



Original Article

Effects of estrogen inhibition formula herbal mixture for danazol-induced precocious puberty in female rats: an experimental study with network pharmacology



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ABSTRACT

Background: This study aimed at determining the effect of the herbal mixture estrogen inhibition formula (EIF) and its possible mechanisms by precocious puberty animal models and network pharmacology-based analysis.

Methods: Precocious puberty animal models were established by a single injection of 300 μ g danazol, then female rats were administered EIF, vaginal openings were monitored, uterus and pituitary indices were determined. The levels of ALP, E2, LH, and FSH were measured using ELISA kits. Real-time PCR was performed to evaluate the mRNA expression of GnRH, UNC5C, and netrin-1 in hypothalamic tissues. We applied network pharmacological analysis to predict potential targets and pathways of EIF.

Results: EIF delayed danazol-induced early vaginal opening. In the onset model, EIF reduced the increased levels of serum ALP, E2, LH, and FSH; as well as mRNA expressions of GnRH, Netrin-1, and UNC5C. Moreover, long-term administration of EIF not only diminished all impaired factors but also had no effect on the normal development of the animals. The gene set enrichment analysis showed that the targets of EIF are mainly associated with the GnRH signaling and ovarian steroidogenesis pathways.

Conclusion: EIF could be used in preclinical research for the treatment of precocious puberty by the inhibition of HPGA pre-maturation.

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1. Introduction

Precocious puberty is defined as the onset of secondary sexual characteristics, which occurs before the age of 8 in girls and 9 in boys. Differential diagnoses based on clinical signs, bone age determination, pelvic echography (for girls), and hormone test, are frequently used to distinguish between central (GnRH-dependent) precocious puberty and peripheral (GnRH-independent) precocious puberty.^{1, 2} Central precocious puberty (CPP) caused by the early activation of the hypothalamic-pituitary-gonadal axis (HPGA) leads

to the release of gonadotropins, which results in the stimulation of gonadal sex steroids that trigger the development of puberty and accelerate bone maturation.³ CPP is more frequent in girls than in boys, with most cases being idiopathic. GnRH agonists, the most widely used therapy for central precocious puberty, improve final adult height, hence, preventing psychosocial problems resulting from emotional stress in affected children.⁴

The increasing number of children with precocious puberty is the result of high-caloric diets, sedentary lifestyles, and early exposure to sexual content from TV or the Internet.⁵ Clinical studies have shown that the prevalence of CPP is higher in overweight and obese girls than girls with normal weight.⁶

In East Asia, herbal remedies are used for the treatment of CPP.⁷ Several pharmacological studies and clinical trials have been performed to investigate the efficiency of herbal medicine in the

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Table 1
List of Primers for Real-Time PCR of the Herbal Mixture.

Gene	Forward (5' -> 3')	Reverse (5' -> 3')
GnRH	ATTCTACTGACTTGGTCGTG	GGAATATGTGCAACTTGCTGT
Netrin-1	AGAGTTTGTGGATCCGTTCC	TTCTTGCACTTGCCCTTCTT
UNC5C	CATCATAAAGCAGGCCGACTC	GACCAGCCACCGTTGACATAG
B-actin	CACCCGCGAGTACAACCTTC	CCCATACCCACCATCACAC

treatment of CPP. This study aimed at investigating the efficacy of the herbal mixture Estrogen Inhibition Formulae (EIF) in CPP treatment, which consists of 12 medicinal plants using precocious puberty female rat models induced by danazol. We also conducted network pharmacology-based analysis of EIF by identifying candidate compounds for bioactivity and predicting their target genes and pathways related to precocious puberty.

2. Methods

2.1. Preparation of herbal mixture

EIF (Estrogen Inhibition Formulae, EIF) is a herb mixture used in traditional Asian medicine and used clinically in the treatment of Precocious puberty.⁸ The mixture prescription is mainly composed of 12 medicinal plants (Supplementary Table 1). The herbal mixtures were boiled with distilled water (DW) at 100 °C for 4 h and then filtered using a 300-meshed filter (50 μm). After condensing the extract for 1 h, it was placed under -70 °C for at least 3 h. The frozen extract was processed to the frozen lyophilization for 72 h and used for animal experiment.

