in the emulsion. CMX contains two times more brevilin A than the conventional liquid extract of brevilin and its International Nomenclature Cosmetic Ingredient ID number is 33849.

2.2. HPLC

To determine the contents of the CMX fraction, 10 μL of filtered samples were injected into a HPLC ultraviolet (UV) system (Thermo Scientific Dionex Ultimate 3000, Thermo Fisher Scientific, Sunnyvale, CA, USA), equipped with a quaternary solvent delivery system, an auto-sampler, and a UV detector. Chromatography separation was carried out on a Supersil column ODS-I (250 mm × 4.6 mm, 5.0 μm). UV at 224 nm was used to screen samples. The mobile phase consisted of 0.1% formic acid in distilled water (A) and methanol (B) at a flow rate of 1.0 mL/min. The isocratic condition was A 55% and B 45%.

Accuracy tests and precision tests were evaluated to confirm whether the measured results were accurate and reproducible for three concentrations (1, 10, and 100 μg/mL). Both intra-day and inter-day test for accuracy and precision were conducted. The intra-day tests were determined by measuring three replicates on a day, and the inter-day tests were conducted for a period of 3 days for each concentration.

2.3. Ultra (U)HPLC-Q-TOF-MS Conditions

UHPLC Q-TOF-MS was performed using an Agilent 6530 Accurate-Mass Q-TOF LC mass spectrometer, equipped with an Agilent 1260 Infinity LC System (Agilent Technologies, Santa Clara, CA, USA). The samples (1 μL) were ionized in electrospray ionization positive ion mode. Chromatographic separation was achieved on a 4.6 × 50 mm I.D. 1.8-μm Eclipse XDB-C18 (Agilent Technologies, Foster City, CA, USA). Solvent A (0.1% formic acid in distilled water) and solvent B (0.1% formic acid in acetonitrile) at a flow rate of 500 μL/min were used for the mobile phase and the solvent gradient system was 15% B at 0–0.1 min, 15% B at 0.1–7 min, 100% B at 7–11 min, and 15% B at 11–20 min. The autosampler and column oven temperatures were 4 °C and 40 °C, respectively. The mass conditions were: gas temperature, 200 °C; pressure of nebulizer, 45 psi; fragmentor voltage, 150 V; and skimmer voltage, 60 V. The mass scan range was set to 50–1000 m/z and scan rate was 1.0 spectra/sec.

2.4. JAK3 Inhibition Assay

The inhibitory action of CMX on JAK3 was evaluated using a commercial luminescent kinase assay kit (V9441, Promega, Fitchburg, WI, USA). The experimental procedure was carried out according to the manufacturer's instruction. The test was performed in 96-well plates and luminescence was measured by GloMax®Navigator (GM2000, Promega, Madison, WI, USA).

2.5. Study Population

Seventy-two patients, 46 ± 0.5 years of age, in good physical and mental health, with mild to moderate vertex balding (II and IV for 4 men according to a modified Norwood-Hamilton classification scale, and 1 and 2 for 68 women according to Ludwig scale), were randomized to treatment groups. Sixty-six patients completed the 24-week study. The exclusion criteria included significant abnormalities on laboratory evaluation or physical examination, prior surgical correction of scalp hair loss, use of topical hair growth drugs or products such as minoxidil within 1 year of the start date. Alterations in hairstyle or dyeing of the hair were not allowed during the study.

2.6. Study Design and Hair Counts

This was a randomized, double-blind, placebo-controlled study to evaluate changes in the hair cycle conducted in Korea by the Korea Dermatology Research Institute (Seoul, Korea). Institutional review board approval (KDRI-IRB-19714-A, approved October 20, 2019) and informed consent were obtained before patients entered the study. Patients were randomized to receive 0.5 mL of CMX (1% brevilin A microemulsion tonic) or matching placebo (1:1) once daily for 24 weeks. Cutaneous irritation was evaluated by a dermatologist. The analysis of the ratio of telogen to anagen hair was conducted using phototrichograms.

The hair count was conducted following earlier studies with minor modifications. Briefly, hair in the target area was clipped for assessment of the total hair count and for differentiation of the growing anagen hairs that lengthen from resting telogen hairs. Three days later, a phototrichogram of the target area was taken for the assessment of anagen hair count, based on the number of hairs that had lengthened over 3 days.

2.7. Network Pharmacological Analyses

Network pharmacological analysis was performed by predicting targets and identifying related pathways of CMX. The predicted targets of CMX were obtained from the traditional Chinese medicine systems pharmacology database and analysis platform (TCMSP) (http://tcmspw.com/tcmsp.php), Bioinformatics Analysis Tool for Molecular mechANism of the traditional Chinese medicine (BATMAN-TCM) (http://bionet.ncpsb.org/batman-tcm/), and traditional Chinese medicine-mesh (TCM-mesh) (http://mesh.tcm.microbioinformatics.org/) [24–26]. Targets included experimentally validated compound-target interactions and predicted interactions based on machine learning methods (support vector machine and random forest for TCMSP, similarity-based method for BATMAN-TCM, and random forest for TCM-mesh). The performance of these methods for the prediction of compound-target interactions has been shown to be reliable.

