Exploring the Mechanism of Complex Lemon-Angelica Sinensis-Boswellia Essential Oil on Anxiety Disorders with Melasma Through Network Pharmacology and Experimental Validation

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Abstract

The incidence rate of melasma is notably high among patients with anxiety disorders. Aromatherapy primarily influences the physiological and psychological states of individuals through the inhalation or application of essential oils, thereby facilitating the treatment or alleviation of various conditions. This study aims to explore the action mechanism of complex lemon-angelica sinensis -boswellia essential oil (CEO) in treating anxiety disorders with melasma. We investigated the active ingredients, targets, and pathways of CEO in relation to anxiety and melasma using network pharmacology. We employed cell assays and conducted nebulized essential oil inhalation tests on CUMS mice to validate the intervention effects of CEO on anxiety. A total of 28 active components, including neryl acetate, 3-butenylphthalide and octyl acetate, and 26 cross-targets, such as ESR1, CCND1 and PIK3CA, were identified. GO and KEGG pathway analyses indicated that these cross-targets were primarily involved in endocrine regulation, cell proliferation, and apoptosis, specifically through PI3K/Akt signaling pathway and calcium signaling pathway. The experimental results demonstrated that CEO significantly reduced the secretion of NO, TNF-a and IL-6, as well as the mRNA expressions of ESR1, CCND1 and PIK3CA in cells compared to the inflammatory cell model. Furthermore, CEO notably decreased the forced swimming immobility time of mice and the levels of IL-1β, ESR1 and CCND1 in hippocampus when compared to model mice. These findings suggest that CEO may regulate ESR1, CCND1 and PIK3CA through its citral, 3-butylphthalate and neryl acetate, thereby influencing endocrine function, cell proliferation and apoptosis, inhibiting inflammation and anxiety-like behavior in CUMS-induced mice.

Introduction

Anxiety disorders are neurological disorders characterized by anxiety, while melasma is a type of melanotic dermatosis that appears on the face. Modern medicine posits that the development of anxiety disorders and melasma is closely linked to endocrine imbalances and mental health[1]. Clinical data indicates a high incidence of melasma among individuals with anxiety, particularly impacting the physical and mental well-being of women, especially those who are pregnant or experiencing menopause[2]. In traditional Chinese medicine, anxiety disorders and melasma fall under the categories of 'Depression syndrome' and 'Liver spot'[3], with 'Liver-Qi stagnation' identified as a primary cause[4]. These conditions often coexist, leading to the term 'anxiety disorders with melasma'. Current treatments for anxiety disorders typically involve medications like benzodiazepines, serotonin reuptake inhibitors, or βadrenergic receptor blockers, while melasma is commonly treated with oral supplements such as vitamin C, vitamin E, and tranexamic acid, as well as topical applications of hydroquinone, kojic acid, azelaic acid, and arbutin cream. Despite the separate treatment approaches for each condition, there is a notable reliance on anti-anxiety medications. Building on the understanding of the underlying causes of both disorders, we propose a treatment approach that focuses on addressing both conditions simultaneously, termed 'treating different diseases together' for anxiety disorders with melasma.

For a long time, people have hoped to achieve safer and more effective therapeutic effects with fewer side effects by developing natural medicines and seeking new ways of administration. The aromatherapy of essential oil has been proven to avoid the first-pass effect and gastrointestinal irritation during the delivery of essential oil to lesion sites[5]. The small molecules of essential oils can act on the central nervous system through sniffing and transdermal absorption, stimulating the release of neurotransmitters and playing a role in regulating moods[6]. Citrus×limon (L.) Osbeck，angelica sinensis (Oliv.) diels and boswellia sacra flück. (accepted scientific name of each plant by MPNS) are all rich in essential oils and has a strong aromatic smells. The efficacy of lemon[7], angelica sinensis[8] and boswellia[9] in treating melasma has been recorded respectively, which includes promoting the blood circulation and removing stasis, etc. In recent years, it has been found that lemon essential oil (LEO)[10], angelica sinensis essential oil (AEO)[11,12] and boswellia essential oil (BEO)[13,14] can significantly improve anxiety disorders. These indicates that the three essential oils have varying degrees of therapeutic effects on anxiety and melasma.

Drawing from previous studies on the therapeutic effects of LEO, AEO and BEO in anxiety disorders and melasma, this research proposes the creation of a composite essential oil (CEO) combining these oils. Employing network pharmacology, the study investigates the potential mechanisms of CEO in treating anxiety disorders with melasma. The intervention effect of CEO on anti-inflammation, and key protein expression was validated through HaCaT cell experiments, while the anti-anxiety effect of inhaled nebulized CEO was evaluated using a classic anxiety disorder animal model (Chronic unpredictable mild stress, CUMS).

Materials and Methods

Database，Reagents and Instruments

Materials and reagents

Experimental mice

30 SPF grade ICR female mice, weighing 18-22g, purchased from Shanghai Slake Experimental Ani-

Materials and reagents	Brand	Lot No.
Limon essential oil (LEO) *Angelica sinensis essential oil (AEO)* *Boswellia essential oil (BEO)*	steam distillation extraction supercritical CO2 fluid extraction	Made by the laboratory
HaCaT immortal human epidermal cells	Beina Chuanglian Biotechnology	339817
Neryl acetate（NA）	Tokyo Chemical Industry Co., Ltd	PPE8F-RN
3-Butylidenephthalide（3-B）	Aladdin Reagent Company	J2009089
Octyl acetate（OA）	Aladdin Reagent Company	D1503133
Methanol (chromatographic purity)	Merck	34885
Fetal bovine serum	BOVOGEN	2011B
DMEM cell culture medium	VivaCell	C3113-0500
TNF-α Elisa Kit	Elabscience	E-EL-H0109c
IL-6 Elisa Kit	Elabscience	E-SOEL-H0001
NO detection kit	Solarbio	BC1475
Penicillin -streptomycin solution	New Saimei	C100C5
Trypsin（＋EDTA）	Full form gold	FG301-01
D-Hanks buffer	Solarbio	H1040
DMSO	Solarbio	D8371
Lipopolysaccharide（LPS）	Solarbio	L8880
Cell counting kit-8（CCK-8）	Biosharp	BS350A
Tyrosinase activity detection kit	Solarbio	BC4055
FastPure Cell/Tissue total RNA isolation kit	Novizan	RC112-01
Revert aid first strand cDNA synthesis kit	Thermo Fisher Scientific - CN	K1622
NovoStart SYBR qPCR SuperMix plus	Novoprotein	E096-01A
Primer synthesis	Sangon Biotech	JX-YW
Diazepam tablet	CSPC Pharmaceutical group	1mg/tablet
Mouse IL-1 β Elisa Kit	Hangzhou multi sciences Co., Ltd	EK201B
Mouse Cyclin- D1 Kit	Shanghai Coibo Biotechnology Co., Ltd	CB10701-Mu
Mouse ESR1 Kit	Wuhan Fine Biotech Co., Ltd.	EM1007

mal Co., Ltd. [License number: SCXK(Shanghai)2022-0004, certificate number: 20220004014596]. The experiment was conducted at Hangzhou Hebio Technology Co., Ltd. [License number: SYXK (Zhejiang) 2020-0013]. The experiment follows the 3R principle and the relevant regulations of the animal ethics committee of laboratory (SLXD20210326012). Feeding conditions: temperature of 22-24℃, humidity of (55±10)%, 12 h/12 h of alternating light/dark in the animal room (with lights on from 8:00 to 20:00), during the feeding period, the mice were free to eat and drink water.