2.2. Identification of chemical constituents and candidate compounds

Chemical constituents of EIF (12 herbs) were retrieved from three network pharmacology databases: TCMSP (<http://sm.nwsuaf.edu.cn/lsp/tcmsp.php>),⁶ TCM-BATMAN (<http://bionet.ncpsb.org/batman-tcm>),⁷ and TCM-mesh (<http://mesh.tcm.microbioinformatics.org/>).⁸ To perform subsequent analysis, we excluded compounds for which Pubchem CID number is not available. The 2D structures of compounds was obtained by converting the CID number into SMILE format using identity exchange service of Pubchem (<https://pubchem.ncbi.nlm.nih.gov/idexchange/idexchange.cgi>). Classification of the EIF compounds into hierarchical taxonomic divisions was performed using Classyfire (<http://classyfire.wishartlab.com/>), a web-based application for automated structural classification of chemical entities.⁹

To select candidate compounds for bioactivity that can act on the central nervous system, the GI absorption and BBB permeability of the constituents of EIF were predicted using SwissADME (<http://www.swissadme.ch/>), a web-based tool to evaluate pharmacokinetics, druglikeness and bioavailability score.¹⁰ The candidate compounds were determined as those with favorable pharmacodynamic parameters (GI absorption, 'high'; BBB permeant, 'Yes').

2.3. Animals and experimental design

Two-day-old Sprague-Dawley female rats and their mothers were purchased from Daehan Biolink (Chungcheongbuk-do, South

Korea) and housed under a 12 h/12 h light/dark cycle at a controlled temperature. The experimental protocol was approved by the Institutional Animal Care and Use Committee at Gachon University (GIACUC-R2019035). The female rats were randomly divided into the following groups: normal, CPP model, CPP model with 200 mg/kg EIF, CPP model with 400 mg/kg EIF, CPP model with 30 μg/kg leuplin (as a positive control), and CPP model with saline. To establish CPP models, PD5 female rats were administered 300 μg of danazol (Sigma) dissolved in 30 μl of vehicle (propylene glycol: ethanol, 1:1, v/v). EIF and leuplin were then suspended in saline and orally administered from PD 15 till the day of sacrifice (Fig. 2A, experimental design). Body weight and vaginal opening were examined daily.

2.4. Whole blood samples and tissue collection

The animals were sacrificed on PD 28 and 44 to evaluate hormonal levels and mRNA expression for the onset and long-term safety effects of EIF, respectively. Whole blood samples were then collected from the heart to evaluate plasma hormone levels. Uteruses and pituitary glands were harvested and weighted. Organ index was determined by dividing organ weight with whole body weight. Hypothalamic tissues were obtained from brains and stored at -80 °C until subsequent use.

2.5. Quantification of blood hormone levels using ELISA

Whole blood samples were collected from the heart, and plasma was obtained after centrifugation and stored at -80 °C until used. E2, LH, FSH, and ALP levels were then determined using ELISA Kits (Abebio, Wuhan, China), following the manufacturer's instructions. A standard curve for each target was created using Curve Expert 1.4 software. The concentrations were determined using linearization.¹¹

2.6. Quantitative real-time polymerase chain reaction

Hypothalamic tissues were homogenized with rotterred homogenizer and total RNA was extracted using the Tri-RNA reagent.¹² The same amount of total RNA was used to prepare complementary DNA (cDNA) using the RevertAid RT First strand synthesis kit (ThermoFisher). Quantitative real-time PCR was performed using the QuantStudio™ Real-Time PCR System. Reaction mixtures were prepared with 1 μl cDNA, 10 μl PowerUp™ Syber™ Green Master Mix (2X, ThermoFisher Scientific), and 1 μl of a set of primers (Table 1). The specific sequences of primers are listed in Table 2. PCR reactions were completed in 40 reaction cycles (denaturation at 95 °C for 2 s and annealing at 60 °C for 20 s) after pre-denaturation for 2 min at 95 °C. Relative mRNA levels for each gene were analyzed using the ΔΔCt value method.¹³

2.7. Construction of compound-target network

Compound-target network is a bipartite network with two types of nodes: compounds and targets. The edges between compounds and targets are defined as compound-target interactions (1 or 0). To construct a network, compound-target interaction information for the bioactive compounds of EIF was retrieved from

Table 2
Significant Enrichment Pathway Related to Precocious Puberty by Targets of EIF (Adjusted *p*-value of ≤ 0.05).