The pathways related to the targets were identified by gene set enrichment analysis (GSEA) using Enrichr (http://amp.pharm.mssm.edu/Enrichr/) [27]. Enrichr computes enrichment by the assessment of multiple gene-set libraries (e.g., gene ontology, Kyoto Encyclopedia of Genes and Genomes (KEGG), and Online Mendelian Inheritance in Man) and calculates adjusted p-values, z-scores, and combined scores for the gene lists of interest (target genes). The combined score is calculated by the logarithm of the multiplication of the p-value and z-score.

A compound–target network is a bipartite network, in which nodes are defined as compounds and targets and the edges between compounds and targets are defined as compound-target interactions (Yes or No). This compound-target network was constructed and visualized based on information about the compounds, targets, and pathways of brevilin A using Cytoscape (https://cytoscape.org/) [28].

2.8. Statistical Analysis

The efficacy of CMX treatment was analyzed in the full analysis set, defined as all randomized subjects who had at least one application of the study treatment. The analysis of the outcome determined the adjusted mean difference between CMX and the placebo in total hair, terminal hair, and anagen hair count changes between the baseline and week 24. The changes in total hair count, terminal hair count, and anagen hair count from the baseline to the study endpoint were analyzed using two-way repeated measures analysis of variance. Enzymatic assay data were analyzed using the two-tailed Mann–Whitney U test. Statistical significance was set at $p \leq 0.05$. All statistical analyses were processed using the Scipy module in Python 3.6 or SPSS 25 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. CMX Analysis

To evaluate the reliability of HPLC analysis method, intra and inter-day accuracy and precision test were evaluated and their results are summarized in Table 1. Four chemical markers in five batches of CMX samples were quantified using HPLC-UV. The representative chromatogram of CMX is shown in Figure 2A. The retention time of M1, M2, M3, and brevilin A were 12.28, 14.21, 16.86, and 18.95 min and the content of each compound in the CMX fraction was 14.92 ± 4.25, 12.24 ± 7.58, 2.75 ± 0.76, and 62.93 ± 17.00 mg/mL, respectively. The content of brevilin A in CMX was two times higher than that of the three others combined and brevilin A covers 67.78% of the total area (Figure 2). The chromatographic results and contents are summarized in Table 2.

Table 1. Intra- and inter-day precision and accuracy of four compounds (n = 3).

Analyte	Concentration (μg/mL)	Intra-Day		Inter-Day	
		Accuracy (%)	Precision (RSD%)	Accuracy (%)	Precision (RSD%)
Arnicolide D	1	85.3	6.0	84.6	13.6
	10	102.0	3.6	96.3	4.9
	100	100.0	0.1	100.1	0.4
Arnicolide C	1	91.2	8.3	112.9	3.5
	10	94.9	3.4	94.4	4.5
	100	100.0	0.1	99.9	1.2
Microhelenin C	1	105.9	4.6	115.3	12.9
	10	102.3	0.3	103.6	2.9
	100	100.0	0.1	99.7	0.7
Brevilin A	1	86.2	7.5	87.9	15.0
	10	100.9	1.3	99.6	3.7
	100	99.9	0.1	99.1	0.9