Experimental apparatus

Network pharmacology analysis of CEO anti anxiety with melasma

Screening of active components and related targets of CEO

Instrument	Manufacturer	Model/Lot No.
Electronic analytical balance	Sartorius	BSA124S
GC -MS spectrometry	Agilent China	8890-5977B
Micro pipetteguns	Eppendorf	10 μL-100 μL
Vertical pressure sterilizer	Shanghai Boxun Industrial Company	YXQ-100SII
Supercentrifuge	Eppendorf	5417R
Ultra cold storage freezer （-80℃）	Thermo Fisher Scientific - CN	902 907 906
CO_2 incubator	Thermo Fisher Scientific - CN	4111FO
Inverted epifluorescence microscope	Olympus	IX73P1F
Multi-functional microplate reader	Molecular Devices	SpectraMax M3
PCR appearance	Eppendorf	6333000073
Quantitative PCR instrument	Beijing Kubo Technology Co., Ltd	Q225
Digital Camera	Canon	500D
Atomizer	Yuyue Medical Equipment Co., Ltd	405E

Table 1. Real-Time PCR Primer sequences

Primer name	Primer Sequence 5'→3'	Molecular weight / bp
PIK3CA	F:AAGAGCCCCGAGCGTTTCTG R:GCCTCACGGAGGCATTCTAA	208
CCND1	F: GATCAAGTGTGACCCGGACT R: CTTGGGGTCCATGTTCTGCT	100
ESR1	F: GTCAGTGCCTTGTTGGATGC R: ACACATTTTCCCTGGTTCCT	308
GAPDH	F: ATCAGCAATGCCTCCTGCAC R:TTCCCGTTCAGCTCAGGGAT	242

The volatile components in LEO, AEO and BEO were obtained by GC-MS analysis and literature supplement. Their relevant targets were obtained by Pubchem, SwissADME and SwissTargetPrediction databases.

Collection of disease targets and the cross- targets between CEO and diseases

The potential targets related to anxiety and melasma were identified through the main keywords "anxiety disorders" and "melasma" in GeneCards, OMIM and DrugBank databases. The cross-targets between CEO and anxiety with melasma were obtained by drawing a Venn diagram. The cross-targets were imported into String database for protein analysis, in which "Organism" was set to "Homo sapiens" and combination score was set to "≥0.40" . Then the protein-protein interaction (PPI) was downloaded in Tsv format and imported into Cytoscape 3.9.0 software for visual analysis.

GO function and KEGG pathway enrichment

Gene ontology (GO) and kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment of cross-targets were analyzed by Metascape database, and the visual analysis were carried out by Bioinformatics Tools.

Constructing of "CEO- component- target-pathway-anxiety with melasma" network

The first 20 KEGG pathways were selected by sorting the p value from small to large, the related cross-targets and associated active components of CEO were imported into Cytoscape 3.9.0 software to construct the "CEO component-target-pathway-anxiety with melasma" network. The network was selected for visualization by Pathway Builder Tool.

Molecular docking between key compounds and core targets

The SDF format of the molecular structure of key compounds was downloaded from PubChem database, and the SDF format was converted to MOL2 format through Open babel. The best protein structure of the core target was obtained through RCSB PDB database and downloaded in PDB format. The molecular docking between the key compound and the core target was performed using Autodock 1.5.6 software, and then the docking results were visualized using Pymol software.

Effect of CEO on HaCaT proinflammatory induction by LPS

Cytotoxicity experiment

HaCaT cells with logarithmic growth phase were taken and inoculated into 96 well plate (100μL/well) at concentration of 5×10^3cells/mL, and cultured at 37℃in a 5% CO2 incubator for 24 h. The original culture medium was discarded, 100μL of 1μg/mL LPS was ad ded to the cells to stimulate cultivation for 4h to obtain model cells, and the sample group added 100μL/well of DMEM medium containing sample. The samples included LEO, AEO, BEO, CEO (prepared from three essential oils in a 1:1:1 ratio), NA, 3-B and OA, and the sample concentration was diluted to 0.25, 0.5 and 1μg/mL with DMEM medium containing 0.04% DMSO. At the same time, a blank setting group (DMEM medium), a control group (DMEM medium containing 0.04% DMSO) were set up. Each group had 6 wells, and after 24 h of cultivation, the culture medium was discarded and cells were washed the twice with PBS. 10μL/well of CCK-8 solution was added to each well, and further cultured in the dark for 4 h. The absorbance at 450 nm was measured. The formula was for calculating cell survival rate $/\%= (A_{\text{sample}} - A_{\text{blank}}/A_{\text{control}} - A_{\text{blank}}) \times 100$.

In subsequent experiments, the drug concentration of the sample was determined based on the cell survival rate.

Determination of tyrosinase activity in cells

HaCaT cells were incubated with sample solution for 48 h, washed with PBS, and then centrifuged after ultrasonic lysis. The supernatant was taken for testing. 50 μL of 1% L-dopa solution was added to the 50 μL supernatant and incubated at 37℃ for 1 h. The absorbance value at 475nm was determined by microplate reader. Tyrosinase inhibition rate%=(^{A}control -^{A}blank)×100/(^{A}sample- ^{A}blank)

Levels of the inflammatory cytokines IL-6, TNF-a and NO

100 μL of HaCaT cells suspension at logarithmic phase were added to 96-well plate (2×10^4 cells/well) and cultured for 24 h. After removing the medium, 100μL of 1μg/mL LPS were added to stimulate for

4 h, and the cells were washed three times with phosphate buffer solution. 100 μL of new DMEM medium containing samples were added to culture for 24 h. Then, the cell supernatant was collected to determine the TNF-α, IL-6 and NO levels according to the instructions of kit.

Determination of mRNA expression of PIK3CA, CCND1 and ESR1

RNA extraction of HaCaT cells were performed by TRIzol and its concentration measured. Reverse transcription was performed using a reverse transcription kit under conditions of 42 ℃ for 15 min and 95 ℃ for 3 min. After completion, it was stored at -20℃ and subjected to fluorescence quantitative amplification using a fluorescence quantitative kit. The reaction system was 20 μL, and the reaction conditions were 94 ℃ for 30 s, one cycle, 94 ℃ for 5 s, 60 ℃ for 30 s, and 40 cycles. The results were analyzed using $2^{-\Delta\Delta Ct}$. The primer sequences are shown in Table 1, synthesized by Shanghai Biotechnology Co., Ltd. Table1 Real-Time PCR Primer sequences

CUMS mouse model and water maze experiment

Grouping and administration: After 7 days of adaptive feeding, mice were randomly divided into a blank group, model group, positive group (100 μg/mL of estazolam solution prepared with 0.8% NaCl solution and administered orally at a dose of 10 mL/kg for 4 weeks), an essential oil group (5% essential oil nebulizer prepared with pure water, and continuously inhaled with nebulized essential oil for 30 min for 4 weeks), and a neryl acetate group (the same as the essential oil group). Each group consists of 6 mice.

Mouse anxiety model: The control group mice were fed normally without any stimulation, while the other four groups were stimulated using CUMS. 7 kinds of stimuli were randomly set up, 2 kinds/day, with each stimulus appearing 8 times, including: swimming in ice water (4℃, 5 min), heat stress (45℃, 5 min), water restriction (24 h), fasting (24 h), tail clipping (1 min), slope feeding, and damp mattress (24 h), for a total of 28 days.

