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Regulation of appetite-related neuropeptides by herbal medicines: research using microarray and network pharmacology

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Abstract

Anorexia means loss of appetite and is a state whereby a desire to eat is either reduced or eliminated resulting in reducing or stopping food intake. Sipjeondaebo-tang (SDT) and Hyangsayukgunja-tang (HYT) are prescriptions known to have appetite-improving effects, but studies on their mechanisms and active components are insufficient. The hypothalamus is the center of appetite control, and various appetite control mechanisms are known. We used mouse hypothalamic neuronal GT1-7 cells as appetite control center cells and analyzed the difference in efficacy between SDT and HYT using microarray and network pharmacology. Microarray analysis showed that SDT and HYT affect the regulation of genes related to appetite control in the digestive tract and central nervous system. Using network pharmacology, we analyzed the differential expression of neuropeptide Y receptors, glucagon, corticotropin-releasing hormone receptors 1, and 5-hydroxytryptamine receptor 4 among the 17 anorexia-related genes selected from the comparative toxicogenomics database and also analyzed the active components that affect gene expression. In conclusion, the appetite-related genes contributed to anorexia control, and the difference in the action mechanism of the two complex prescriptions could be explained.

Keywords Sipjeondaebo-tang, Hyangsayukgunja-tang, Anorexia, Quality evaluation, Herbal medicine

Introduction

Anorexia means loss of appetite and is a state whereby a desire to eat is reduced or eliminated resulting in reducing or stopping food intake [1]. Anorexia has various causes such as nausea, vomiting, side effects of medication, psychological factors, and aging [2, 3]. Appetite is the psychological desire to eat, associated with sensory experiences such as the sight and smell of food or cognitive, emotional, social situations, and cultural conventions. Appetite is regulated by interactions between peptide hormones in the digestive tract or adipose tissue and the hypothalamus [4]. The hypothalamus is the center of appetite control, and various appetite control mechanisms are known. The hypothalamus regulates short- and long-term dietary intake by synthesizing numerous anorectic and orexigenic neuropeptides. The function and structure of several hypothalamic peptides, including

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melanin-concentrating hormone (MCH), cocaine- and amphetamine-regulated transcript (CART), orexins, neuropeptide Y (NPY), melanocortins, and agouti-related peptide (AGRP) have been studied in rodent models. In addition, peripheral neuropeptides, including bombesin, amylin, peptide YY (PYY3-36), ghrelin, and cholecystokinin (CCK), govern essential gastrointestinal processes, such as absorption, secretion, and motility, offer feedback to the central nervous system on nutrition availability, and may help regulate food intake [5].

Sibjeondaebo-tang (SDT) and Hyangsayukgunja-tang (HYT) are commonly used prescriptions for anorexia but have different components. SDT is a frequently prescribed herbal medicine comprising 10 herbs (*Astragali Radix, Panax ginseng radix, Atractylodes Rhizoma Alba, Poria sclerotium, Rehmanniae Radix, Angelicae Gigantis Radix, Paeonia Radix, Cnidii Rhizoma, Glycyrrhizae Radix et Rhizoma, and Cinnamomi Ramulus*) in Korea, Japan, and China [6]. SDT is also called Shi-Quan-Da-Bu-Tang in China and Juzen-taiho-to in Japan. SDT is used to treat both qi and blood deficiency syndromes by balancing Yin and Yang, and is also widely used for treating chronic illnesses by restoring physiological function and improving immunity [7]. HYT (named as "Xiang Sha Liu Jun Zi Tang" in Chinese) has been used for various digestive disorders, such as gastric flatulence, anorexia, nausea, and vomiting. HYT is commercially available and comprises 14 herbs: *Cyperi Rhizoma, Atractylodis Rhizoma Alba, Poria Sclerotium, Pinelliae Tuber, Citri Unshius Pericarpium, Amomi Fructus Rotundus, Magnoliae Cortex, Amomi Fructus, Ginseng Radix Alba, Aucklandiae Radix, Aipiniae Oxyphyllae Fructus, Glycyrrhizae Radix et Rhizoma, Zingiberis Rhizoma Crudus, and Zizyphi Fructus* [8].

