



Article

Unraveling the Cold Property of Gardeniae Fructus: Material Basis and Biological Mechanisms

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Abstract

In traditional Chinese medicine (TCM), Gardeniae Fructus is classified as a “cold” herb, a property that is increasingly explained by modern research showing that it can alleviate related disorders through modulation of the central nervous and endocrine systems, energy metabolism, and gut microbiota. This study aimed to elucidate the material foundation and biological mechanisms underlying its cold property. Chemical components of Gardeniae Fructus were separated via multi-stage extraction and characterized by GC-MS and LC-MS, yielding four distinct fractions: aliphatic, iridoid, crocin, and polysaccharide. In a rat model of heat syndrome induced by levothyroxine sodium, administration of the total extract or individual fractions over 15 days modulated central nervous, endocrine, and energy metabolism indicators, with the iridoid, crocin, and polysaccharide fractions demonstrating significant cold properties. Gut microbiota analysis revealed that the total extract, polysaccharide, and iridoid fractions notably reshaped microbial structure, reducing Firmicutes and Lactobacillus abundance. These findings indicate that the iridoid, crocin, and polysaccharide fractions may be key material bases for the cold property of Gardeniae Fructus, with the iridoid fraction exhibiting the strongest effect.

Keywords: Gardeniae Fructus; cold property; material basis; scientific basis



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1. Introduction

Gardenia jasminoides Ellis, belonging to the genus *Gardenia* of the Rubiaceae family, is the mature and dried fruit of the plant commonly known as Gardeniae Fructus (Zhizi) [1]. Gardeniae Fructus has a long history as a typical traditional Chinese medicine (TCM) used in clinical practice and has shown good clinical efficacy, such as Longdan Xiegan Pill [2], Zhizi Jinhua Pills [3], and Zhizi chi Decoction [4]. In terms of the Four Temperatures (a classification of herbs based on their thermal properties in TCM), the property of Gardeniae Fructus has been relatively stable throughout historical records, being classified as cold [5]. Throughout the historical records of traditional Chinese medicines, Gardeniae Fructus is recognized as an herbal medicine with unique efficacy in clearing heat and removing fire, and it is one of the many heat-clearing herbs that demonstrates significant efficacy in heat syndrome (in TCM, refers to a pattern of illness characterized by excessive heat in the body,

with symptoms such as fever, thirst, constipation, restlessness, and others) [6]. However, the scientific basis of its cold property, the material components responsible, and their biological mechanisms remain poorly defined.

With the rapid development of modern research technologies in recent years, the scientific connotations of TCM theories have been gradually uncovered [7]. To continuously advance the modernization of TCM theories, numerous researchers have conducted in-depth studies on the theory of medicinal properties of Chinese herbs [8]. Recent research suggests that the cold property of herbs correlate with measurable effects on physiological systems, including the neuro-endocrine axis, energy metabolism, and gut microbiota [9]. Animal models, such as the levothyroxine-induced heat syndrome model, provide a validated platform for investigating these correlates [10,11]. Therefore, this study aimed to elucidate the material basis and multi-system biological mechanisms underlying the “cold” property of Gardeniae Fructus. We hypothesized that its cold nature is attributable to specific fractions that counteract heat syndrome by modulating these key systems.

To test this, we first separated Gardeniae Fructus into four major fractions (aliphatic, iridoid, crocin, and polysaccharide). We then systematically evaluated the effects of the total extract and each fraction in a rat heat syndrome model, assessing general physiological status, central nervous and endocrine functions, energy metabolism pathways, and gut microbiota composition. Our work aims to clarify the relationship between the components and their activities in Gardeniae Fructus, providing a scientific framework for understanding its foundational TCM property.

2. Results

2.1. Preparation Method for Fractionated Components of Gardeniae Fructus

Based on an extensive literature review and preliminary experiments, the main chemical constituents of Gardeniae Fructus are known to include aliphatic, polysaccharides, iridoid, and crocin fractions. Given the plant’s diverse traditional applications and the need for precise quantitative separation of pharmacologically active substances (considering bioavailability and specific proportional requirements), a fractional extraction protocol was adopted. Following extraction, the solution underwent concentration, alcohol precipitation, macroporous resin chromatography, polyamide chromatography, and drying, ultimately separating into four distinct fractions. The yields were as follows: total extract 47.33%, crocin fraction 3.89%, iridoid fraction 8.32%, polysaccharide fraction 15.46%, and aliphatic fraction 12.39%.

2.2. Mass Spectral Analysis of the Aliphatic, Iridoid and Crocin Fractions

After methyl ester derivatization of the aliphatic fraction, GC-MS analysis was performed to obtain the total ion chromatogram (TIC) of Gardeniae Fructus aliphatic components, the relative abundance of each compound was estimated using the peak area normalization method (Figure 1). Mass spectra of all peaks were matched against the NIST 17.0 library. Following the exclusion of impurity peaks, the major chemical constituents were identified. The relative percentage content of each compound in the petroleum ether extract was calculated using the peak area normalization method and comparatively analyzed (Table 1).

Table 1. Results of GC-MS qualitative analysis of chemical components in aliphatic fraction of Gardeniae Fructus.

No.	Molecular Formula	TR/Min	Peak Area %	Chemical Name
1	C ₂₀ H ₄₂	13.745	0.29	Eicosane
2	C ₁₇ H ₃₂ O ₂	13.890	0.39	Palmitoleic acid
3	C ₁₇ H ₃₄ O ₂	14.259	11.04	Palmitic acid
4	C ₁₈ H ₃₄ O ₂	15.562	0.38	8-heptadecene

Table 1. Cont.