Water maze test: After 4 weeks of administration, a water maze experiment was conducted and continuously administered during this period. The water maze was divided into four quadrants, with the platform about 1cm below the water surface and the water temperature maintained at 25 ± 1 ℃. (1) Positioning navigation experiment: Swimming time and trajectory of the mice finding the platform within 120 s were recorded. The mice that did not find the platform were guided to stay on the platform for 15 s. Continuous training for 5 days. (2) Space exploration experiment: On the 6th day, the platform was dismantled and the time of mice to reach the target quadrant and the number of times to cross the platform were recorded within 5 min. After the experiment, the mice were anesthetized and the hippocampus was removed, and the relevant proteins were measured according to the instructions of the ELISA kit.

Data processing

The results were expressed as x±s . The differences between the groups were compared using one-way ANOVA. $P<0.05$ indicates a significant difference between the two groups.

Results

Network pharmacology analysis results

Components and of related targets number of CEO

Based on the GC-MS of three essential oils, a total of 30 components in LEO, 10 components in AEO, and 14 components in BEO were screened. Other components in essential oils were supplemented through literature, including D-limonene, α-pinene, and linalool in LEO[15,16] and β-basil in BEO [17,18], as shown in Table 2. 37 active components and 394 potential corresponding targets were obtained by Pubchem, SwissADME and SwissTargetPrediction

Cross-targets between CEO and anxiety disorders with melasma

After searching, merging and removing duplicate targets, 1120 targets for anxiety disorders and 1609 targets for melasma were obtained. The cross-targets of CEO and diseases were shown in Figure 1. *CEO* had 88 cross-targets with anxiety disorders and 105 cross-targets with melasma, and a total of 26 cross-targets among the three. The 26 cross-targets were uploaded to String database, the free target CHRM3 was deleted, and the PPI network was constructed, as shown in Figure 2. The network consisted of 25 nodes and 59 edges, and there were 9 core targets'degree value greater than the average (4.72), as shown in Table 3.

Table 2. Basic information of active components of CEO

Component	CAS	Source	Component	CAS	Source
Phenetole (-)-Clovene Tetradecane a-Terpineol	103-73-1	*LEO*	Butylphthalide	6066-49-5	*AEO*
γ-Cadinene	39029-41-9		(+)-Cyclosativene	22469-52-9	
(-)-γ-Elemene	3242-08-08		1-Octanol	111-87-5	
But-2-enoic acid cyclohexyl ester	16491-62-6		Eucalyptol	470-82-6	*BEO*
3-Tert-butylpyridine	38031-78-6		Octyl acetate	112-14-1	
Selina-3,7(11)-diene	6813-21-4		*β-Ocimene*	13877-91-3	
Neral	106-26-3		3-Carene	13466-78-9	
Citral	141-27-5		(-)-Bornyl acetate	5655-61-8	
3-Cyclohexene-1carboxylicacid	54162-90-2		6-Methyl-5-hepten-2yl-tiglate	1000383-64-5	
Neryl acetate (3-Benzoyloxy-3,4dihydro-2H-pyran-2-yl) methyl benzoate	141-12-8 1000193-34-		(1r,3e,7e,11r,12r)-4,8,12,15,15-	70000-19-0	
Isocaryophyllene	118-65-0		Linalool	78-70-6	*LEO*
cis-a-Bergamotene	18252-46-5		*D-Limonene*	138-86-3	*BEO*
N-phenyl-3-methyl-	121190-27-0				*LEO*
4pentenamide			*a-Pinene*	80-56-8	AEO

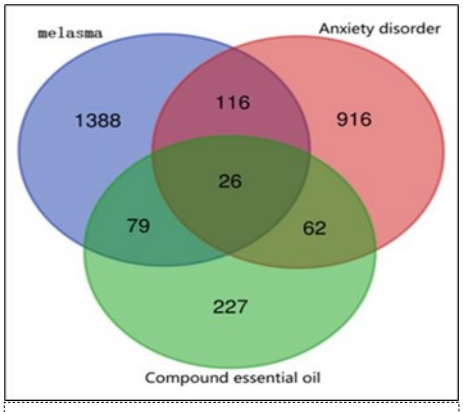


Figure 1. Cross-targets between CEO and anxiety with melasma

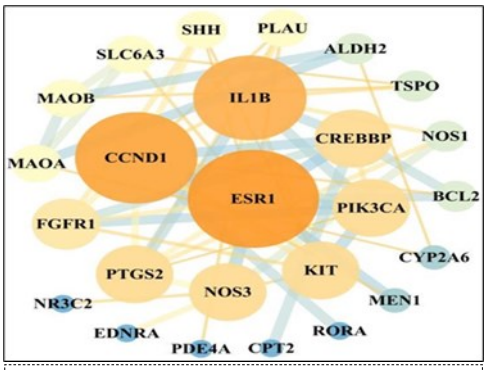


Figure 2. Protein-protein interaction network

Table 3. Core targets with top degree value

Cross-targets	Protein name	Uniprot ID	Degree value
ESR1	Estrogen receptor	P03372	13
CCND1	G1/S-specific cyclin-D1	P24385	12
IL-1β	Interleukin 1β	P01584	11
CREBBP	CREB-binding protein	Q92793	7
PIK3CA	Phosphatidylinositol 4,5-bisphosphate -3-kinase catalytic subunit alpha iso-form	P42336	7
KIT	Mast/stem cell growth factor receptor	P10721	7
NOS3	Endothelial nitric oxide synthase	P29474	7
PTGS2	Prostaglandin G/H synthase 2	P35354	7
FGFR1	Fibroblast growth factor receptor 1	P11362	6

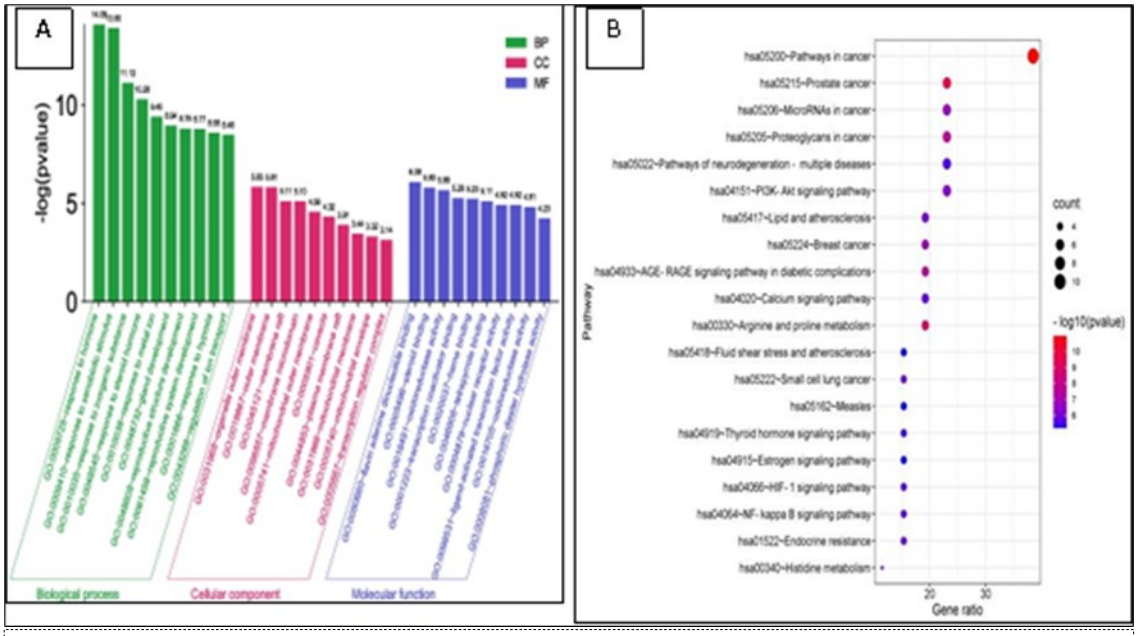


Figure 3. Enrichment diagram of GO function(A) and KEGG pathway(B)