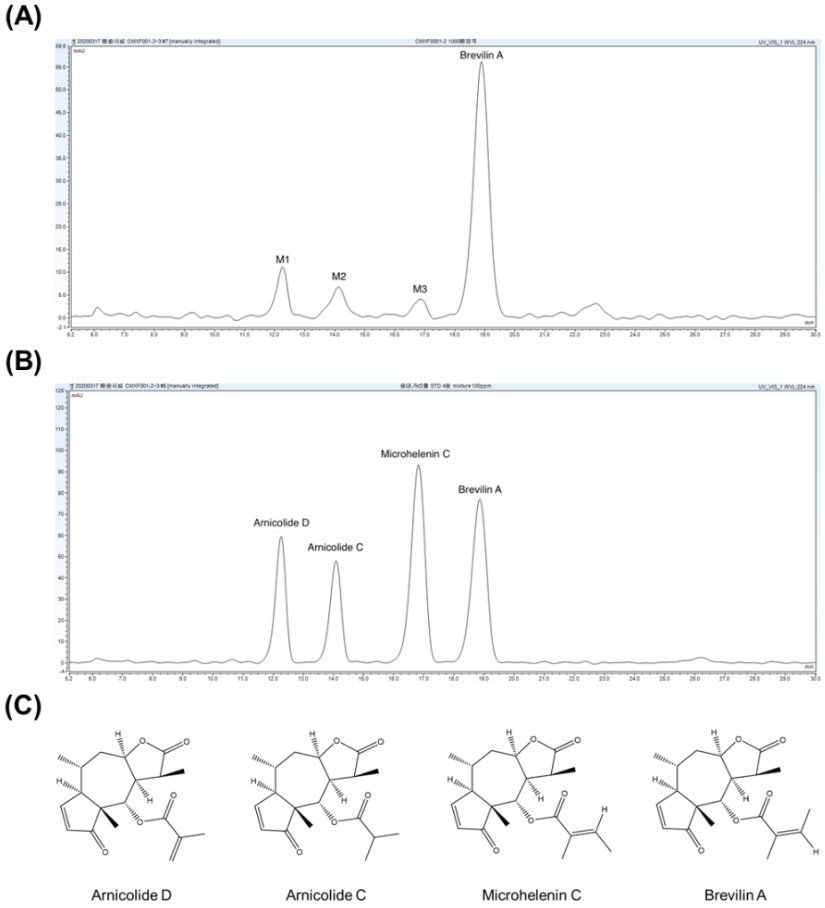

Figure 2. HPLC chromatogram of CMX and chemical structures of active compounds. (**A**) HPLC chromatogram of CMX sample. (**B**) HPLC chromatogram of standard mixtures of arnicolide D, arnicolide C, microhelenin C, and brevilin A. (**C**) Chemical structures of arnicolide D, arnicolide C, microhelenin C, and brevilin A. CMX, emulsion extract of brevilin A from *Centipeda minima*; HPLC, high-performance liquid chromatography.

Table 2. Chromatographic results and contents of CMX (n = 5).

Compounds	M1	M2	M3	Brevilin A
RT (min)	12.28	14.21	16.86	18.95
Content (mg/mL)	14.92 ± 4.25	12.24 ± 7.58	2.75 ± 0.76	62.93 ± 17.00

(CMX, emulsion extract of brevilin A from *Centipeda minima*; RT, retention time).

3.2. Compound Identification by LC-Q-TOF

To identify the major peaks of CMX shown in Figure 2, UHPLC-Q-TOF-MS with a positive ionization mode was equipped. The molecular weights of M1, M2, and M3 were 355.1514, 357.1665, and 369.1670 Da in the form of $[M+Na]^+$, respectively (Figure 3). The molecular weight and retention time of M1 were identical to those of arnicolide D, with high accuracy, which indicated that M1 is arnicolide D. For the same reasons, M2 and M3 were identified as arnicolide C and microhelenin C. The detailed mass information and analytical error (ppm) are summarized in Table 3. In addition, the retention times of these identified compounds were compared with the standards of arnicolide D, arnicolide C, microhelenin C, and brevilin A for additional identification by HPLC-UV method and the comparative chromatograms are shown in Figures 2 and 3.