No.	Molecular Formula	TR/Min	Peak Area %	Chemical Name
5	C ₁₈ H ₃₆ O ₂	16.024	0.54	Heptadecanoic acid
6	C ₁₉ H ₃₄ O ₂	18.133	48.78	Linoleic acid
7	C ₁₉ H ₃₆ O ₂	18.264	17.58	Elaidic acid
8	C ₁₉ H ₃₈ O ₂	18.746	6.74	Methyl stearate
9	C ₅₄ H ₁₁₀	18.940	0.52	n-Tetrapentacontane
10	C ₂₀ H ₃₈ O ₂	21.082	0.15	cis-10-Nonadecenoic acid, methyl ester
11	C ₁₉ H ₃₄ O ₂	24.534	0.78	E, E, Z-1,3,12-Nonadecatriene-5,14-diol
12	C ₁₉ H ₃₆ O ₃	25.348	0.29	methyl 8-[(2R,3S)-3-octyloxiran-2-yl] octanoate
13	C ₂₁ H ₄₀ O ₂	25.731	0.47	11-Icosenoic acid methyl ester
14	C ₂₁ H ₄₂ O ₂	27.272	1.34	arachidic acid
15	C ₂₃ H ₄₆ O ₂	36.596	0.46	Methyl 20-methyl-heneicosanoate
16	C ₂₆ H ₅₂ O ₂	40.423	0.19	Methyl 18-methyl-tetracosanoate
17	C ₃₀ H ₅₀	41.795	1.30	Squalene
18	C ₂₈ H ₄₈ O	46.492	0.39	Campesterol
19	C ₂₉ H ₄₈ O	46.885	0.26	Stigmasterol
20	C ₂₉ H ₅₀ O	47.651	0.88	γ-Sitosterol
21	C ₃₂ H ₅₂ O ₂	47.922	0.41	Lupeol acetate
22	C ₄₂ H ₆₃ O ₃ P	49.106	6.81	tris(2,4-ditert-butylphenyl) phosphate

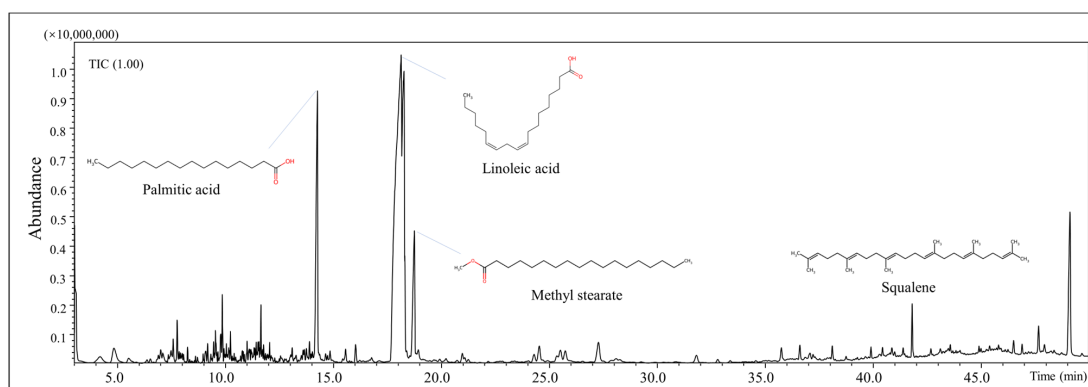


Figure 1. Total ion current chromatogram of aliphatic fraction of Gardeniae Fructus.

Samples from the iridoid and crocin fractions were analyzed by UPLC-MS with positive/negative ion switching mode, yielding TIC of iridoid fraction shown in Figure 2 and crocin fraction shown in Figure 3. Using an in-house Gardenia Fructus-specific mass spectral database for preliminary screening, we tentatively identified 17 compounds in the iridoid fraction and 19 compounds in the crocin fraction (Tables 2 and 3), detailed ion fragment information is provided in Supplementary Materials Tables S1 and S2.

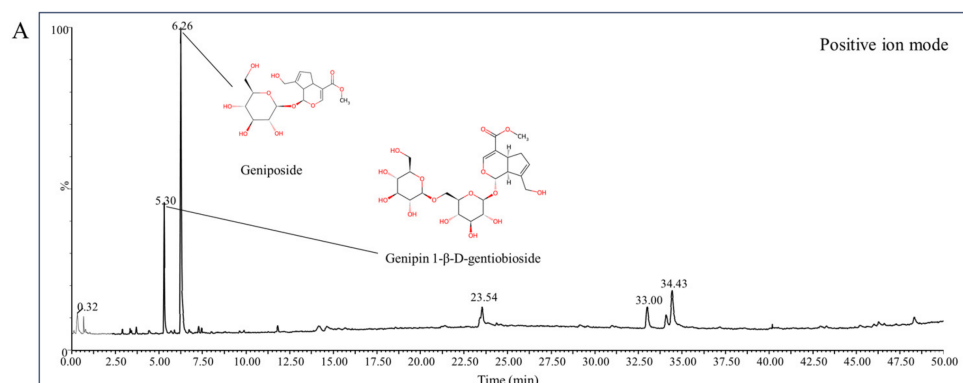


Figure 2. Cont.

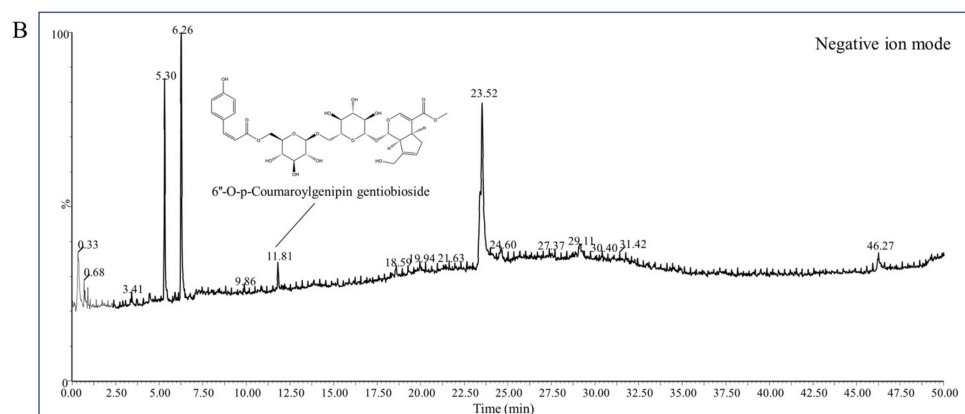


Figure 2. TIC diagrams of the iridoid fraction of Gardeniae Fructus. (A) TIC diagrams under positive ion modes. (B) TIC diagrams under negative ion modes.