Term	Overlap	Adjusted <i>P</i> -value	Odds Ratio	Combined Score	Genes
GnRH signaling pathway	8/91	7.18E-10	32.56	744.44	MAP2K1;SRC;GNAS;GNRH1;PTK2B;GRB2;SOS1;EGFR
Ovarian steroidogenesis	7/50	4.15E-10	51.85	1217.24	INSR;CYP1A1;GNAS;CYP19A1;IGF1R;INS;CYP17A1

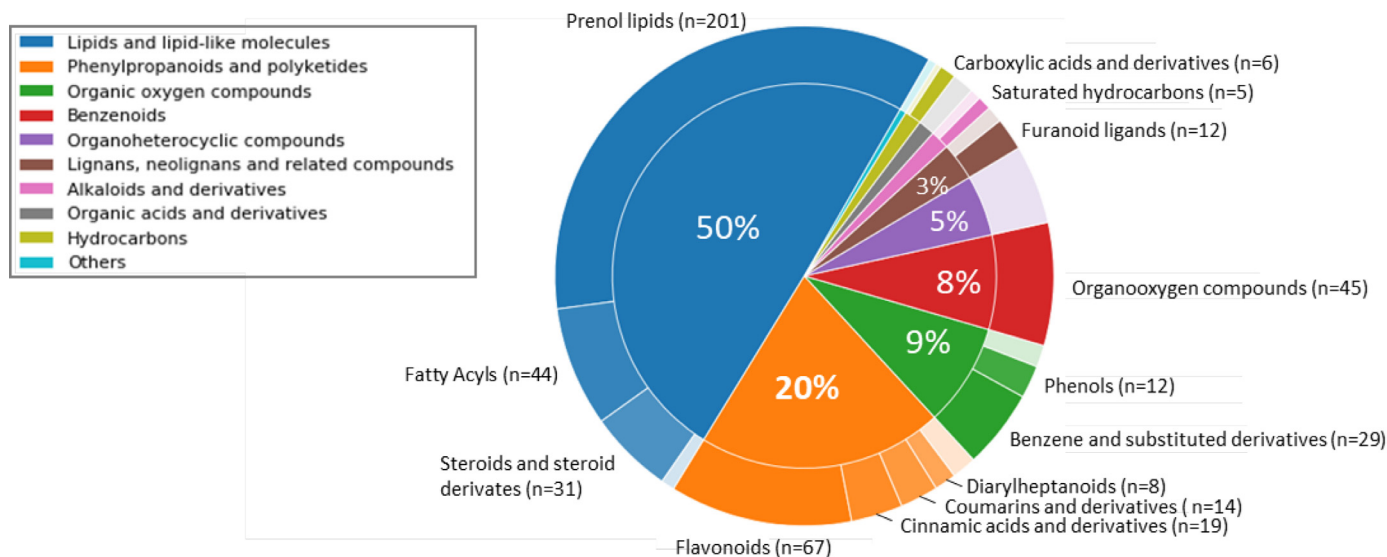


Fig. 1. Distribution of compounds in estrogen inhibition formula across different chemical superclasses and classes obtained from ClassyFire. The inner circle and outer circle represent the proportions of superclasses and classes, respectively.