Herbal medicine preparations are widely used owing to their long history and safety. They are effective in treating complex syndrome-type diseases owing to the complex efficacy of their various components. However, these preparations have many drawbacks, such as non-specific and weak efficacy, resulting in efficacy evaluation difficulties. Therefore, microarray and bioinformatic methods should be used that would facilitate the analysis of complex herbal prescriptions. In this study, we used mouse hypothalamic neuronal GT1-7 cells as appetite-regulating cells and analyzed the differences in the related genes by SDT and HYT using microarray.

Methods

Preparation of herbal prescriptions

SDT and HYT were prepared from a chopped herbal mixture by Hanpoong Pharmaceutical Co., Ltd. (Jeonju, Korea) and the Korea Institute of Oriental Medicine, respectively. Briefly, the mixture was added to 125 mL of distilled water and decocted at 90–100℃ for 3 h. The extract was filtered through filter paper with a 5 μm pore size. The filtrate was concentrated using an evaporator, and the remaining mass was vacuum-dried to obtain a powder. The powder was dissolved in dimethyl sulfoxide (DMSO) for in vitro experiments.

Cell cultures and viability

GT1-7 cells were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM; Corning, Manassas, VA, USA) supplemented with 10% fetal bovine serum (FBS; Atlas Biologicals, Fort Collins, CO, USA) and 1% antibiotics (100 U/mL penicillin and 100 U/mL streptomycin; Gibco BRL, Carlsbad, MD, USA). The cells were seeded at a density of 2×10^4 cells/well on a 96-well plate. GT1-7 cells were incubated at 37 °C in a 5% CO_2 humidified incubator. The cells were treated under various concentrations (25, 50, 100, and 200 μg/mL) for 24 h. Cell viability was measured using an EZ-cytox assay kit from DoGenBio (Seoul, Korea) at 450 nm using a microplate reader.

RNA extraction

GT1-7 cells were treated with 25 and 200 μg/mL SDT and HYT, respectively. After 24 h, total RNA was isolated using the RNeasy mini kit following the manufacturer's protocol (Qiagen Inc., Valencia, CA, USA). Subsequently, the purity was quantified by measuring the 260/280 ratio between 1.8 and 2.1. In addition, the integrity number (RIN) was over 7.

Microarray data collection and analysis

Cell samples were collected and subjected to total RNA extraction for use in the Clariom™ S Assay platform for mice. Following the manufacturer's instructions, the extracted total RNA was converted into cDNA using the GeneChip Whole Transcript (WT) Amplification kit. Subsequently, the sense cDNA was fragmented and biotin-labeled using terminal deoxynucleotidyl transferase (TdT), facilitated by the GeneChip WT Terminal labeling kit. Approximately, 5.5 μg of the biotin-labeled DNA target was hybridized to the Affymetrix GeneChip Array and maintained at a consistent temperature of 45 °C for 16 h. After hybridization, the arrays were processed through a wash-and-stain cycle on a GeneChip Fluidics Station 450, followed by scanning with a GCS3000 Scanner (Affymetrix). Probe cell intensity data were generated and converted into a CEL file via the Affymetrix® GeneChip Command Console® Software. Furthermore, the Affymetrix Power Tools and R 3.3.3 software facilitated data analysis, allowing comprehensive examination and interpretation of the microarray data.

Analysis of the association between microarray data and anorexia

To compile a list of genes perceived to be associated with anorexia, we gathered list of genes from the toxicogenomics database (CTD) [9], where genes are curated by their associations with diseases in terms of markers, mechanisms, or therapeutics. We analyzed the fold-change in anorexia-associated genes in the microarray compared with that in the control. Genes with an absolute fold-change value ≥ 1.5 were presumed to be differentially expressed genes (DEGs). To infer the compound causing changes in the expression of anorexia-associated genes, we reconstructed a prescription-herb-compound-target network focusing on anorexia-associated genes that were differentially expressed. To build the network, information about the herb-component relationship was obtained from TM-MC, and compound-target information was gathered from curations by Hwang et al. [10], which included data from ChEMBL [11], BindingDB [12], STITCH [13], Herbal Ingredients' Targets Database [14], and the Traditional Chinese Medicine Integrated Database [15].