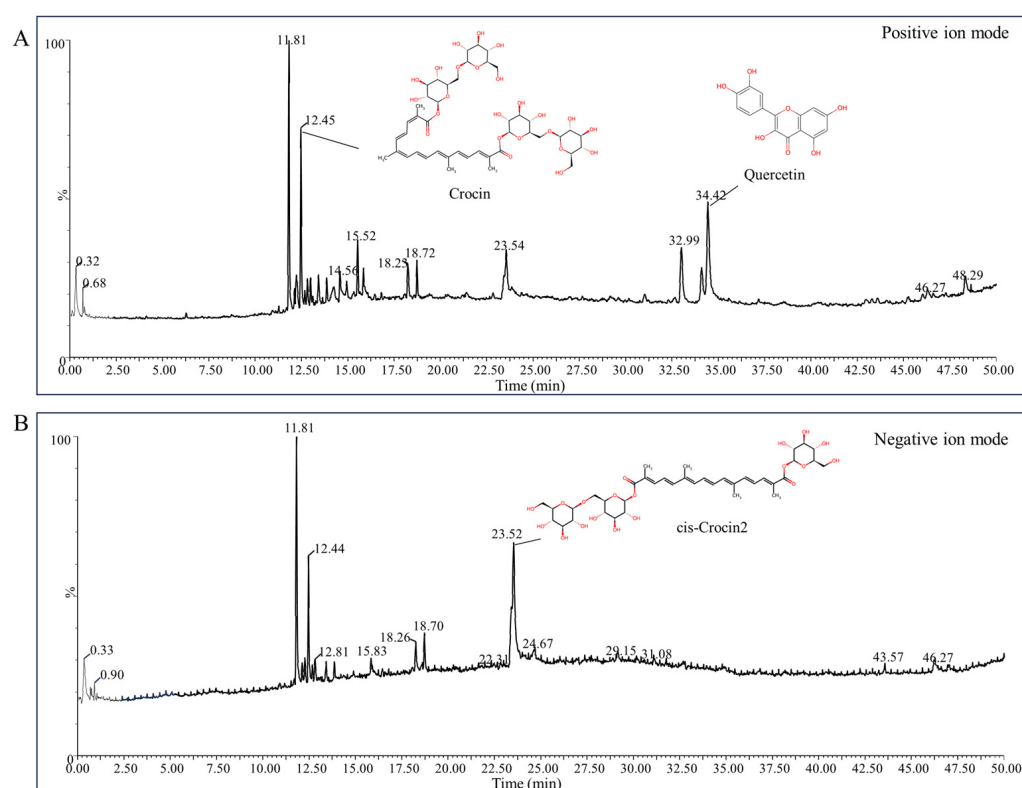


Figure 3. TIC diagrams of the crocin fraction of Gardeniae Fructus. (A) TIC diagrams under positive ion modes. (B) TIC diagrams under negative ion modes.

Table 2. Results of LC-MS qualitative analysis of chemical components in iridoid fraction of Gardeniae Fructus.

No.	TR/Min	Chemical Name	Molecular Formula
1	2.41	Geniposidic acid	C ₁₆ H ₂₂ O ₁₀
2	2.91	Feretoside	C ₁₇ H ₂₄ O ₁₁
3	3.34	Gardenoside	C ₁₇ H ₂₄ O ₁₁
4	3.69	Deacetylasperulosidic acid	C ₁₇ H ₂₄ O ₁₁
5	4.42	jasminoside B/D	C ₁₆ H ₂₆ O ₈
6	5.31	Genipin 1-β-D-gentiobioside	C ₂₃ H ₃₄ O ₁₅
7	5.90	1-O-sinapoyl-beta-D-glucose	C ₁₇ H ₂₂ O ₁₀
8	5.68	Genipin	C ₁₁ H ₁₄ O ₅

Table 2. Cont.

No.	TR/Min	Chemical Name	Molecular Formula
9	6.25	Geniposide	C ₁₇ H ₂₄ O ₁₀
10	6.73	Sinapoyl-β-D-glucoside	C ₁₇ H ₂₂ O ₁₀
11	7.27	jasminoside A/E	C ₁₆ H ₂₆ O ₇
12	7.43	jasminoside A/E	C ₁₆ H ₂₆ O ₇
13	7.46	Jasminoside O/T	C ₂₁ H ₃₄ O ₁₁
14	8.01	2-methyl-L-erythritol-4-O-(6-O-trans-sinapoyl)-β-D-glucopyranoside	C ₂₃ H ₃₂ O ₁₃
15	11.71	jasminoside H	C ₂₂ H ₃₆ O ₁₂
16	11.81	6''-O-p-Coumaroylgenipin gentiobioside	C ₃₂ H ₄₀ O ₁₇
17	23.52	cis-crocin 2/trans-crocin 2	C ₃₂ H ₄₄ O ₁₄
18	32.99	ethoxyphenyl-5-hydroxy-7-methoxy-4H-chromen-4-one	C ₁₇ H ₁₃ O ₇
19	34.42	Quercetin	C ₁₅ H ₁₀ O ₇

Table 3. Results of LC-MS qualitative analysis of chemical components in crocin fraction of *Gardeniae Fructus*.