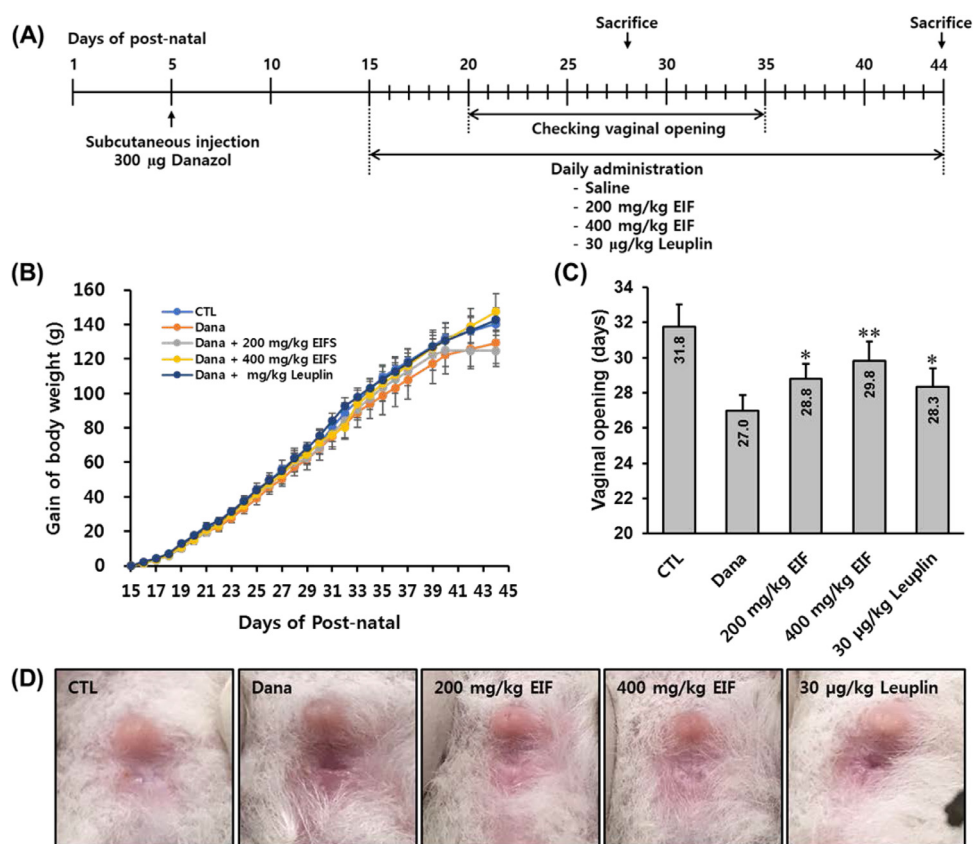


Fig. 2. EIF herbal mixture delayed the day of vaginal opening in danazol-treated female rats. (A) Schematic representation of experimental timeline. Five-day-old rats were injected with 300 µg of danazol to induce CPP. At 15 days old, the mice were divided into the following treatment groups: saline, EIF herbal mixture (200 and 400 mg/kg), and leuplin (30 µg/kg). Body weight and vaginal opening were examined daily. The rats were sacrificed on days 28 and 44 to collect blood, and uterus, pituitary gland, and hypothalamus samples. (B) The increased body weight of rats in the tested groups from day 15–44. (C) Treatment with the EIF herbal mixture delayed the day of vaginal opening. (D) The representative vaginal pictures of rats in the experimental group on day 28. The vagina completely opened in the group only treated with danazol. **p* < 0.05, ***p* < 0.01, and ****p* < 0.001 (vs. the saline-treated group).

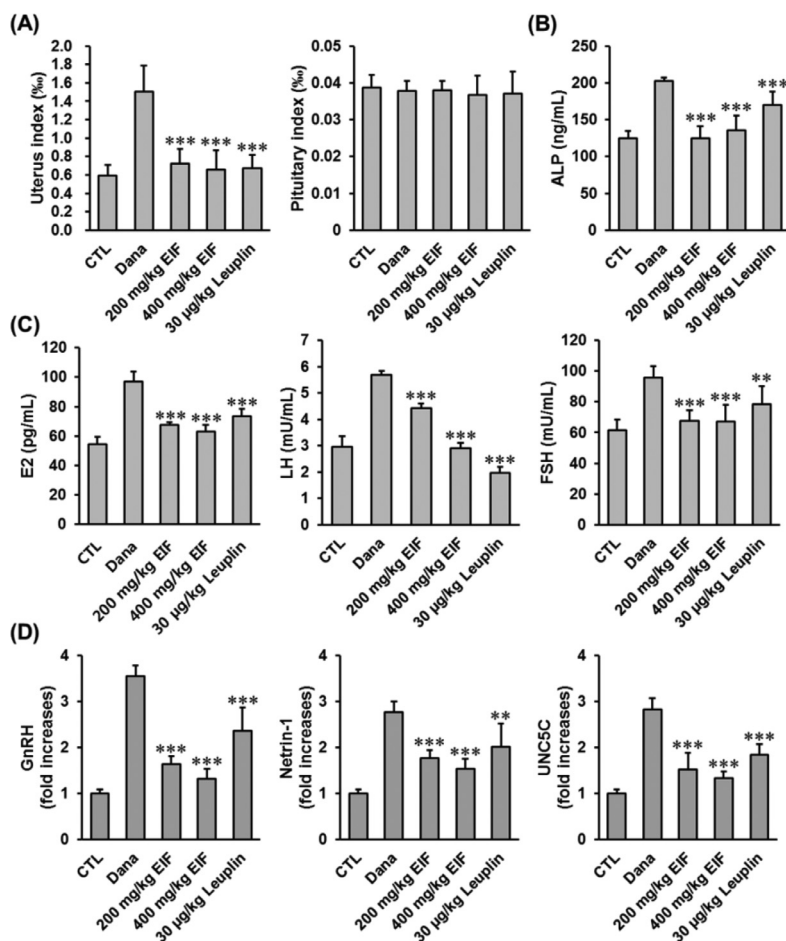


Fig. 3. The onset effects of EIF in CPP. (A) Effects of EIF herbal mixture on uterus and pituitary indexes in CPP rats. (B) Effects of EIF herbal mixture on serum ALP levels in CPP rats. (C) EIF herbal mixture reduced serum E2, LH, and FSH levels in CPP rats. E2, LH, and FSH levels were measured using ELISA Kits. (D) The EIF herbal mixture suppressed the expression of GnRH, Netrin-1, and UNC5C in the hypothalamic tissues of CPP rats. Relative levels of mRNA for each gene were determined using qRT-PCR. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ (vs. the saline-treated group).