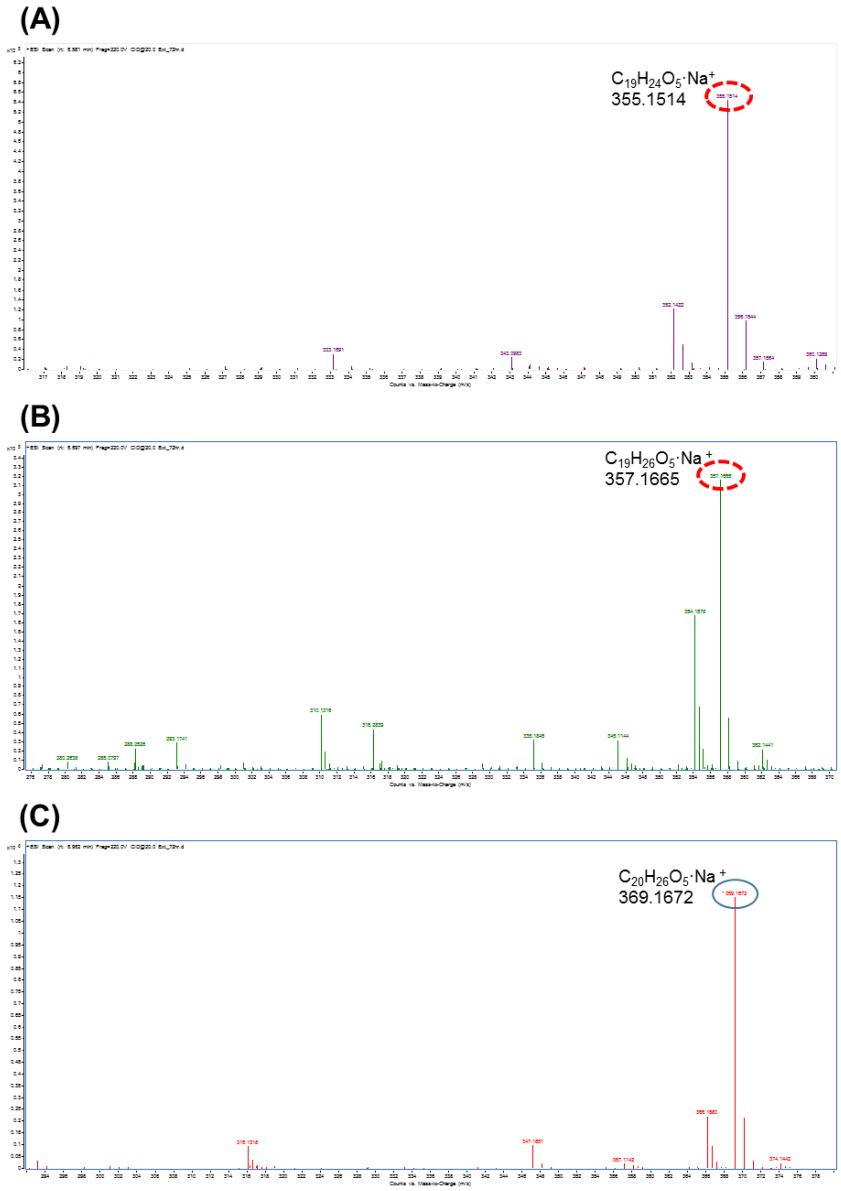

Figure 3. LC-Q-TOF mass spectrum of (**A**) arnicolide D, (**B**) arnicolide C, and (**C**) microhelenin C in CMX. CMX, emulsion extract of brevilin A from *Centipeda minima*; LC-Q-TOF, liquid chromatography-quadrupole-time of flight.

Table 3. LC-Q-TOF in the positive ion mode of CMX.

Compounds	Formula [M+Na]+	Theoretical Mass (Da)	Measured Mass (Da)	Error (ppm)
M1	$(C_{19}H_{24}O_5{\cdot}Na)^+$	355.1516	355.1514	0.56
M2	$(C_{19}H_{26}O_5{\cdot}Na)^+$	357.1672	357.1665	1.96
M3	$(C_{20}H_{26}O_5{\cdot}Na)^+$	369.1672	369.1672	0

(CMX, emulsion extract of brevilin A from *Centipeda minima*; LC-Q-TOF, liquid chromatography-quadrupole-time of flight).

3.3. JAK3 Inhibition Assay

An enzymatic assay was performed to evaluate the inhibitory effect of CMX on JAK3. We compared the effect of brevilin A and CMX in this test. Staurosporine (2.3 μg/mL) and tofacitinib (5 μg/mL) were used as reference drugs. CMX showed more potent inhibition of JAK3 than brevilin A at equal concentrations (Figure 4).