No.	TR/Min	Chemical Name	Molecular Formula
1	11.81	6''-O-p-Coumaroylgenipin gentiobioside	C ₃₂ H ₄₀ O ₁₇
2	12.10	6'-O-trans-sinapoyl	C ₃₄ H ₄₄ O ₁₉
3	12.24	6'-O-trans-Feruloyl genipin gentiobioside	C ₃₃ H ₄₂ O ₁₈
4	12.45	Crocin-1	C ₄₄ H ₆₄ O ₂₄
5	12.66	6'-O-trans-Sinapoyl jasminoside B	C ₂₇ H ₃₆ O ₁₂
6	12.80	6'-O-trans-Sinapoyl jasminoside L	C ₂₇ H ₃₆ O ₁₂
7	13.38	6'-O-sinapoylgeniposide	C ₂₈ H ₃₄ O ₁₄
8	13.85	Crocin 2	C ₃₈ H ₅₄ O ₁₉
9	15.83	6'-O-trans-cinnamoylgenipin gentiobioside	C ₃₂ H ₄₀ O ₁₆
10	16.79	6'-O-p-coumaroyl geniposide	C ₂₆ H ₃₀ O ₁₂
11	18.23	Crocin 3	C ₃₂ H ₄₄ O ₁₄
12	18.70	Cis-crocin 1	C ₄₄ H ₆₄ O ₂₄
13	23.52	cis-crocin 2/trans-crocin 2	C ₃₂ H ₄₄ O ₁₄
14	32.99	ethoxyphenyl-5-hydroxy-7-methoxy-4H-chromen-4-one	C ₁₇ H ₁₃ O ₇
15	34.42	Quercetin	C ₁₅ H ₁₀ O ₇

2.3. Effects of *Gardeniae Fructus* Total Extract and Its Fractions on the General Status of the Heat Syndrome Model

Results showed that the body weight of the Model group was significantly lower than that of the control group. Although no significant differences were found between the treatment groups and the Model group, an increasing trend was observed in the polysaccharide and crocin groups (Figure 4A). As illustrated in Figure 4B, anal temperature was significantly higher in the Model group compared to the control. In contrast, all treatment groups, including total extract, iridoid, aliphatic, crocin, and polysaccharide, showed a significant reduction in anal temperature compared to the Model group ($p < 0.05$). Notably, animals in the total extract and iridoid groups exhibited signs of loose feces, soiled perianal fur, and dark green urine. These behavioral and excretory changes (loose feces, dark green urine) were recorded as qualitative clinical observations, consistent with traditional descriptions of cold herb overdose in TCM, which often manifests as gastrointestinal and metabolic side effects. While not quantified in this study, their consistent occurrence in groups receiving the total extract and iridoid fraction aligns with the biochemical and microbial evidence of excessive cooling collectively supporting the concept of cold property.

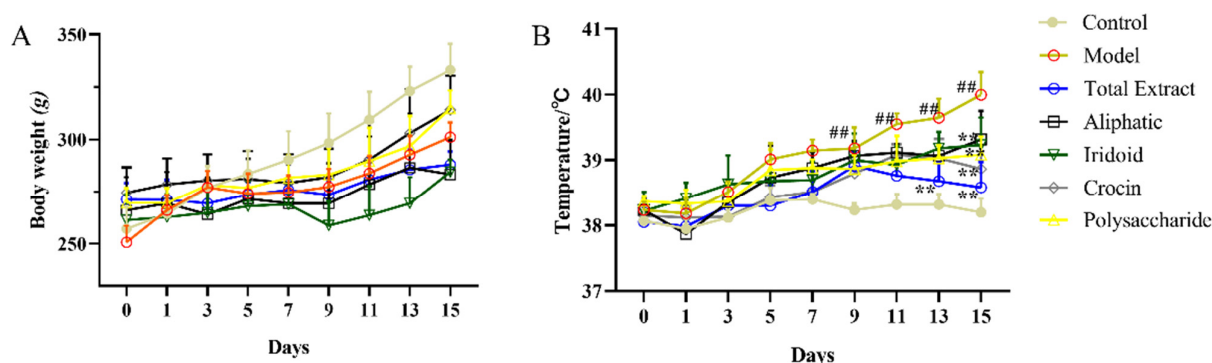


Figure 4. Effects of Gardeniae Fructus total extract and its fractions in general status of heat syndrome model rats. **(A)** Trends in body weight changes among treatment groups during the administration period in heat syndrome model rats. **(B)** Temperature trends during the dosing period in heat syndrome model rats. ## $p < 0.01$ vs. Control group. ** $p < 0.01$ vs. Model group.

2.4. Effects of Gardeniae Fructus Total Extract and Its Fractions on the Central Nervous and Endocrine Systems of the Heat Syndrome Model

As shown in Figures 2 and 3, significant differences were observed in the Model group compared to the control across multiple systems, confirming successful establishment of the heat syndrome model. Regarding central nervous function, levels of NE, DA, AVP, 17-OHCS, and AchE were significantly increased, while 5-HT was decreased (Figure 5A–F). Endocrine markers, including TSH, T3, and T4, were also markedly elevated (Figure 5G–I). Compared with the Model group, the total extract group showed a significant increase in 5-HT and reductions in NE, 17-OHCS, DA, T3, and TSH. The iridoid group exhibited increased 5-HT and decreased AchE, 17-OHCS, NE, DA, T3, and TSH. In the aliphatic group, 17-OHCS was reduced. The crocin group displayed increased 5-HT and decreased AchE, 17-OHCS, AVP, T3, TSH, and T4. Finally, the polysaccharide group showed significant reductions in AchE, 17-OHCS, T3, and TSH. Together, these results indicate that the heat syndrome model induced a state of central nervous excitation and endocrine hyperactivity, characterized by elevated monoamine neurotransmitters (NE, DA), AVP, and thyroid hormones (T3, T4). Gardeniae Fructus total extract and its active fractions (notably iridoid and crocin) effectively reversed this excitatory state, primarily by increasing 5-HT levels and reducing AchE activity, NE, DA, 17-OHCS, and thyroid hormone levels, highlighting their significant cooling properties via modulation of the neuro-endocrine axis.