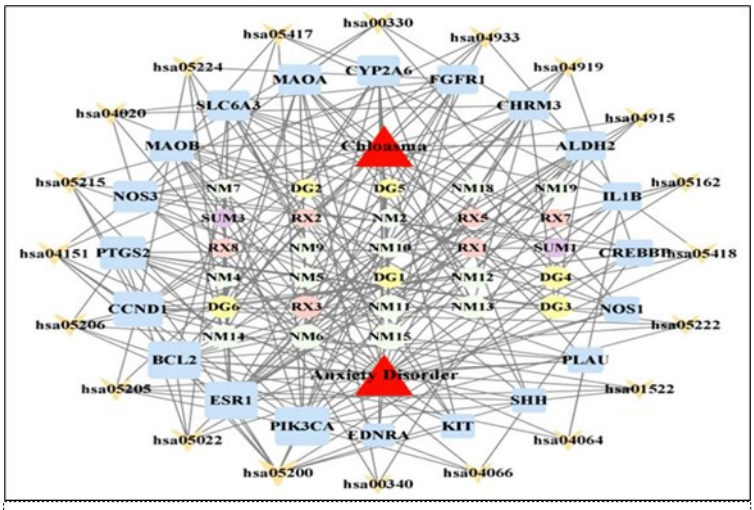


Figure 4. "*CEO*- components- targets- pathways - anxiety with melasma" network

Note: 20 KEGG，28 active components and 20 cross-targets were shown with orange graphs, circles, blue squares，respectively.

Table 4. Molecular docking energy of active compounds with core targets. (KJ/mol)

Molecule name	ESR1 (7NFB)	CCND1 (2W96)	PIK3CA (7L1C)
Neryl acetate	-19.87	-14.64	-16.11
Citral	-16.01	-15.27	-17.36
3-Butylidenephthalide	-19.12	-21.21	-20.79
Octyl acetate	-16.82	-11.59	-11.51
Tranexamic acid	-21.38	-17.15	-20.71
Diazepam	-26.19	-23.43	-24.73

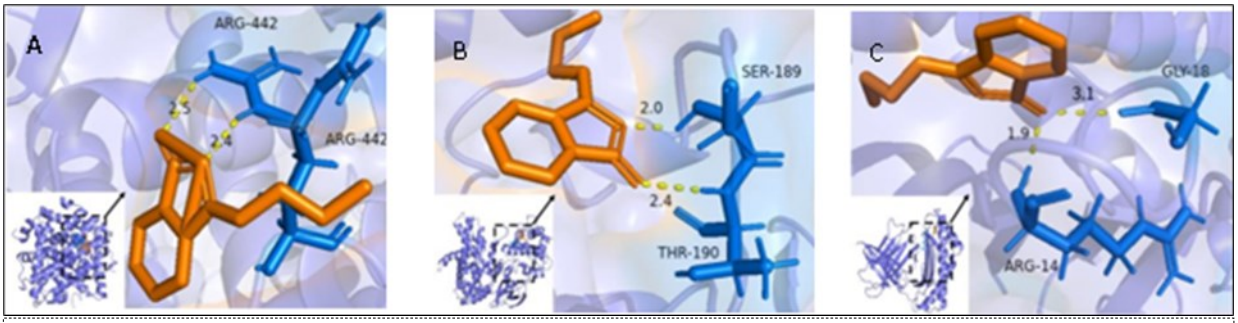


Figure 5. Molecular docking of 3-butylidenephthalide with ESR1(A), CCND1(B) and PIK3CA (C).

Note: 3-butylidenephthalide was depicted in orange, protein was depicted in blue (the code represents amino acid residues), hydrogen bond was depicted in yellow dashed line (the number represents the binding distance).

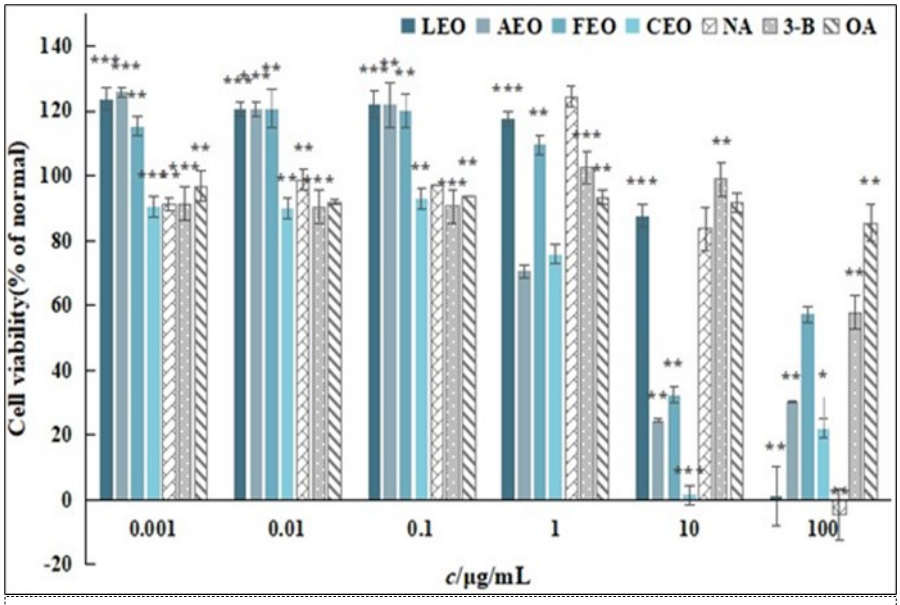


Figure 6. Effect of CEO and key compounds on HaCaT cell viability

Note: Compared with control cells,*p<0.05,**p<0.01,***p<0.001.