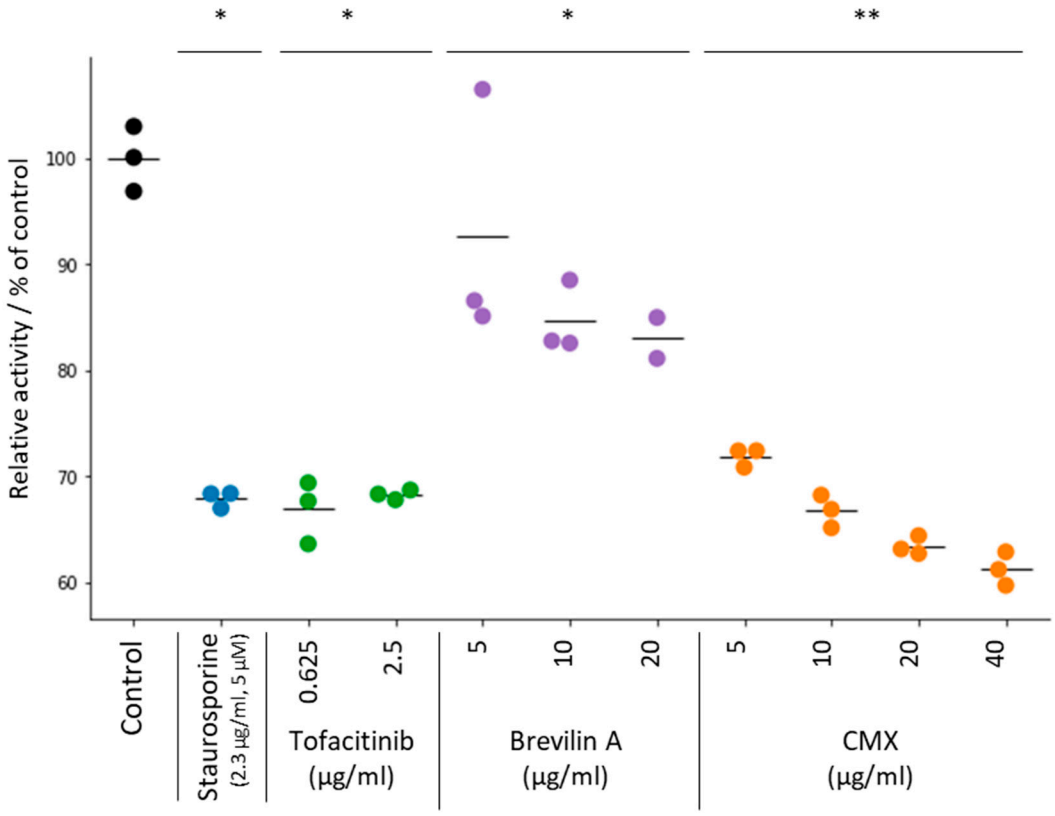

Figure 4. Comparison of inhibitory effect of staurosporine, tofacitinib, and CMX on JAK3 kinase. * $p < 0.05$ versus control group; ** $p < 0.01$ versus control group; statistical significance was determined using two-tailed Mann–Whitney U test. CMX, emulsion extract of brevilin A from *Centipeda minima*; JAK, Janus kinase.

3.4. Study Population

Seventy-two patient with active mild to moderate hair loss in the vertex area enrolled in the study. Thirty-two (1 man and 31 women, 88.9%) placebo-treated subjects and thirty-four (1 man and 33 women, 94.4%) CMX-treated group completed the 24-week study. The subject ages ranged from 37–54 years (mean ± standard deviation, 46.6 ± 8.5 years) (Table 4). Age, total hair count, terminal hair count, and anagen hair count were similar at baseline. Significant differences in total hair count, terminal hair count, and anagen hair count from the baseline to 24 weeks were observed between the placebo and CMX subjects ($p < 0.001$ for total hair count, terminal hair count, and anagen

hair count, respectively; Figure 5). The CMX group showed higher total hair count, terminal hair count, and anagen hair count than the placebo group, with means 2.4 versus −1.1, 3.7 versus 0.6, and 4.2 versus 0.6, respectively (Figure 5A,B,D,E,G,H). Similar improvements to CMX in total hair count, terminal hair count, and anagen hair count were observed in categorical change. Among patients in the CMX group, 8, 17, and 19 (23.6%, 50.0%, and 55.9%) patients had significant improvement (3 >) in total hair count, terminal hair count, and anagen hair count at 24 weeks, whereas only of 1, 4, and 7 (3.0%, 11.8%, and 20.6%) patients in the placebo group exhibited improvements (Figure 5C,F,I), respectively.