2.5. Effects of Gardeniae Fructus Total Extract and Its Fractions on Energy Metabolism of the Heat Syndrome Model

Energy metabolism was markedly altered in the Model group. Key substance metabolism indicators, including PDH, SDH, LDH, PYGL, and GSK-3 were significantly increased, along with energy metabolism-related markers such as AMPK, Na^+/K^+ -ATPase, and cAMP (Figure 6A–H). Compared to the Model group, the total extract, iridoid, crocin, and polysaccharide groups all showed reductions in LDH, PYGL, GSK-3, Na^+/K^+ -ATPase, and AMPK. The total extract and crocin groups also exhibited decreased PDH. In the aliphatic group, GSK-3 and AMPK were decreased. To further investigate the regulatory impact on energy metabolism, we examined the mTOR/PGC-1 α signaling pathway using Western blot analysis. The results revealed that, compared to the control, the Model group exhibited significantly increased protein levels of PGC-1 α , along with a notable decrease in P-mTOR/mTOR. In comparison to the model group, the total extract, iridoid, crocin, and polysaccharide groups all exhibited significant down regulation of PGC-1 α . In contrast, the aliphatic group displayed a significant increase in PGC-1 α expression. Furthermore, the total extract, iridoid, and crocin groups demonstrated a marked upregulation

of P-mTOR/mTOR (Figure 6I–K). The data collectively demonstrate that heat syndrome drove a hypermetabolic state, marked by increased activity of key enzymes (PDH, SDH, LDH, PYGL) and energy regulators (AMPK, $\text{Na}^+\text{-K}^+\text{-ATPase}$, cAMP). The iridoid, crocin, and polysaccharide fractions robustly counteracted this state, primarily through down-regulating the mTOR/PGC-1 α signaling pathway, thereby suppressing both material and energy metabolism, which constitutes a core mechanism of their cold property.

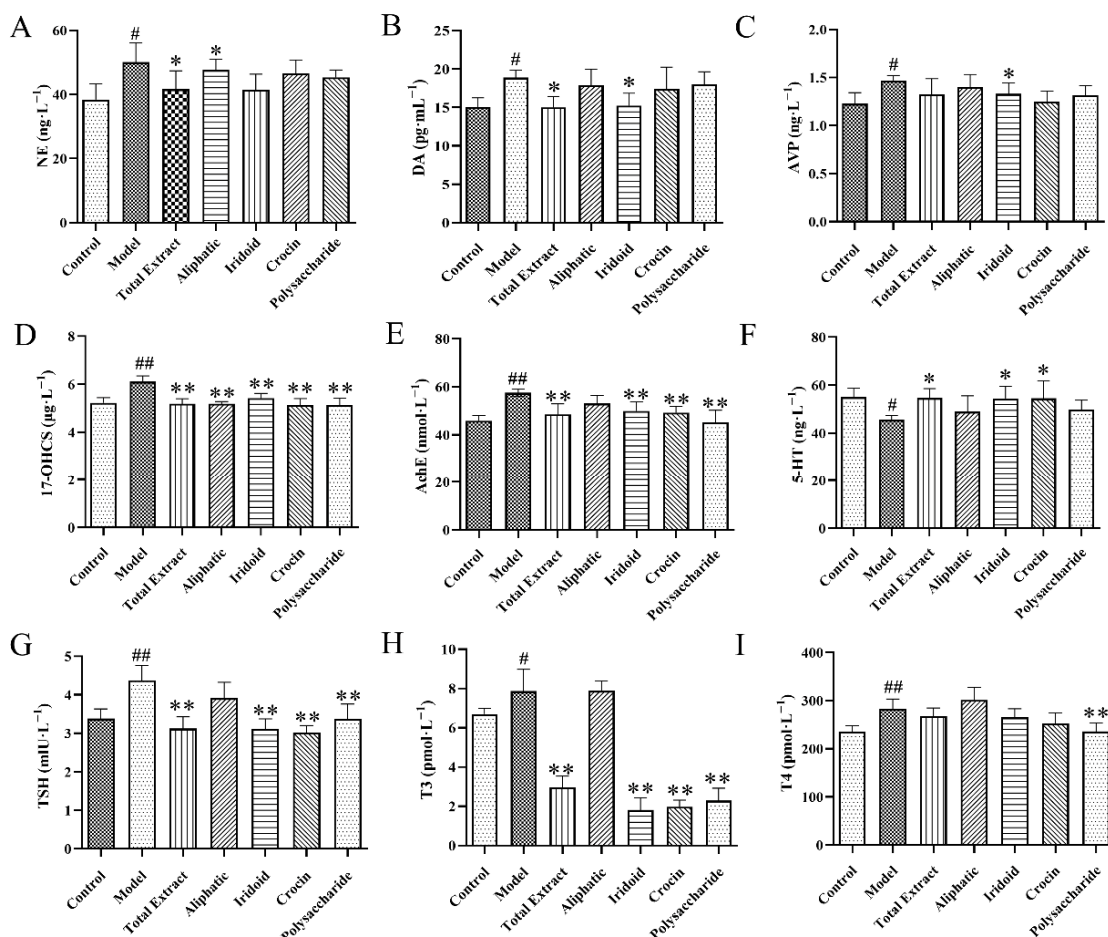


Figure 5. Effects of Gardeniae Fructus total extract and its fractions on the central nervous and endocrine systems of heat syndrome model rats. (A–F) NE, DA, AVP, and 5-HT in brain tissue, as well as 17-OHCS and AchE in plasma, were measured to evaluate the central nervous system changes. (G–I) The plasma levels of TSH, T3, and T4 were detected as characteristics of the endocrine system changes. ## $p < 0.01$, # $p < 0.05$ vs. Control group. * $p < 0.05$, ** $p < 0.01$ vs. Model group.