TCMSP, BATMAN-TCM, and TCM-mesh. This included experimentally validated interactions or predicted interactions based on the 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 predictive methods for compound–target interactions have been proven to be reliable.^{6–8}

To understand the mechanisms of EIF on precocious puberty, we retrieved precocious puberty-related genes and their functionally associated genes from the Entrez Gene database¹⁴ and string v 11.0,¹⁵ respectively. Then, we identified common targets between retrieved target genes from the Entrez Gene database and predicted targets from TCMSP, BATMAN-TCM, and TCM-mesh. After integrating information about bioactive compounds of EIF, their targets and related pathways, we constructed and visualized a network using Cytoscape 3.7.1 (<https://cytoscape.org/>).¹⁶

2.8. Gene set enrichment analysis

Gene set enrichment analysis (GSEA) based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database was performed to identify potential pathways related to precocious puberty using Enrichr.¹⁷ Enrichr computes enrichment by assessing multiple gene-set libraries (e.g., gene ontology, KEGG, and Online Mendelian Inheritance in Man) and generates 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 multiplication of the p -value and Z -score.

2.9. Statistical analysis

Data were presented as mean \pm SEM. The Student's t -test was used for the comparison of two groups with multiple comparison correction (Bonferroni correction). Statistical significance was determined at the level of $p < 0.05$, $p < 0.01$, and $p < 0.001$ (vs. the saline-treated group).

3. Results

3.1. Description of herbal formula and chemical constituents

We identified 567 chemical constituents of EIF from three databases – TCMSP, TCM-BATMAN, and TCM-mesh – including 34 in *Coix lachryma-jobi* L., 69 in *Curcuma zedoaria* Rosc., 75 in *Artemisia capillaris* Thunb., 72 in *Curcuma longa* L., 92 in *Citrus reticulata*, 28 in *Polyporus umbellatus* (Pers.) Fries, 133 in *Forsythia suspensa* (Thunb.) Vahl, 48 in *Agastache rugosa* (Fisch. et Meyer) O. Kuntze, 71 in *Perilla frutescens* var. *acuta* Kudo, 68 in *Citrus aurantium* L., 14 in *Zizyphus jujuba* Mill., and 51 in *Atractylodes lancea* (Thunb.) DC. To describe chemical compositions, the compounds of EIF with a chemical structure were classified by Classyfire. The result showed that compounds of EIF are distributed across 12 superclasses and 59 classes of ClassyFire. Among the