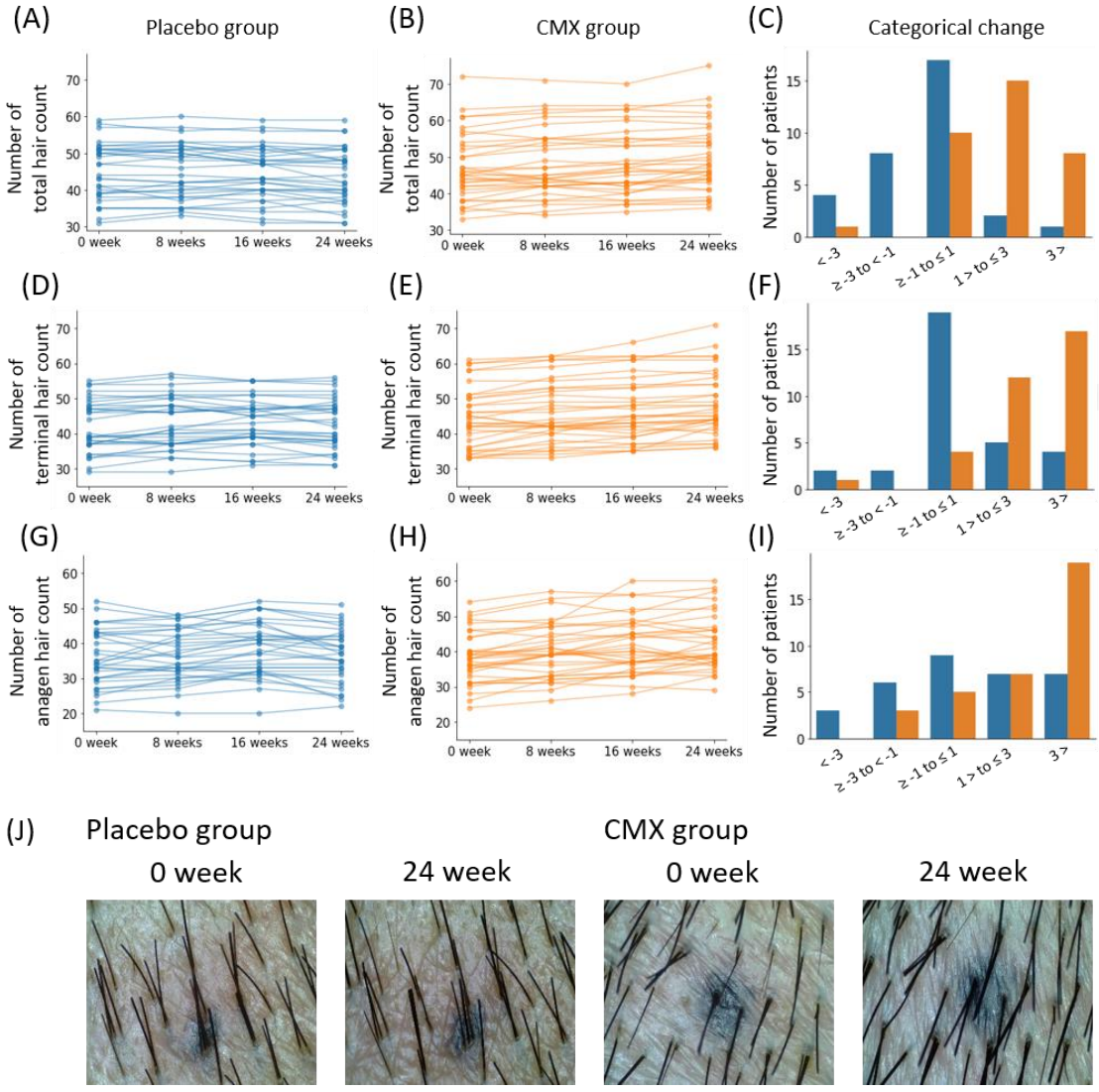

Figure 5. Hair count change and categorical change. Changes in total hair (**A**,**B**), terminal hair (**D**,**E**), and anagen hair (**G**,**H**) counts. Each point indicates the value of each patient. Categorical changes in total hair (**C**), terminal hair (**F**), and anagen hair (**I**) counts from the baseline to 24 weeks in subjects treated with CMX (n = 34) or placebo (n = 32). Representative macrophotographs of the scalp area at week 0 and week 24, for Placebo group and CMX group (**J**). CMX, emulsion extract of brevilin A from *Centipeda minima*.

Table 4. Baseline characteristics of placebo group and CMX group.

	Placebo Group (n = 32)	CMX Group (n = 34)
Age (mean ± SE)	46.9 ± 4.0	46.2 ± 4.7
Baseline hair count (mean ± SE)		
Total hair count	45.0 ± 7.8	47.0 ± 9.1
Terminal hair count	42.4 ± 7.4	44.6 ± 8.6
Anagen hair count	36.0 ± 8.1	38.1 ± 7.3

3.5. Network Pharmacological Analysis

We conducted a network pharmacological analysis to investigate the underlying mechanisms of brevilin A, the active compound of CMX. We identified 40 target genes of brevilin A from three network pharmacology databases: TCMSP, BATMAN-TCM, and TCM-mesh [24–26]. These targets are either experimentally validated or predicted by machine learning algorithms. To test whether these targets were significantly associated with the JAK-STAT signaling pathway and its related pathways, GSEA was performed based on KEGG [29]. The related pathways, obtained from KEGG, were: pathways involved in apoptosis and the cell cycle, cytokine–cytokine receptor interactions, the mitogen-activated protein kinase (MAPK) signaling pathway, the phosphatidylinositol-3-kinase (PI3K)-Akt signaling pathway, and the ubiquitin-mediated proteolysis pathway. Thus, not only the JAK-STAT signaling pathway but also cytokine–cytokine receptor interactions and the MAPK signaling pathway, which had high combined scores and low p-values for the targets of brevilin A (Table 5). This suggests that the effects of brevilin A in the amelioration of hair loss are mediated by the JAK-STAT signaling pathway and related pathways. The potential mechanisms of brevilin A, focusing on the JAK-STAT signaling pathway and related pathways were summarized, using KEGG mapper (Figure 6).

Table 5. Enrichment analysis of pathways related to JAK-STAT signaling pathway by the targets of brevilin A.

Term	Overlap	Adjusted *p*-Value	Odds Ratio	Combined Score	Genes
JAK-STAT signaling pathway	4/162	0.0016	12.66	103.88	*IL10*; *IL4*; *STAT3*; PDGFB
Cytokine-cytokine receptor interaction	5/294	0.0015	8.72	72.21	*IL10*; *IL4*; *CX3CR1*; *TGFB2*; *TNFSF11*
MAPK signaling pathway	5/295	0.0015	8.69	8.70	*CACNA1I*; *TGFB2*; *PDGFB*; *PRKCA*; *CACNA1G*

(JAK, Janus kinase; MAPK, mitogen-activated protein kinase; STAT, signal transducer and activator of transcription).