2.6. Effects of Gardenia Fructus Total Extract and Its Fractions on Gut Microbiota Diversity and Composition in Heat Syndrome Model

High-throughput sequencing of rat fecal samples on the Illumina platform yielded 3,065,692 high-quality sequences after processing. These sequences were clustered into OTUs based on similarity. The Venn diagram (Figure 7A) shows OTU distribution across groups, indicating sufficient sequencing depth. Species number decreased in the Model group compared to the control. The total extract and iridoid groups had the lowest species richness among all treatments, while other fractions remained relatively similar. The top 10 genera, including *Lactobacillus* and *Lachnospiraceae* group, are shown in Figure 7B. Their total relative abundance exceeded 70% in the Model group but decreased in all treatment groups, with the total extract, iridoid, and polysaccharide groups dropping to approximately 60%. A sparse curve analysis confirmed the reliability of the diversity estimates (Figure 7C). Principal coordinates analysis (PCA) analyzed OTU data of each

group and reflected the distance and difference between samples on the two-dimensional coordinate map. As shown in Figure 7D, the result indicated that the samples from Model group were separated from control group, total extract group, iridoid group and polysaccharide group were far away from the Model group. Those results showed that the total extract group, iridoid group and polysaccharide fractions altered the gut microbiota structure in rats with heat syndrome model.

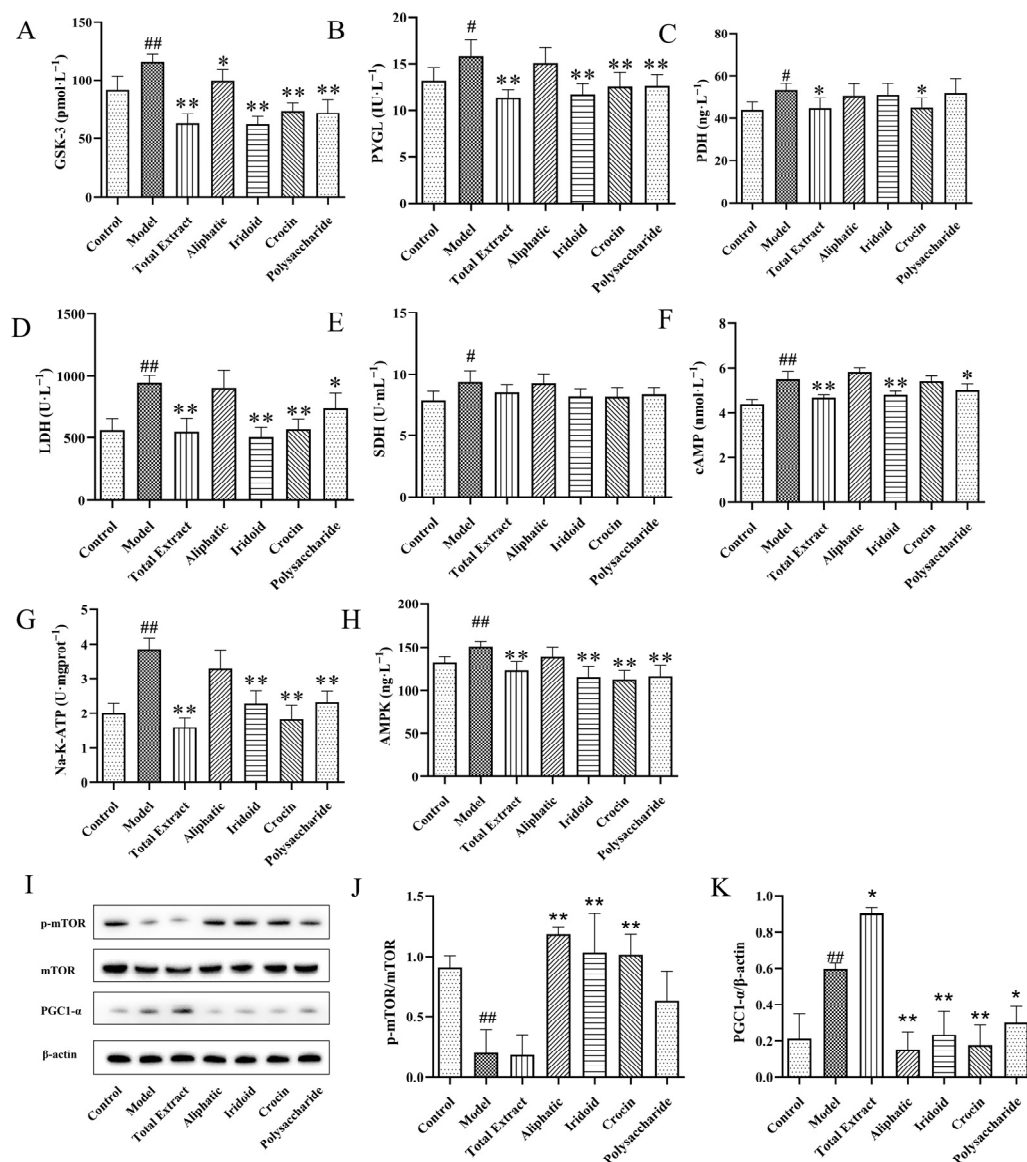


Figure 6. Effects of Gardeniae Fructus total extract and its fractions on energy metabolism of heat syndrome model rats. (A–H) PDH, SDH, and LDH enzymes, GSK-3, PYGL and cAMP in plasma, as well as AMPK and Na⁺-K⁺-ATPase in liver tissue, were measured to evaluate the energy metabolism changes. (I–K) Detection of alterations in key energy metabolism pathways—the mTOR/PGC-1α pathway. ### $p < 0.01$, # $p < 0.05$ vs. Control group. * $p < 0.05$, ** $p < 0.01$ vs. Model group.