Statistical analysis

The data were performed using the GraphPad software (GraphPad Prism 5, USA). The results were analyzed using one-way analysis of variance to determine differences between the treatment and control groups. All data are presented as mean ± standard error of the mean. Values of $^*p < 0.05$ were statistically significant.

Results

Cell viability in GT1-7 cells

We first investigated cytotoxicity under varying concentrations (25, 50, 100, and 200 μg/mL) of SDT and HYT in the GT 1–7 cell line. As shown in Fig. 1, both samples showed no cellular toxicity, even at high concentrations. SDT showed viability in 100 ± 0.28, 100.50 ± 0.06, 100.34 ± 0.48, 100.45 ± 0.49, 100.17 ± 0.20% (25, 50, 100, and 200 μg/mL) and HYT in 100 ± 0.28, 98.04 ± 0.51, 99.55 ± 0.19, 99.94 ± 0.50, 100.56 ± 0.49%, 25, 50, 100, and 200 μg/mL, respectively. Therefore, 25 and 200 μg/mL concentrations were selected for further study (Fig. 1).

Enrichment analysis

We conducted a pathway enrichment analysis based on the KEGG pathway [16] for DEGs and found that DEGs in samples treated by 'HYT' or 'SDT' were related to multiple pathways associated with digestive systems (including pancreatic secretion) and appetite regulations from the central nervous system (including apelin signaling pathway), and olfactory and nasal infection (olfactory transduction and Staphylococcus aureus infection). More specifically, pancreatic secretion was associated with DEGs in the high-dose group treated with 'HYT' or 'SDT', while salivary secretion was associated with the high-dose 'SDT' group. Serotonergic synapses were associated with DEGs in both the low-and high-dose 'SDT' groups, and the apelin signaling pathway was associated with DEGs in the high-dose 'SDT' group. The top 20 terms were selected based on the high-dose 'SDT' group; however, notably this does not imply that 'SDT' was more

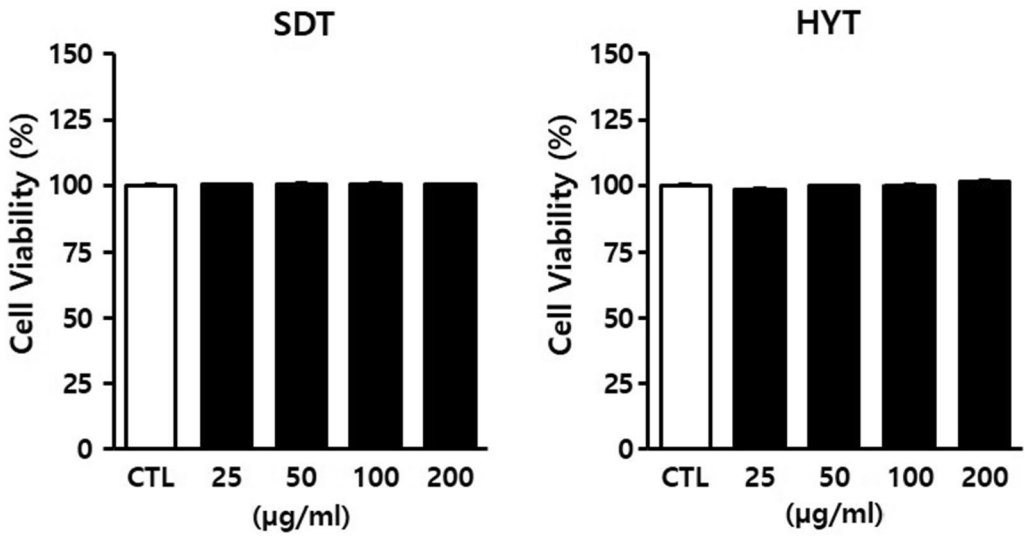

Fig. 1 SDT and HYT cell viability in GT1-7 cells. The cells were treated with various SDT and HYT concentrations for 24 h. Cell viability was determined colorimetrically using EZ-Cytox assay at absorbance 450 nm. The data are expressed as the means ± SD (n = 3). $^*p < 0.05$ compared to control group

effective over 'HYT' in regulating the digestive system or appetite-related CNS regulations (Table 1).