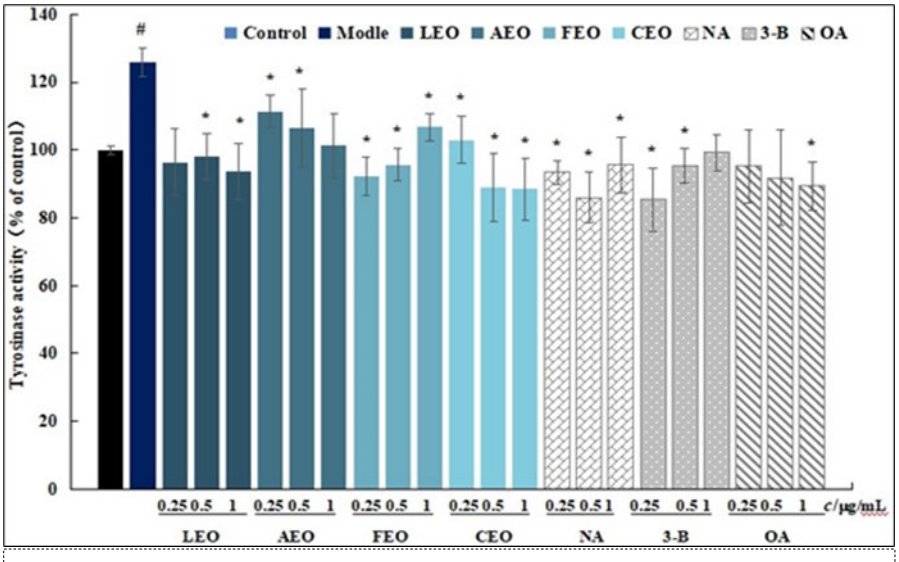


Figure 7. Effect of CEO and key compounds on tyrosinase activity in HaCaT cells

Note: Compared with control, #p<0.05, compared with modle, *

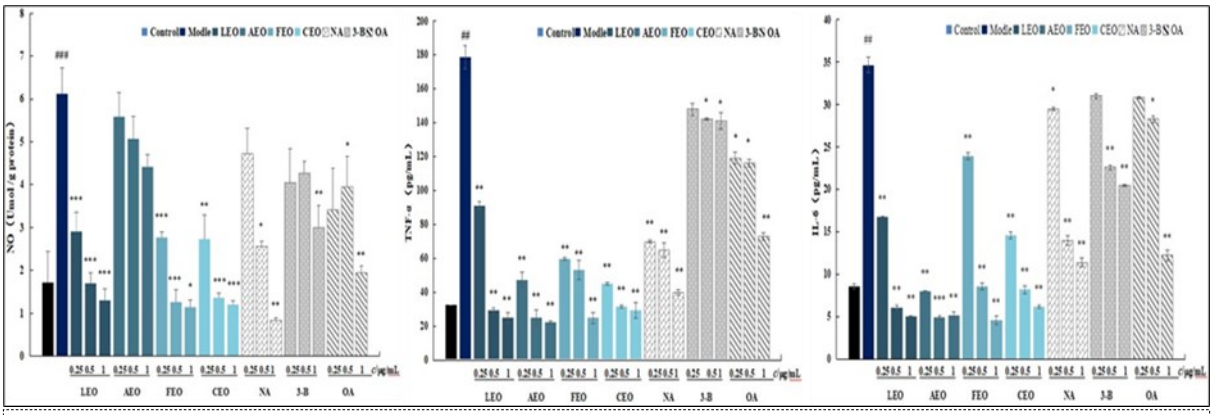
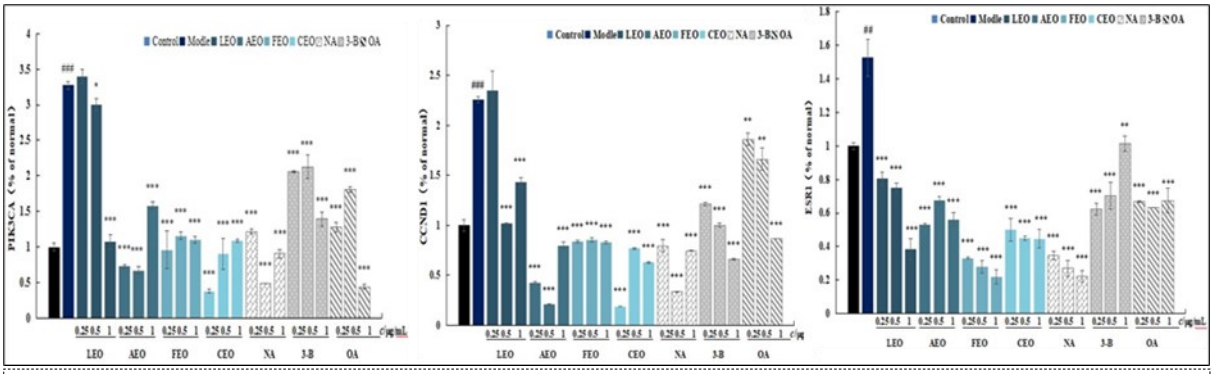


Figure 8. Effects *CEO* and key compounds on the levels of NO, TNF-α and IL-6 in LPS-induced HaCaT cells

Note: Compared with control, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$; compared with modle, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.



Note: Compared with control, ##p＜0.01, ###p＜0.001;compared with modle, *p＜0.05，**p＜0.01，***p<0.001.

Table 5. The results of the water maze experiment on mice (x±sd）

Indicator	control group	modle group	positive drug group	*CEO group*	NA group
Escape latency/s	58.27±	103.08±	84.22±	83.43±	87.87±
Swimming time in Remove target quadrant /s platform	30.05± 10.45	17.58±13.68	31.02±4.64	25.26±3.51	33.01±5.98
Number of platform crossing /time	3.67±0.58	0.33±0.58##	3.00± 0.00**	2.00± 0.00**	1.67±1.15

Note: Compared with the control group, the modle group had # p<0.05, # # p<0.01. Compared with the modle group, the positive drug group, CEO and NA group had *p<0.05, ** p<0.01, respectively

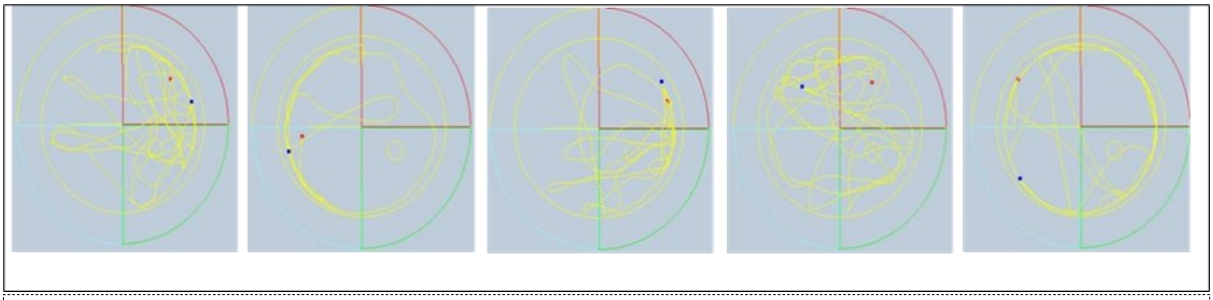


Figure 10. Activity trajectory diagram of mice in space exploration experiments

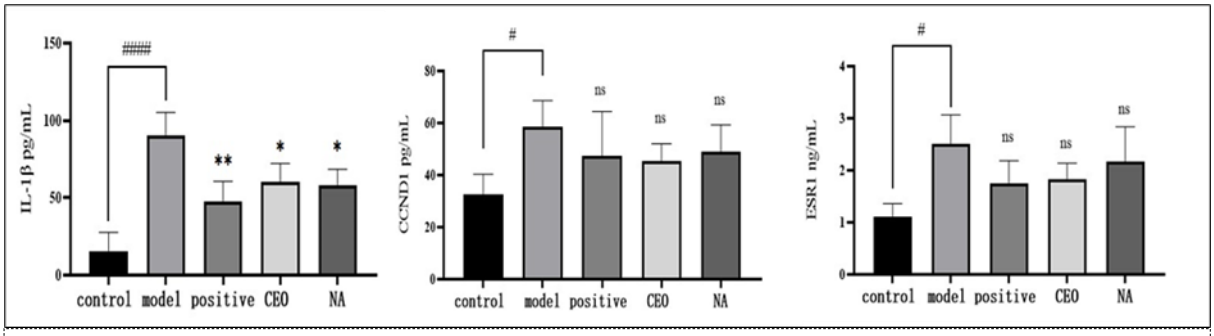


Figure 11. Content of IL-1β, CCND1 and ESR1 in mice hippocampus

Note: Model vs.control, # p<0.05, ####p<0.0001; administration vs. model , *p<0.05, **p<0.01.