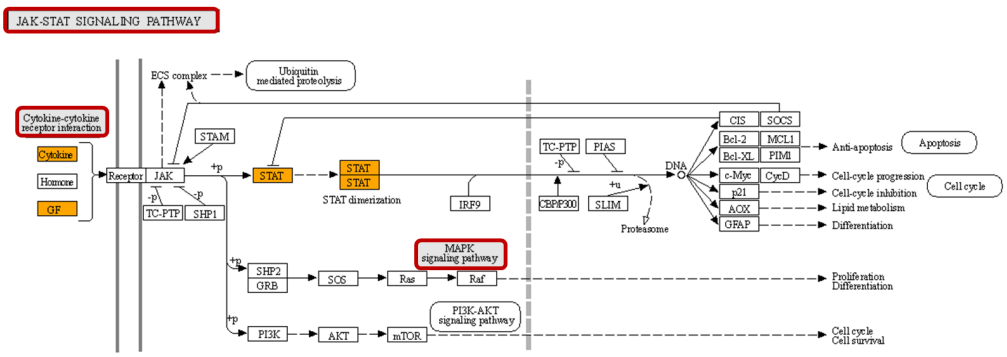

Figure 6. JAK-STAT signaling pathway (hsa04630) and the targets of brevilin A. The pathway maps were constructed using KEGG mapper. Round square and square represent pathways and gene targets, respectively. Red-rimmed round squares and orange-colored boxes indicate significantly associated pathways (adjusted *p*-value < 0.05) for targets of brevilin A and targets predicted to interact with the compounds of brevilin A, respectively. JAK, Janus kinase; STAT, signal transducer and activator of transcription.

To elucidate the compound-target interactions, we constructed and visualized the compound-target network between brevilin A and its target genes (Figure 7). There were 5, 5, and 4 related targets for the MAPK signaling pathway, cytokine–cytokine receptor interactions, and the JAK-STAT signaling pathway, respectively. PDGFB, IL10, and IL4 were involved in the JAK-STAT signaling pathway and related pathways. These results imply that the effect of brevilin A is exerted by the simultaneous modulation of multiple targets related to pathways that are closely related to hair loss.

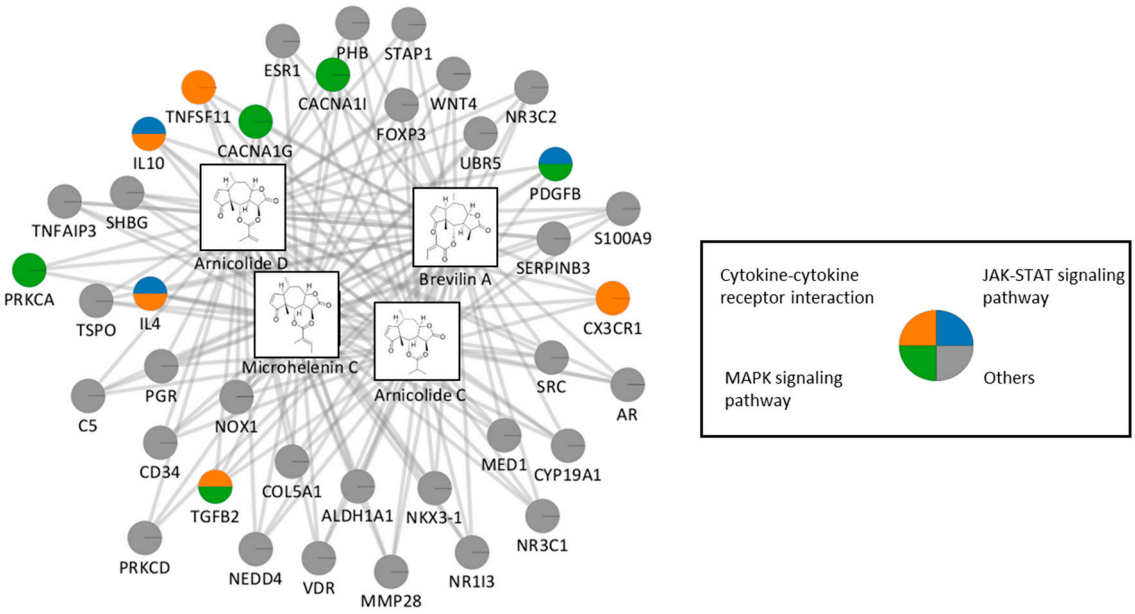

Figure 7. Compound-target network of CMX. Rectangles and circles represent the compounds and targets, respectively. Lines between compounds and targets indicate the interactions between them. CMX, emulsion extract of brevilin A from *Centipeda minima*.