Association with anorexia

We identified 17 anorexia-related genes, including Tnf, Il1b, Tac1, and Npy, from the CTD. Among them, tachykinin 1 (Tac1), Gcg, Crhr1, Crhr2, Htt4, and Ifna2 were differentially expressed in cell lines treated with 'SDT' or 'HYT'. Specifically, Npy5r, Npy1r, and Npy6r, which were associated with Npy, showed increased expression in the low-dose 'SDT' group. While the expression of Tac1 decreased in the high-dose 'SDT' group, the expression of Crhr2 and Ifna2 increased. Il2ra, which was associated with Il2, tended to decrease in all the experimental groups. In the high-dose 'HYT' group, a decrease in the expression of Gcg and Crhr1, which was not substantial in the other groups, was observed (Table 2, Additional file 1: Fig. S1).

Compound on anorexia-associated DEGs

To infer which components within the prescription caused changes in the expression of anorexia-related genes, we analyzed a prescription-herb-compound-target network focused on anorexia-associated DEGs. As a result, we found that linolenic acid, roemerine, falcarindiol, palmitic acid, glycine, retinal, beta-ionone, aporheine, adenosine, thymidine, or oleic acid were associated with Gcg, Npy5r, Npy1r, or Htr4 (Fig. 2 and Additional file 1: Table S1). Naphthalene that is a kind of polycyclic aromatic hydrocarbons and known as a toxic carcinogen was omitted from the analysis.

Discussion

Herbal preparations are widely used owing to their long history of treating illnesses and safety. SDT has been reported in studies and clinical cases to improve symptoms of anorexia caused by cancer, liver toxicity, and hemodialysis [17–20]. HYT is a modified prescription that improves the efficacy of Yukgunja-tang (YT). YT, also called Rikkunshi-to in Japan, is a traditional prescription used to treat upper gastrointestinal symptoms, such as anorexia, nausea, dyspepsia, and gastroesophageal reflux [21–23]. HYT and YT have been used to improve the condition of patients with cancer [23–25] and Parkinson's disease [26] having anorexia. SDT is known to promote gastrointestinal motility to improve symptoms of anorexia [27, 28], and YT also improves gastrointestinal motility and symptoms of anorexia by increasing the level of ghrelin in plasma [29]. Most studies on the improvement of anorexia symptoms using SDT and YT focused on gastrointestinal motility; however, the difference in

Table 1 Top 20 terms in enrichment analysis based on the high-dose 'SDT' and 'HYT' groups. Bolded terms indicate referred terms for the indication of SDT and HYT. Bolded p-values indicate significant relationship between the corresponding pathways and the drugs.