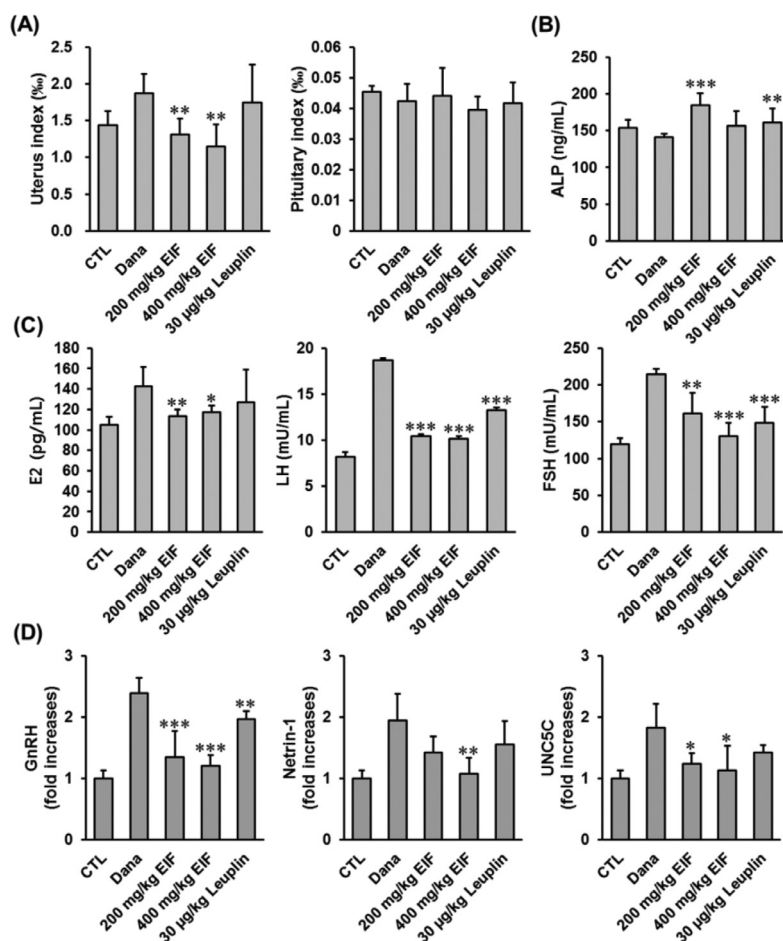


Fig. 4. The long-term safety effects of EIF in CPP. (A) Effects of EIF herbal mixture on uterus and pituitary indexes in CPP rats. (B) Effects of EIF herbal mixture on serum ALP levels in CPP rats. (C) EIF herbal mixture reduced serum E2, LH, and FSH levels in CPP rats. E2, LH, and FSH levels were measured using ELISA Kits. (D) The EIF herbal mixture suppressed the expression of GnRH, Netrin-1, and UNC5C in the hypothalamic tissues of CPP rats. Relative levels of mRNA for each gene were determined using qRT-PCR. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ (vs. the saline-treated group).

12 superclasses, lipid and lipid-like molecules, phenylpropanoids and polyketides, and benzenoids are the top three superclasses with 272, 113, and 46 compounds, respectively (Fig. 1). Among 59 classes, prenol lipids, flavonoids, and organooxygen compounds are the top three classes with 197, 67, and 42 compounds, respectively.

3.2. The day of vaginal opening, organ index of uterus and pituitary

EIF (200 or 400 mg/kg) was administered to the rats by oral gavage and its therapeutic effects were evaluated. The results revealed treatment with danazol decreased body weight, while treatment with EIF and leuplin increased weight (Fig. 2B). Vaginal opening was noticed after postnatal day (PD) 27 in danazol-injected rats, while it was significantly delayed in the 200 mg/kg EIF, 400 mg/kg EIF, and leuplin groups (Fig. 2C and D).

The uterus index significantly increased in the danazol-treated group, but markedly decreased in the EIF- and Leuplin-treated groups, almost as same as in the control group (Figs. 3A and 4A). However, there was no significant difference in the pituitary indexes of the different experimental groups.

Danzol administration at the onset of experiments resulted in significantly increased serum ALP levels, whereas treatment with EIF ameliorated this increase (Figs. 3B and 4B) in rats at the age of 28 days.

3.3. Effect of EIF on hormone levels in serum of the rats

For the onset effects, the levels of serum LH, FSH, and E2 were significantly higher in the danazol-treated group compared with the control group, and were dose-dependently reduced by treatment with 200 and 400 mg/kg EIF (Fig. 3C). Similarly, LH, FSH, and E2 levels on PD 44 were significantly increased in the danazol-treated group and dose-dependently reduced by treatment with EIF (especially LH levels) as showed in Fig. 4C.

3.4. Effect of EIF on hypothalamic GnRH, netrin-1, and UNC5C mRNA expressions

At the onset of precocious puberty, the expression of GnRH, netrin-1, and UNC5C mRNAs in hypothalamic tissues of danazol-treated rats increased significantly (Fig. 3D). However, compared with the control group, the expression of these genes was significantly reduced by continuous administration of EIF, in a dose dependent manner (Fig. 3D). Furthermore, additional sets of experiments were carried out to evaluate the long-term effect of EIF on danazol-induced precocious puberty rats. Even though the levels were not higher than the onset levels of precocious puberty animals, we observed that on PD 44, the expression of GnRH, netrin-1, and UNC5C mRNAs in hypothalamic tissues had normalized (Fig. 4D). Contrary, the mRNA expression of these genes was suppressed in the EIF-treated groups (Fig. 4D).