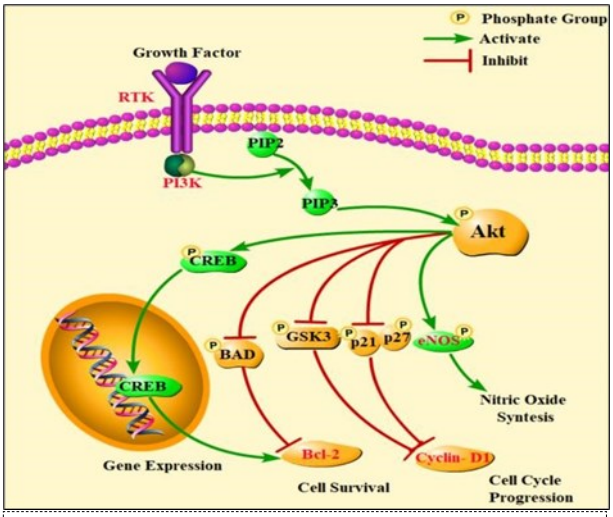


Figure 12. Regulation of PI3K/Akt signal pathway by active components of CEO.

Note: The protein encoded by cross-targets is shown in red text.

Enrichment analysis of GO function and KEGG pathway

GO function and KEGG pathway enrichment of 26 cross-targets were analyzed by Metascape database. List all kinds of top items according to p value were sorted from small to large.

Figure 3 showed the top 10 items in GO functional analysis. GO contained: 403 biological processes (BP) were mainly related to response to hormones, xenobiotic stimulus, inorganic substance, etc. 15 cell composition (CC) were mainly related to organelle outer membrane, outer membrane, membrane raft, etc. 23 molecular function (MF) were mainly related to flavin adenine dinuctide binding, steroid binding, oxidoreductase activity, etc.

KEGG pathway enriched 69 signaling pathways, among which the top 20 KEGG pathways enriched 20 targets, accounting for 77% of 26 cross-targets, as shown in Figure3. The 20 signaling pathways were mainly related to 7 signaling related pathways (such as PI3K/Akt and calcium signal pathway, etc.), 6 cancer related pathways (such as pathways in cancer, prostate cancer, etc.), and 2 amino acid metabolism related pathways (including arginine and proline metabolism, histidine metabolism, etc.).These pathways also involved nervous system diseases.

"CEO- components- targets- pathways - anxiety with melasma" network

The network was constructed for 28 active components, 20 cross-targets and 20 KEGG pathways, as shown in Figure 4.

The network consisted of 70 nodes and 215 edges. The degree values of neryl acetate, lavandulyl acetate, neral, N-phenyl-3-methyl-4-pentenamide and citral in LEO, 3-butenylphthalide, senkyunolide A and E-ligustilide in AEO, octyl acetate in BEO were greater than 4. The degree values of PIK3CA, CCND1, BCL2 and ESR1 were greater than 19. The degree values of pathways in PI3K/Akt signaling pathways, pathways of neurodegeneration- multiple diseases, calcium signaling pathway, etc. were greater than 5.

Results of molecular docking

The targets with high degree value in PPI network were compared with the targets enriched in PI3K/Akt signal pathway, then ESR, CCND1 and PIK3CA were selected as the core targets to verify molecular docking with four key components, including neryl acetate, citral, 3butylidenephthalide and octyl acetate. Tranexamic acid for melasma[19] and diazepam for anxiety disorders[20] were selected as positive controls, as shown in table 4. The results of molecular docking showed that the 4 key components of CEO all had strong binding force with 3 core targets, and their binding energy were less than -5 KJ/mol. But their binding energy was weaker than the positive control drugs.

Taking 3-butylidenephthalide as an example, it was connected with amino acid residues of the target protein through hydrogen bonds, such as ARG-442 of ESR1, THR-190 and SER-189 of CCND1, ARG-14 and GLY-18 of PIK3CA, respectively, as shown in Figure 5.

Cell experiment results

The effect of CEO and key compounds on the survival rate of HaCaT cells

0.001~100 μg/mL of essential oil was added to HaCaT cells and cultured for 24 h to detect cell viability, as shown in Figure 6. Compared with control cells, cell growth showed varying degrees of inhibition when the concentration of essential oils and key compounds exceeds 10 μg/mL. When the concentration was less than or equal to 1μg/mL, they did not damage to HaCaT cells and had a promoting effect on the activity of HaCaT cells. So for subsequent research, 1μg/mL was chosen as the maximum experimental concentration for samples.

Effect of CEO and key compounds on tyrosinase in HaCaT cells

The effect on intracellular tyrosinase was shown in Figure 7. Compared with the control cell group, the tyrosinase activity in the induced model group was significantly increased ($p<0.05$). Compared with the model group, the tyrosinase in the sample group decreased in varying degrees, and the inhibition rate of tyrosinase increased with the increase of drug concentration. The content of tyrosinase in 1μg/mL CEO group was 11.51% less than that in control cells. Inhibition of tyrosinase activity is the main strategy to reduce melanin production and avoid skin pigmentation

Effects of CEO and key compounds on cellular inflammatory factors

The experimental results (Fig. 8) showed that the levels of NO, TNF-α and IL-6 in the model group increased significantly compared to the control group after LPS inducting ($p<0.01$~0.001), indicating the successful modeling of inflammatory HaCaT cells. Compared with the model group, the levels of NO, TNF-α and IL-6 in each sample groups were reduced to varying degrees, and the degree of reduction in the essential oil group was more than that in the key compounds group, all showing a dose-dependent relationship. 1 μg/mL LEO, AEO，BEO, CEO and neryl acetate could reduce the level of NO, TNF-α, and IL-6 to control cells.

Effect of CEO and key compounds on mRNA expression of key targets

Compared with the control group, the mRNA levels of PIK3CA, CCND1 and ESR1 in the model group were significantly increased with $p<0.01$, indicating successful modeling of inflammatory HaCaT cells. The CEO and key component groups effectively inhibited the mRNA expression levels of PIK3CA, CCND1 and ESR1 in inflammatory cells. Except for the low concentration groups of 0.25~0.5μg/mL LEO, 3-butylidenephthalide and octyl acetate, AEO, BEO, CEO and neryl acetate could regulate the mRNA levels of PIK3CA and CCND1 to control cell levels. Except for the 1μg/mL 3-butylidenephthalide treatment group, all other samples groups were able to regulate mRNA levels of ESR to control cell levels，as show in Figure 9. The cell experiment supported the results obtained in section "3.1" .

Mice experiment results

Effect of CEO on spatial learning and memory in CUMS mice

After 28 days of CUMS stimulation modeling on mice, the water maze test was conducted on the mice, and the results were shown in Table5 and Figure 10

Compared with the control group, the escape latency of the model group was significantly prolonged, the swimming time of mice was significantly shortened, the number of times mice crossed the target was significantly reduced, and the swimming trajectory of mice was sparse, indicating that after 28 days of CUMS stimulation, mice anxiety model was successfully established.

Compared with the model group, the escape latency of mice in each treatment group was between the control group and the model group, and the swimming time and cross platform frequency increased. The dense movement trajectories of mice in the essential oil group and positive group indicated that inhaling atomized CEO could help improve the anxiety state of mice induced by CUMS stimulation, and the effect of the CEO group on improving mice anxiety was better than that of the neryl acetate group.

Detection results of related proteins in mice hippocampus

Estrogen receptor(ESR1), cyclin D1 (CCND1), and interleukin-1β (IL-1β) were measured in mice hippocampus, and the results were shown in Figure 11.

Compared with the control group, the levels of IL-1β, CCND1 and ESR1 in the mice hippocampus of anxiety group increased ($p<0.05$, $p<0.0001$), indicating successful modeling. Compared with the

anxiety group, the levels of IL-1β ($p<0.05$, $p<0.01$), CCND1, and ESR1 in each treatment group decreased. CEO and neryl acetate downregulated the expression of IL-1β, CCND1 and ESR1 proteins in the hippocampus of CUMS stimulated mice, thereby alleviating anxiety symptoms in mice.