Terms	HYT 25 mg/mL		HYT 200 mg/mL		SDT 25 mg/mL		SDT 200 mg/mL	
	P-value	Overlap	P-value	Overlap	P-value	Overlap	P-value	Overlap
Olfactory transduction	**7.72E-68**	128	**1.14E-54**	136	**2.94E-88**	180	**1.28E-58**	148
Pathways in cancer	**3.36E-05**	27	**3.05E-06**	36	**3.46E-04**	33	**5.03E-11**	48
PI3K-Akt signaling pathway	**1.93E-05**	22	**1.99E-03**	22	**2.59E-04**	26	**3.18E-10**	37
Neuroactive ligand-receptor interaction	**7.31E-06**	24	**6.72E-06**	29	**4.09E-09**	37	**1.72E-07**	34
Herpes simplex virus 1 infection	**1.72E-02**	18	**6.67E-03**	24	**1.29E-03**	28	**1.83E-07**	37
Staphylococcus aureus infection	**3.68E-09**	18	**3.05E-06**	17	**1.16E-05**	17	**2.35E-07**	19
Calcium signaling pathway	**1.71E-02**	12	**5.75E-03**	16	5.70E-02	14	**2.73E-06**	24
Human papillomavirus infection	**5.45E-05**	21	**1.58E-02**	19	**7.46E-03**	22	**8.79E-06**	29
Aldosterone synthesis and secretion	2.10E-01	5	1.06E-01	7	3.16E-01	6	**6.17E-05**	14
JAK-STAT signaling pathway	**4.03E-02**	9	**6.59E-03**	13	**3.15E-02**	12	**6.17E-05**	18
cGMP-PKG signaling pathway	**7.92E-03**	11	8.94E-01	6	7.89E-01	7	**6.43E-05**	18
Kaposi sarcoma-associated herpesvirus infection	**9.48E-05**	16	6.61E-01	8	**3.97E-03**	17	**1.37E-04**	20
Pancreatic secretion	5.22E-02	7	**4.46E-03**	11	1.21E-01	8	**1.61E-04**	14
Human T-cell leukemia virus 1 infection	**1.97E-02**	12	2.24E-01	11	**1.99E-02**	16	**1.61E-04**	21
Salivary secretion	3.33E-01	4	5.95E-02	7	1.00E + 00	3	**2.17E-04**	12
Drug metabolism - other enzymes	6.97E-02	6	7.45E-02	7	**2.24E-02**	9	**4.36E-04**	12
Hematopoietic cell lineage	**2.84E-02**	7	**1.16E-02**	9	5.91E-01	5	**5.04E-04**	12
Serotonergic synapse	7.88E-02	7	5.37E-02	9	**2.59E-04**	15	**5.43E-04**	14
Human cytomegalovirus infection	4.31E-04	16	6.94E-01	9	1.64E-01	13	5.68E-04	20
Apelin signaling pathway	3.66E-01	5	4.94E-01	6	7.14E-01	6	7.84E-04	14

Table 2 Fold-change of anorexia-associated genes in 'SDT' and 'HYT' treated cell lines compared to the control. Bolded gene symbols and fold-change values indicate significant changes in gene expression (|fold-change| > 1.5). Inference scores represent the strength of the inferred association between anorexia and the gene in the CTD database

Symbol	Category	Inference score	Fold-change				Note
			SDT 25 mg/mL	SDT 200 mg/mL	HYT 25 mg/mL	HYT 200 mg/mL	
Tnf	mechanism	238.84	1.02	−1.31	−1.13	−1.01	
Il1b	mechanism	228.3	−1.17	1.41	−1.30	−1.32	
Tac1	mechanism	119.12	−1.17	**−1.75**	−1.11	1.01	
Npy	mechanism	109.76	−1.01	−1.01	−1.03	−1.01	
Npy5r			**1.68**	−1.08	−1.11	1.09	Related to NPY
Npy1r			**1.93**	−1.00	1.41	1.09	Related to NPY
Npy6r			**1.99**	1.00	1.18	−1.11	Related to NPY
Il2	mechanism	103.27	1.42	1.41	1.17	1.40	
Il2ra			−1.50	**−1.85**	**−1.70**	**−1.85**	Related to IL2
Crh	mechanism	99.37	−1.03	1.01	1.00	1.14	
Il1rn	therapeutic	83.17	1.02	1.24	−1.09	1.05	
Tnfrsf1a	therapeutic	74.18	−1.01	−1.15	1.09	1.35	
Gcg	mechanism	56.98	−1.00	−1.35	−1.28	**−2.26**	
Crhr1	mechanism	45.93	−1.23	−1.23	−1.17	**−1.54**	
Gip	mechanism	42.47	−1.07	−1.43	−1.06	−1.41	
Cck	mechanism	37.71	−1.18	1.03	1.02	−1.41	
Crhr2	mechanism	31.94	1.01	**1.54**	−1.33	1.02	
Htr4	mechanism	30.24	−1.09	1.40	**1.56**	−1.03	
Pyy	mechanism	26.85	−1.31	−1.40	−1.44	−1.31	
Ifna2	mechanism	16.2	−1.06	**1.52**	−1.10	1.04	
Ucn2	mechanism	9.16	−1.05	−1.09	−1.15	1.11	

the mechanism of action of these two herbal medicines remains unclear.