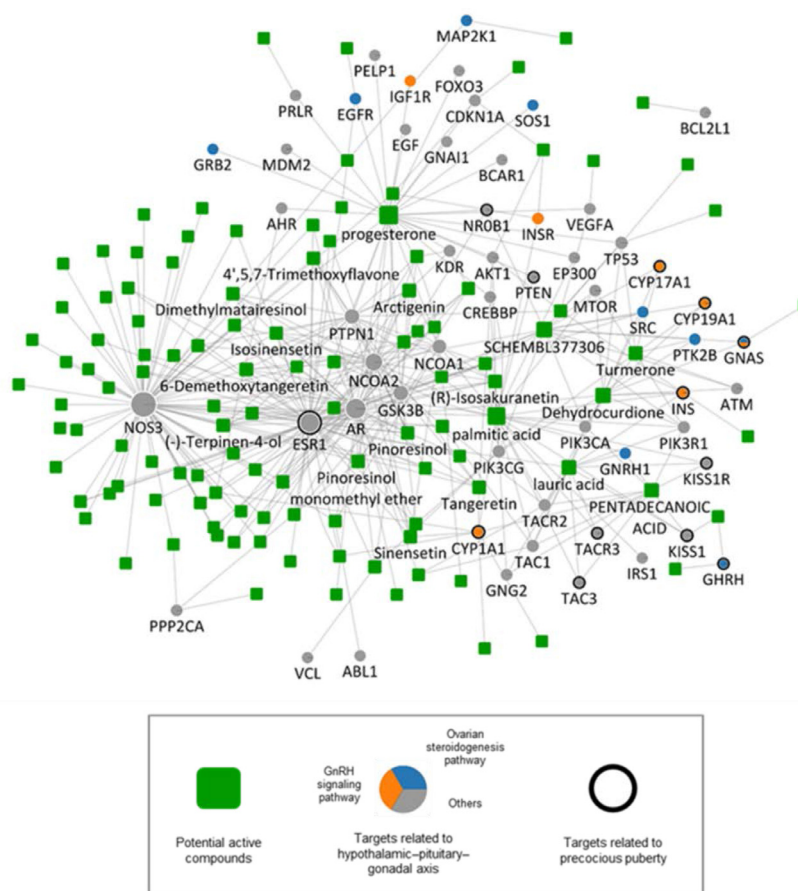


Fig. 6. Compound-target network of EIF. Rectangles represent the compounds, and circles represent the targets, and the edge indicates the interaction between the compound and the target. The targets of EIF were colored to indicate the pathways related to precocious puberty (GnRH signaling pathway and ovarian steroidogenesis pathway). The size of each node is proportional to its degree (the number of edges). Genes directly associated with precocious puberty are bordered in the network.

3.5. Identifying bioactive constituents, target genes and pathways

In order to identify candidate compounds for bioactivity, we consider two pharmacokinetic parameters of the compounds: human intestinal absorption (HIA, also called GI absorption) and blood-brain barrier (BBB) permeability. Among the 567 compounds in the EIF, 27.3–87.5% of the compounds in each superclass met our screening criteria, resulting in 248 compounds as candidate compounds for bioactivity (Supplementary Table 3).

We identified 1910 target genes of candidate compounds by using network pharmacology databases. These targets are either experimentally validated or predicted by machine learning algorithms. Among GSEA results for those target genes, we focused on the GnRH signaling pathway and ovarian steroidogenesis pathway, which are closely related to precocious puberty. The result showed that these two pathways had the high combined scores and low *p*-values, which indicates that the targets of EIF are significantly associated with these pathways (Table 2). The potential mechanisms of EIF related to the precocious puberty pathways were summarized using KEGG Mapper (Fig. 5).

3.6. Construction of compound-target network

We constructed and visualized the compound-target network between the candidate compounds and their target genes that are related to or functionally connected with the precocious puberty. The network consisted of 137 nodes and 399 edges, in which nodes denote the candidate compounds or precocious puberty-related

targets (88 and 49, respectively) and edges represent the interactions between compounds and targets (Fig. 6). The numbers of related targets for the GnRH signaling pathway and ovarian steroidogenesis were 8 and 7, respectively. We prioritize targets of the EIF using a degree, one of the key topological parameters. Among the 49 targets of EIF, the key regulators of HPGA activation such as ESR1 and AR showed second- and third-highest degree distributions (56 and 44, respectively), which suggests that the mechanisms of EIF are closely related to modulating the function of HPGA.