Discussions

Using network pharmacology as a tool, of, the active components of CEO in the treatment of anxiety with melasma were screened, including neryl acetate, citral, 3-butylphthalide and octyl acetate,etc. Neryl acetate in LEO is accompanied by lemon and lavender-like aroma[21] and plays the role of anti-anxiety[22,23]. Clinical olfactory and animal experiments have found that citral in LEO can act on the brain to alleviate depression through the olfactory system. Citral also has a stronger tyrosinase inhibition effect than limonene and plays a leading role in whitening effect of LEO[24]. 3-Butylidenephthalide is a phthalide component in AEO. Research[25] has shown that phthalide components in angelica sinensis can improve the disease of melasma by promoting blood circulation and removing blood stasis[26]. And 3-Butylidenephthalide is an effective inhibitor of norepinephrine and 5-HT reuptake, which can cross the blood-brain barrier and improve brain blood microcirculation, and have neuroprotective effects on the central nervous system[27]. Octyl acetate is the main component

of BEO, which has anti-inflammatory, blood-activating and analgesic effects[30]. Therefore, it is speculated that CEO may play a role in the treatment of anxiety with melasma through aromatic esters to soothe emotions, aldehydes to whitening, antidepressant to promote blood circulation, phthalides to remove blood stasis and neuroprotection.

PPI network analysis showed that CEO mainly treated anxiety with melasma through estrogen receptor (ESR), cyclin-D1(CCND1) and interleukin 1β (IL-1β). ESR is the target of estrogen, which can up-regulate the expression of B-cell lymphoma-2 (BCL-2) in astrocytes by acting on nuclear ERα, thus inhibiting neuronal apoptosis[31]. Estrogen can also act on ESR in melanocytes through PKA pathway, enhance cAMP level, and up-regulate expression of cAMP response element binding protein (CREB), microphthalmoid related transcription factor (MITF) and tyrosinase (TYR), then stimulating melanogenesis[32]. The expression of CCND1 is closely related to the proliferation of melanocytes and can lead to the occurrence of melanoma[33,34]. When the nervous system is damaged, the expression of CCND1 in neurons increases to promot the production of neurogliosis[36]. Studies[37] have shown thatinhibiting the expression of IL-1β and other inflammatory mediators in the hippocampus can indirectly protect against neurotoxic injury. And stimulation of IL-1β can also cause the activation of melanocytes, resulting in the formation of melasma[38]. These showed that CEO mainly regulates anxiety with melasma through hormone stimulation, regulation to cell apoptosis and immune inflammation.

GO showed that targets were enriched in hormone response and steroid binding, which further explained the important role of estrogen. Then the expression of proteins such as BCL-2, CREB, MITF and TYR will be up or down, which plays a role in the treatment of anxiety with melasma. PI3K/Akt signaling pathway plays an important role in improving anxiety[41] and melanoma[42], mainly involved in neuronal apoptosis, neuroinflammation, oxidative stress and other mechanisms[43]. More and more evidences show that the regulation (activation or inhibition) of PI3K/Akt signaling pathway by traditional Chinese medicine can not only help prevent neurodegenerative diseases, but also slow down their progress[44]. The mechanism is closely related to the activation of PI3K and phosphorylation of Akt. In Figure 12, Akt promotes eNOS phosphorylation to produce No. NO can stimulate TYR and increase secretion of melanin[45]. In addition, the production of NO and tyrosinase activity in female skin are significantly higher than those in men[46], indicating that estrogen can promote the expression of NO by stimulating eNOS. In mice with anxiety disorder, the concentration of NO increased significantly[47]. Akt can also promote the expression of BCL-2. BCL-2 is an important anti-apoptotic protein[48]. Up-regulating BCL-2 can reduce the production of oxygen free radicals in melanocytes, thus inhibit melanocyte apoptosis[49]. Besides, it can also inhibit hippocampal neuronal apoptosis and protect the central nervous system[50]. Therefore, it is speculated that CEO can act on the corresponding targets of PI3K/Akt signal pathway and regulate the activity of the targets through active components such as neryl acetate and citral, thereby reducing the NO secretion of eNOS, promoting cell proliferation by Cyclin-D1, and the anti-apoptotic effect of BCL-2.

HaCaT cells and CUMS mice experimental datas showed that treating inflammatory HaCaT cells with essential oils and key compounds led to a reduction in inflammatory factors (NO, TNF-α, and IL-6) and in the mRNA expression levels of PIK3CA, ESR1 and CCND1. Inhalation of atomized essential oils had a positive impact on anxiety-like behavior in CUMS mice. The Swimming time and cross platform frequency of mice were significantly increased, and at the same time, the levels of ESR1, CCND1 and IL-1βin the hippocampus of CUMS mice were suppressed. At the same dose, the effect of CEO on tyrosinase, inflammatory factors, related protein down-regulation and anti-anxiety properties in mice exceed that of single essential oils and even exceed the key compound neryl acetate. CEO is a mixture of monomers, it is speculated that there may be a synergistic mechanism between these components, which requires further exploration and in-depth research.

Conclusions

This article explores the possible mechanism of action of CEO on anxiety disorder with melasma. CEO may be that 28 active compounds such as neryl acetate, citral, 3-butylidenephthalide and octyl acetate, etc. act on 20 potential cross- targets such as ESR1, CCND1 and PIK3CA, etc. in the KEGG pathways such as PI3K/Akt signaling pathway, calcium signaling pathway etc., regulateing endocrine, cell proliferation and apoptosis, oxidative stress, inflammatory response and neuronal damage. Some of the research results have been verified by cell experiments and CUMS mouse experiments. It pro-

vides a foundation and reference for further in-depth research in the future.

Competing interests / COI statement

The authors declare no conflict of interest in the research, authorship, and/or publication of this article.

Authors' contributions

Liping Liu was responsible for designing the research for this project. Xu Xu and Shengdong Wang conducted the primary experiments, performed network pharmacology analysis, analyzed data, and wrote the original draft. Chang Liu participated in the cell experiments. All authors have read and agreed to the published version of the manuscript.

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Data Availability

All data generated or analysed during this study are included in this article. Further enquiries can be directed to the corresponding author.

Ethics Consent

This article does not contain any experimental studies with human. The mouse experiment has been approved by the ethics committee of the relevant institution.