Although herbal preparations are effective in treating complex syndrome-type diseases owing to the complex efficacy of their various components, these preparations have many drawbacks, such as non-specific and weak efficacy, making efficacy evaluation difficult. Therefore, we attempted to detect appetite regulation related to multiple genes by SDT and HYT using microarrays. We also used network pharmacological analysis to identify genes involved in improving anorexia in mouse hypothalamic neuronal GT1-7 cells.

Anorexia is associated with several neurotransmitters and neuropeptides in the hypothalamic feeding center, in which corticotropin-releasing hormone (CRH), serotonin (5-Hydroxytryptamine (5HT)), and brain-derived neurotrophic factor play a pivotal role [30]. NPY neurons in the hypothalamic arcuate nucleus are crucial for feeding regulation. Expression and secretion of NPY in the hypothalamus increase during fasting [31], and injection of NPY potently stimulates food intake [32]. Glucagon has been known to be related to glucose metabolism that causes satiety in many studies [33–35]. The anorexic action of glucagon inhibits food intake by sensing blood glucagon levels in the hepatic portal vein [36]. In addition, patients with anorexia frequently have glucose intolerance [37]. TAC1 is related to TAC precursor 1, and a study comparing neural stem cells between anorexia nervosa patients and controls reported that differential TAC1 receptor expression and an aberrant tachykinin neuropeptide signaling pathway may underlie eating disorders [38]. Analysis of SDT- and HYT-treated samples confirmed that appetite control and digestive system genes are related to gene expression. In addition, SDT confirmed that the improvement of anorexia symptoms is related to the NPY hormone in the hypothalamus, as a result of gene expression analysis. In addition, HYT and SDT also improved anorexia symptoms by suppressing gene expression and reducing loss of appetite for internal material such as corticotropin-releasing hormone and serotonin. In addition, active components related to Gcg, Npy5r, Npy1r, or Htr4 genes are predicted to be linolenic acid, roemerine, falcarindiol, palmitic acid, glycine, retinal, beta-ionone, aporphine, adenosine, thymidine, and oleic acid. Among these compounds, palmitic acid and oleic acid have studied in relation to appetite regulation [39, 40].