To evaluate the richness and diversity of the gut microbiota, α -diversity analysis was conducted. The Sobs and Ace indices were used to assess microbial abundance, while the Shannon and Simpson indices reflected diversity, with higher Shannon or lower Simpson values indicating greater diversity. The Coverage index, which exceeded 0.99 for all samples, demonstrated adequate sequencing depth for reliable analysis. Compared to the control group, the Model group, total extract group, and iridoid group exhibited significantly reduced Sobs values. Additionally, the Ace index was notably decreased in

the total extract and iridoid groups relative to the Model group. Shannon and Simpson indices revealed a significant decline in diversity in the Model group compared to controls, while the total extract and polysaccharide groups showed a significant increase in diversity compared to the Model group (Figure 7E–H).

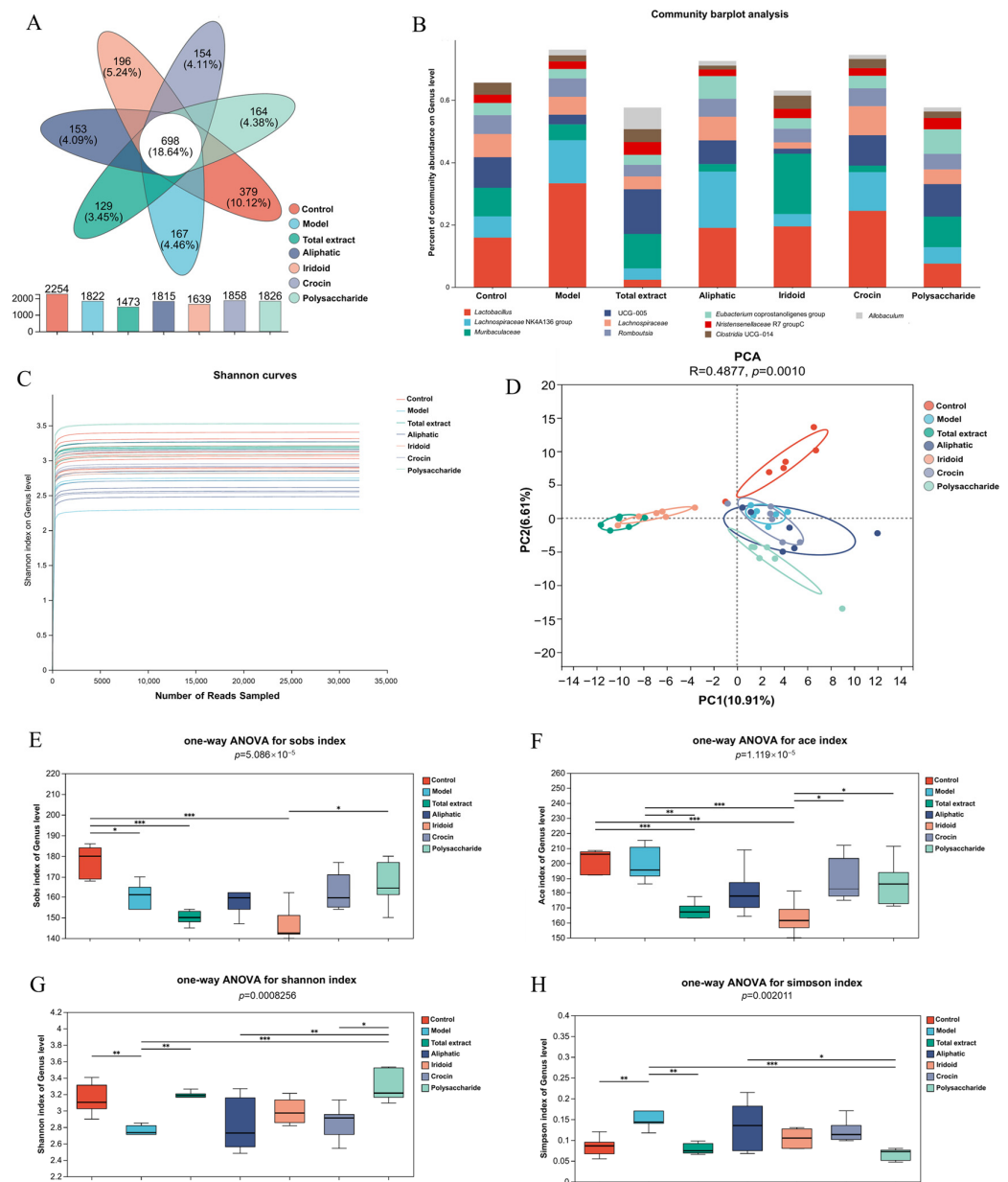


Figure 7. Effects of Gardeniae Fructus total extract and its fractions on the gut microbiota of heat syndrome model rats. (A) Venn diagram of Genus. (B) Bar diagram of community. (C) Dilution curve. (D) PCA scatter plot. (E–H) Mure alpha diversity Sobs, Ace, Shannon, Simpson, Coverage index. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

To further investigate differences in gut microbiota composition among the groups, a linear discriminant analysis effect size (LEfSe) was performed (Figure 8 and Supplementary Materials Figure S1). The analysis revealed distinct microbial biomarkers for each group: The control group was characterized by a high abundance of *Spirochaetia*, *Spirochaetaceae*, and *Treponema*. The model group was predominantly associated with *Lactobacillales*, *Lactobacillaceae*, and *Lactobacillus*. The total extract group was marked by *Erysipelotrichales*, UCG-005, and *Erysipelotrichaceae*. The aliphatic group was enriched with *Lachnospiraceae*, *Lachnospirales*, and *Clostridia*. The iridoid group showed significant representation of *Bac-*

teroidales, Bacteroidota, and Bacteroidia. The crocin group featured *Firmicutes*, *Lachnospiraceae*, and *Faecalibacterium*. The polysaccharide group was dominated by *Oscillospirales*, *Oscillospiraceae*, and *Veillonellales-Selenomonadales*.