References

1. M. Athar, et al. Prevalence and awareness of melasma during pregnancy. International Journal of Dermatology, (2006)
2. A. Shi. Study on the relationship between clinical characteristics and depression and anxiety in female patients with melasma. Liaoning: China Medical University, (2020)
3. Z. Liu, et al. Network Pharmacology: a New opportunity for Modernization of traditional Chinese Medicine. Acta Pharmacologica Sinica, (2012)
4. Y. Ye. Clinical study of embedded needle combined with traditional Chinese medicine in the treatment of melasma of liver depression and qi stagnation. Guangdong: Guangzhou University of traditional Chinese Medicine, (2021)
5. Y. Zhong, et al. Sedative and hypnotic effect of compound Anshen essential oil inhaled and GC-MS analysis of its chemical constituents, Research and Development of Natural products, (2019)
6. K. Zhang. The anxiolytic effect of cedarwood essential oil and its mechanism, Shanghai: Shanghai Jiao Tong University, (2019)
7. J. Hu, et al. Study on the inhibitory effect of Lemon essential Oil on tyrosinase activity in Vitro, Proceedings of the 10th China Cosmetics Symposium, (2014)
8. JH Chen， et al. Effects of Extract of Angelica sinensis on Melanin Synthesis in Co-culture System of Human
9. Melanoma and Keratinocytes, Chinese Journal of Experimental Traditional Medical Formulae, (2012)
10. Dontje KWEA ,et al. The Therapeutic Potential of Essential Oils in Managing Inflammatory Skin Conditions: A Scoping Review, Pharmaceuticals, (2024)
11. S. Wang, et al. Effects of lemon essential oil on anxiety behavior and cognitive ability of off spring autistic rats, Research and Development of Natural products, (2020)
12. L. Min, et al. The effects of angelica essential oil in social interaction and hole-board tests. Pharmacology, Biochemistry and Behavior,(2005)
13. Y. Xie, et al. Application characteristic of Danggui (Angelicae sinensis Radix) in records of Chi-

nese medicine with reference to western medicine, Journal of Shandong University of traditional Chinese Medicine, (2021)

14. D. Liu, et al. Research progress on chemical constituents and pharmacological effects of frankincense, Chinese Herbal Medicine, (2020)
15. R. Ha, et al. Research progress of chemical constituents, pharmacological effects and prediction and analysis of quality markers of frankincense, Chinese Journal of traditional Chinese Medicine, (2021)
16. B. Zhang, et al. Studies on the chemical composition, antioxidation and antibacterial activity of essential oil from lemon peel, Food Industry Science and Technology, (2015)
17. W. Zhao, et al. GC-MS analysis of volatile components in essential oil from lemon peel, Food Industry Science and Technology, (2009)
18. L. Yang, et al. GC-MS Analysis of volatile Oil from Luxiang and frankincense and study on Antibacterial activity of Helicobacter pylori, Chinese Journal of traditional Chinese Medicine, (2021)
19. Y. Wang, et al. GC-MS combined with network pharmacology and molecular docking to explore the analgesic active components and mechanism of frankincense volatile oil, World Science and Technology-Modernization of traditional Chinese Medicine, (2021)
20. J. Li, et al. Research progress of tranexamic acid in the treatment of melasma, Chinese Journal of New drugs and Clinic, (2013)
21. N. Authier, et al. Benzodiazepine dependence: focus on withdrawal syndrome, Annales Pharmaceutiques Françaises, (2009)
22. L. Géraldine, et al. Neryl acetate, the major component of Corsican Helichrysum italicum essential oil, mediates its biological activities on skin barrier. PloS one,(2023)
23. T. Mizuho, et al. Interspecies comparison of chemical composition and anxiolytic-like effects of lavender oils upon inhalation, Natural Product Communication, (2011)
24. Y. Zhang, et al. Natural volatile oils derived from herbal medicines: A promising therapy way for treating depressive disorder, Pharmacological research, (2020)
25. T. Hang. Imaging observation of human brain area activated by aromatic substances through olfactory pathway, Anhui: Anhui Medical University, (2014)
26. S. Song, et al. Experimental study on the effect of total phthalide of angelica sinensis on promoting blood circulation and removing blood stasis, Chinese Herbal Medicine, (2012)
27. H. Li. Clinical observation of compound angelica sinensis injection acupoint injection combined with photon rejuvenation instrument and Q-switched laser in the treatment of facial melasma, Journal of Hubei University of traditional Chinese Medicine, (2019)
28. W. Gong. Study on antidepressant effect and mechanism of angelica sinensis sinensis and its active components, Shanxi: Shanxi University, (2020)
29. M.Y.Liu, et al. Effect and mechanism of Chuanxiong Rhizoma on permeability of blood-brain barrier, Chinese Traditional and Herbal Drugs, (2024)
30. P. O. Kumar, et al. Comparison of Volatile Constituents Present in Commercial and Lab-Distilled Frankincense (Boswellia carteri) Essential Oils for Authentication. Plants,(2022)
31. Q. Liu, et al. Study on the synthesis and kinetics of octyl acetate, Chemical Industry for Daily use, (2016)
32. X. Li. Study on the neuroprotective mechanism of isopsoralen on spinal cord through estrogenα receptor,
33. Shanghai: Chinese people's Liberation Army Naval Medical University, (2018)
34. W. Hong, et al. Advances in etiology and pathogenesis of melasma, Skin Diseases and venereal Diseases, (2021)
35. T. Lin, et al. Study on proteins related to extracellular signal-regulated kinase pathway in malignant melanoma and common nevus, Chinese Journal of Dermatology, (2004)

36. Q. Tang, et al. G6PD regulates the cycle progression of human melanoma cells through cyclin D1/D2, China Journal of Biochemistry and Molecular Biology, (2011)
37. L. Ying, et al. Effects of glycyrrhizin on the contents of SOD, MDA and NO in melasma mouse model and the proliferation of melanoma A375 cells, Chinese Journal of Gerontology, (2014)
38. G. Xiu, et al . Effects of hyperbaric oxygen therapy on neurological function and CyclinD1 expression in rats with traumatic brain injury, Journal of Clinical Neurosurgery, (2018)
39. Z. Zhu, et al. Analysis of serum IL-1β, IL-6 and IL-10 levels in patients with anxiety disorder, International Journal of Psychiatry, (2019)
40. L. He. Guide to the application of freckle whitening skin care products in melasma, Chinese Journal of Dermatology and venereology, (2022)
41. P. Li, et al. Study on the effect and mechanism of paeoniflorin on depression and anxiety-like behavior induced by Bayk8644 in rats, Laboratory Animals and Comparative Medicine, (2020)
42. X. Wang. Association between calcium signal transduction pathway gene polymorphism and specific survival time of cutaneous melanoma, Jilin: Jilin University, (2020)
43. N. Shan, et al. Xiaoyao Powder improved anxiety and depression behavior of VaD mice by regulating mPFC-BLA myelin function through PI3K/AKT/mTOR pathway, Journal of Nanjing University of traditional Chinese Medicine, (2022)
44. W. Qin, et al. Research progress of autophagy and PI3K/Akt/mTOR signaling pathway in drug resistance of melanoma, Oncology Medicine, (2022)
45. F. F. Liu, et al. Regulating PI3K/Akt Signaling Pathway by Traditional Chinese Medicine to Improve Cognitive
46. Impairment：A Review. Chinese Journal of Experimental Traditional Medical Formulae, (2024)
47. A Cianciulli, et al. Microglia Mediated Neuroinflammation: Focus on PI3K Modulation. Biomolecules. (2020)
48. A. Y. Lee. Recent progress in melasma pathogenesis, Pigment Cell & Melanoma Research, (2015)
49. D. Dinesh, et al. Antianxiety-Like Activity of Gallic Acid in Unstressed and Stressed Mice: Possible Involvement of Nitriergic System, Neurochemical Research, (2011)
50. K. Hisamoto, et al. Estrogen Induces the Akt-dependent Activation of Endothelial Nitric-oxide Synthase in Vascular Endothelial Cells, Journal of Biological Chemistry, (2000)
51. T. Michał, et al. Bcl-2-proteins and neurotrophins as important factors for the survival of peripheral neurons in transgenic animals. Postepy biochemii,(2022)
52. L. Cai. Effects of ferulic acid based on Caspase-3, Bcl-2 and Bax on antioxidant protection and apoptosis of epidermal melanocytes, Guangdong: Jinan University, (2016)
53. R. Wang. Effect and mechanism of phloretin on anxiety behavior of CRS rats, Modern Preventive Medicine, (2021)