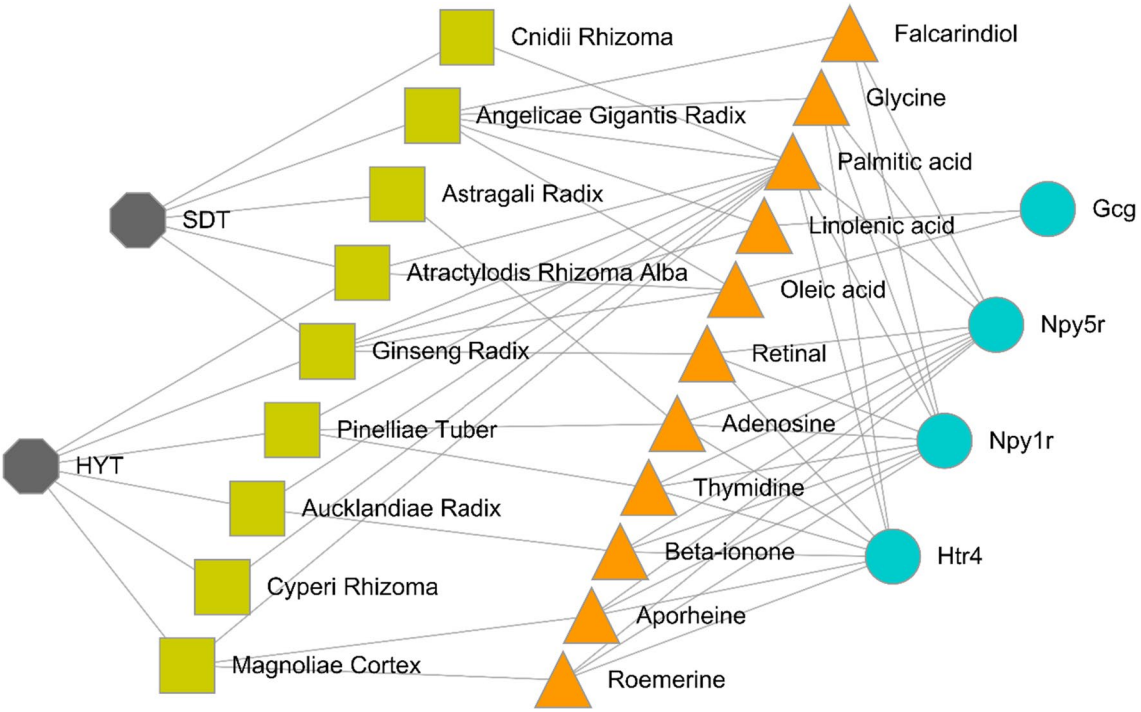

Fig. 2 A prescription-herb-compound-target network focused on anorexia-associated differently expressed genes. Pentagons, squares, triangles, and circles indicate prescriptions, herbs, compounds, and genes, respectively. Edges between nodes indicate associations between entries corresponding to the nodes

In the enrichment analysis, it is found that the targets are associated with genes that would be related to the olfactory and nasal infection such as olfactory transduction and Staphylococcus aureus infection. A temporary or long-term loss of smell can be caused by respiratory infections such as COVID-19, the common cold, and flu that infect olfactory support cells or the lining of the nose and throat [41]. Also, the olfactory decline that often accompanies aging is believed to negatively affect eating pleasure, appetite, food intake, and subsequently nutritional status [42]. Indeed, the olfactory receptors are considered to play a role in regulating appetite and provide opportunity to enhance appetite-related traits, such as feed intake and weight gain [43]. There are possibilities that the drug would be promote the activity of olfactory system and indirectly affects the appetite.

In contrast, this study has a limitation of difficulties in understanding the complex neurophysiological mechanism aspects of appetite regulation by complex emotions such as fear and depression through the interaction of the hypothalamic nerve and the neural network. In addition, our microarray analysis was derived from cell-based and computational experiments, and we believe that the efficacy of these results must be demonstrated through animal experiments.

In conclusion, our experimental results confirmed SDT and HYT efficacies in improving anorexia through gene expression patterns. These results are useful for evaluating the complex efficacy of various active components of herbal medicines and their prescriptions. In addition, it can be predicted in the form of an in vitro test compared to the evaluation method using clinical and animal tests, which is costly and time-consuming. Therefore, the experimental method combining network pharmacology and microarray can be used as a useful tool for analyzing multiple efficacies of herbal medicines and prescriptions and selecting active components.

Abbreviations

SDT	Sipjeondaebo-tang
HYT	Hyangsayukgunja-tang
MCH	Melanin-concentrating hormone
CART	Cocaine- and amphetamine-regulated transcript
NPY	Neuropeptide Y
AGRP	Melanocortins and agouti-related peptide
PYY	Peptide YY
CCK	Cholecystokinin
TdT	Terminal deoxynucleotidyl transferase
DEGs	Differentially expressed genes
CRH	Corticotropin-releasing hormone
5-HT	5-Hydroxytryptamine (serotonin)

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13765-023-00826-x.

Additional file 1: Figure S1. Volume plots for each sample. Each dot indicates a single gene. The x-axis and y-axis indicate volume and log2 fold change, respectively. The volume (intensity) of the expression value is defined as the geometric mean of the expression values of the two groups. **Table S1.** Compounds associated with anorexia-associated differently expressed genes.

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Not applicable.

Author contributions
KSK, CEK, and JYP conceived and designed the experiments; DJ and MJL performed the experiments; DJ, MJL, JHL, and MSS analyzed the data; and JHL and DJ wrote the manuscript. All authors have read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article and its supplementary information files.

Declarations

Competing interests
The authors declare no competing interests.

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