4. Discussion

4.1. Summary of the main results

-We found that treatment with EIF delayed the day of vaginal opening as well as reduced the uterus index in CPP female SD rats model induced by danazol injection. Interestingly, EIF was more effective than leuplin, the reference drug. Danazol stimulation at the onset of experiments resulted in significantly increased serum ALP levels, whereas treatment with EIF ameliorated it. Our results reveal that EIF administration maintains E2, LH, and FSH serum levels as a normal control compared with CPP rats, with long-term safety. Besides, the mRNA expression of netrin-1 and UNC5C genes in hypothalamic tissues was suppressed in the EIF-treated groups. These results suggest that EIF can be a potent therapeutic for precocious puberty.

4.2. Agreement and disagreement with other studies or reviews

Medicinal plants are commonly used as the adjuvant therapy for the treatment of CPP. Thus, some previous studies also used herbal mixtures as the research subjects in the precocious puberty model rats. Tian et al. mentioned that the nourishing “Yin”-removing “Fire” Chinese herbal mixture containing 10 medicinal plants (*Rehmannia glutinosa*, *Scrophularia buergeriana*, *Aemarrhena asphodeloides*, *Cortex Phellodendri*, *Paeonia suffruticosa* Andr., *Alisma plantago-aquatica* L. var. *orientale* Sam., *Prunella vulgaris* L., *Carapax et Plastrum Testudinis*, *Fructus hordei germinate*, and *Gentiana scabra* Bge) efficiently delayed pre-maturation in precocious puberty rats.¹⁸ In the followed studies, Sun et al. reported that this herbal mixture suppressed the hypothalamic kisspeptin expression in danazol-induced precocious rats.¹⁹ The study of He et al. showed that the effects of the nourishing “Yin”-removing “Fire” Chinese herbal mixture on precocious puberty may act through hypothalamic Lin28/let7 pathway expression in the precocious puberty rat model.²⁰ In the present study, we established the herbal formula EIF and found that it inhibited the danazol-induced precocious puberty on rats by decreasing the expression of netrin-1 and UNC5C, which promotes the production and secretion of GnRH from GnRH neurons in the hypothalamus. CPP is caused by the activation of the hypothalamic-pituitary-gonadal axis (HPGA) and disruption of hormonal levels at the early stages of sexual development, herbal mixtures are an attractive therapeutic option for the treatment or prevention of these abnormalities.

4.3. Implication for practice

EIF has potent therapeutic effects in precocious puberty, with long-term safety. It needs more further research about mechanism of action, toxicity, pharmacokinetic, and bioavailability on animal models. If the results of preclinical testes passed, EIF could be considered to apply for clinical trial.

4.4. Implication for research

The results of network pharmacological analysis suggested that the effect of EIF on precocious puberty may caused by the interaction with GnRH signaling pathway and ovarian steroidogenesis pathway that related to HPGA. With this orientation, the continuing studies could limit the target genes to evaluate the efficacy of EIF on suppressing the activation of HPGA, as well as assess the correlation between the pharmacological analysis and experiment.

4.5. Limitation of study

Our studies initially evaluate the effect of EIF on treatment precocious puberty by using animal model. To achieve the reliable conclusion about the efficacy of EIF, the repeated testes are needed. In addition, the related cell experiments should be performed to confirm the results from the studies on rats. The mechanism of action of EIF on HPGA stimulation also needs to be clarified.

4.6. Conclusion

The administration of the EIF herbal mixture delayed vaginal opening. It also led decreased E2, LH, FHS serum levels, which are responsible for the onset of precocious puberty in animals. In addition, real-time PCR results suggests that EIF prevents the pre-maturation of the HPGA, which is a major cause of CPP in developing animals. Further, the long-term administration of EIF had no effect on normal sexual maturation. Network pharmacology-based analysis revealed that the therapeutic effects of EIF are exerted via the pathways that closely related to precocious puberty. Therefore,

the results of this study suggest that the medicinal herbal mixture EIF could be applied in preclinical research for the treatment of precocious puberty and the inhibition of HPGA pre-maturation is a possible mechanism.

Author contributions

Conceptualization: KSK and HLL. Methodology: KSK, HLL, and CEK. Software: WYL. Formal Analysis: SL, KC, and JH. Investigation: JHS, TAT, and JYB. Writing – Original Draft: TAT and JHS. Writing – Review & Editing: KSK, SCP, CEK, and HLL. Supervision: KSK and HLL.

Conflict of interest

The authors declare no conflict of interests.

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Ethical statement

The experimental protocol was approved by the Institutional Animal Care and Use Committee at Gachon University (GIACUC-R2019035).

Data availability

The data will be made available upon request.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.imr.2020.100708](https://doi.org/10.1016/j.imr.2020.100708).

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