4. Discussion

Our study evaluated the stimulatory effect of the extract of C. minima on hair regrowth in a clinical trial combined with network pharmacology-based analysis. HPLC-UV and UHPLC-Q-TOF-MS detected four compounds, arnicolide D, arnicolide C, microhelenin C, and brevilin A, in CMX. Among them, brevilin A was the main component in CMX and the content of brevilin A was more than double than that of the other compounds combined. Previous studies on the chemical constituents of C. minima also mentioned that brevilin A is one of the main sesquiterpene lactones in this plant [22,30].

Initially, a kinase assay was performed to investigate the inhibitory effect of CMX on JAK3 kinase activity. At the same concentrations (5, 10, and 20 μg/mL), CMX showed a better effect on the suppression of JAK3 activity than the liquid extract of brevilin A. At 10 μg/mL, CMX had the equivalent effect as staurosporine (2.3 μg/mL) and tofacitinib (0.625 and 2.5 μg/mL). Staurosporine is a nanomolar inhibitor of protein kinase C and an anticancer drug [31]. Tofacitinib is a JAK1 and JAK3 inhibitor used for the management of alopecia areata [9].

The effect of CMX on hair regrowth was evaluated by a clinical trial with 72 patients who had mild to moderate vertex balding. After 24 weeks, the CMX-treated group (n = 34) showed the stimulation of hair growth in total hair count, terminal hair count, and anagen hair count, which was not observed in the placebo group (n = 32). The anagen hair count is the most important index to evaluate a therapeutic effect on alopecia areata. The treatment with CMX significantly increased the number of patients who had an improved hair growth condition by >3 in total hair count.

We also conducted a network pharmacological analysis to explore the underlying mechanisms of brevilin A, the main constituent of CMX. Brevilin A was involved in the JAK-STAT signaling pathway and related pathways, such as cytokine–cytokine receptor interactions, the MAPK signaling pathway,

and the PI3K-AKT signaling pathway via multiple target genes. The JAK-STAT signaling pathway is vital for the stimulation of CD8αβ+ NKG2D+ T cell infiltration, which characterizes alopecia areata. The binding of extracellular ligands, such as interferons (IFNs), ILs, and other cytokines, to their specific receptors on the cell surface activates intracellular JAK proteins, which leads to the phosphorylation of STAT3 proteins. After activation by the phosphorylation of two monomers, STAT3 dimerizes, STAT3 proteins translocate into the nucleus to promote the transcription of target genes [10]. In the pathogenesis of alopecia areata, the activation of the JAK-STAT signaling pathway results in the production of IL-15 and IFN-γ in a feedback loop to maintain CD8αβ+ NKG2D+ T cell infiltration into the hair follicle [6,9]. Based on the visual compound-target network of brevilin A, the most targeted genes by brevilin A related to the JAK-STAT signaling pathway were STAT3, IL4, IL10, and PDGFB.

Previous studies have mentioned that brevilin A is involved in the regulation of the JAK-STAT signaling pathway of cancer cell lines as a STAT3 inhibitor [12,13], which supports our results from the network pharmacological analysis. Brevilin A is the main component of CMX and its content, quantitated by HPLC, was 62.93 ± 17.00 mg/mL. The inhibitory effect of CMX on JAK3 was demonstrated through the kinase assay, as CMX had a better effect than brevilin A at the same concentrations. To evaluate the efficacy of CMX as a JAK3 inhibitor to treat alopecia areata in humans, 72 patients with baldness received treatment for 24 weeks. The anagen hair count significantly increased with the CMX treatment. Finasteride and minoxidil are commonly used to treat hair loss. However, both have side effects. Finasteride is a 5α-reductase inhibitor for the treatment of androgenetic alopecia; however, it may increase the incidence of sexual dysfunction and risk of depression [32]. Minoxidil is a piperidinopyrimidine derivative and, as its effect does not depend on a hormone factor, it can be used for the treatment of androgen- and non-androgen-dependent hair loss. The long-term application of minoxidil can cause scalp pruritus and scaling [33]. CMX could be used as a potential treatment option for alopecia patients who experience the side effects of other synthetic drugs.

5. Conclusions

We evaluated the effects and mechanisms of CMX on hair loss using a framework that integrated an in vitro investigation, a clinical study, and a network pharmacology-based analysis. The clinical study showed that total hair count, terminal hair count, and anagen hair count were significantly higher in the CMX group than in the placebo group, which suggested that CMX is an effective treatment for patients with mild to moderate vertex balding. Moreover, the network pharmacology-based approach identified the gene targets of CMX and their potential mechanisms, focusing on the JAK-STAT signaling pathway, which elucidated the underlying mechanisms of CMX. This study suggests that the medicinal herbal extract CMX can be useful in the treatment of mild to moderate vertex balding and results in favorable effects on hair quality that contribute to the visible improvements in hair growth observed in treated patients.

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