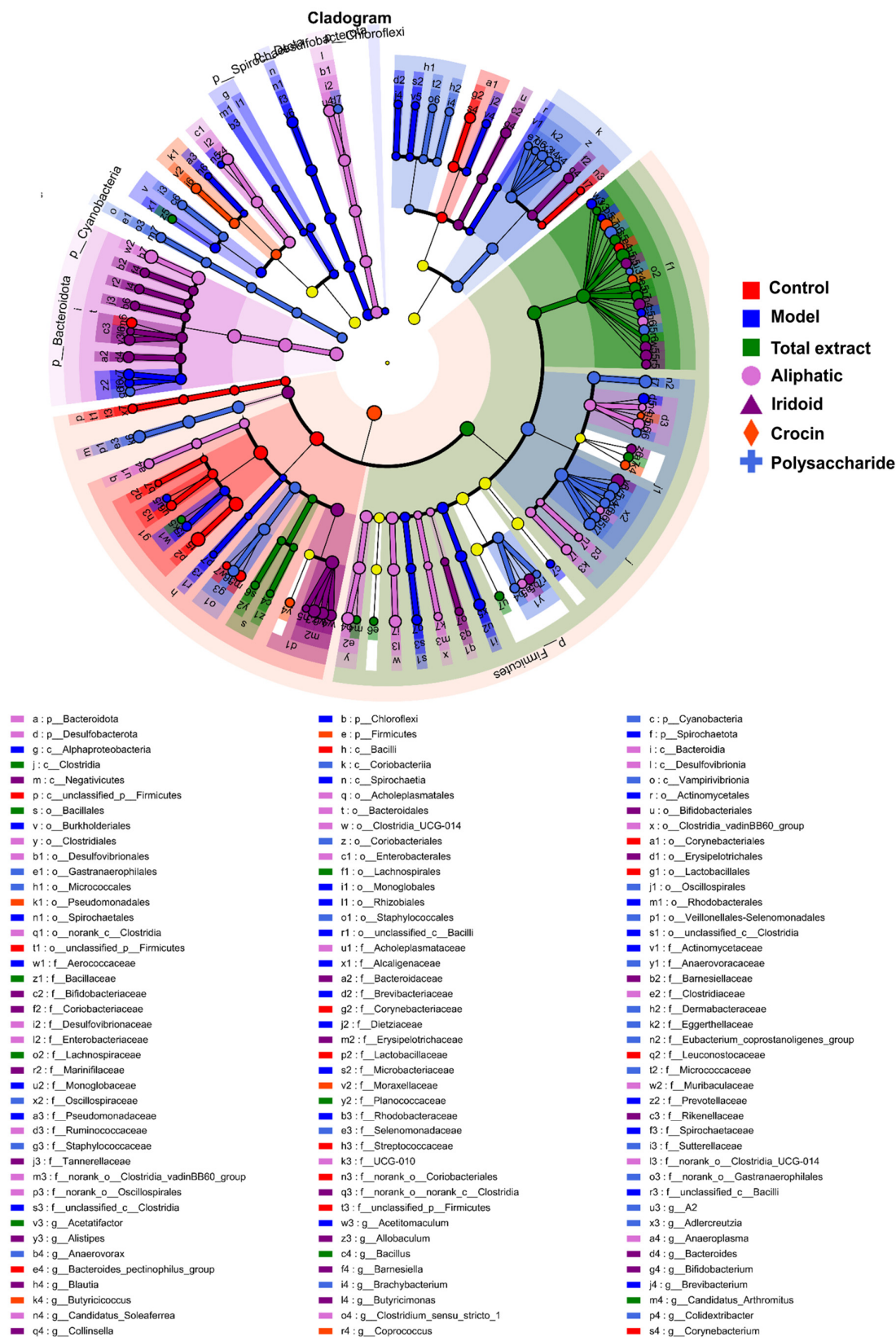


Figure 8. LEfSe analysis of differential species in the gut microbiota of model animals treated with *Gardeniae Fructus* total extract and its four major components.

3. Discussion

In traditional Chinese medicine, heat syndrome refers to febrile symptoms caused by external heat pathogens or internal yin-yang imbalance [12]. It is often characterized by elevated body temperature, irritability, dark urine, and dry stool. A substantial body of modern research indicates that the clinical manifestations of heat syndrome are closely related to hormone levels, energy metabolism, and gut microbiota [13–15]. The theory of herbal properties suggests that the nature of Chinese herbs influences the human physiological functions, thereby affecting therapeutic efficacy or adverse reactions. As a cold-natured traditional Chinese medicine, *Gardeniae Fructus* is widely used in China for the treatment of heat syndrome. Our preliminary research also suggests that the scientific basis of its cold nature may be related to the central nervous system, endocrine system, energy metabolism, and intestinal flora. Among its components, the iridoid fraction of *Gardeniae Fructus* may represent the primary substance responsible for its cold properties.

This study aims to clarify the essence of TCM heat syndrome by using an appropriate animal model to enhance clinical translation. Common modeling methods include administering heat-inducing herbs, chemical drugs, heat exposure, or high-energy diets [12]. Among these, the hyperthyroidism model induced by levothyroxine sodium is widely used in heat syndrome research due to its alignment with human clinical features, hormonal changes, low mortality, and short modeling time [16]. Hyperthyroidism model, characterized by elevated thyroid hormone levels, directly drives a systemic hypermetabolic state, manifested as increased body temperature, weight loss, nervous excitation, and enhanced energy expenditure [17,18], which closely parallels the heat syndrome manifestations described in TCM [19]. While we acknowledge that this model reflects a hormonally driven form of heat and may not capture all etiologies of TCM heat syndrome, it offers a controlled and mechanistically interpretable system for evaluating the cooling properties of *Gardeniae Fructus* fractions across key physiological axes.

Under heat syndrome conditions, symptoms such as dark yellow urine and fever may be associated with dysregulation of water balance, thermoregulation, and blood flow in the central nervous system, as well as hyperactivity of the endocrine axes. This study found that heat syndrome was associated with significantly elevated levels of several central nervous system-related indicators, suggesting a state of neural excitation. Key markers include: AVP, an endogenous antipyretic neurotransmitter involved in water reabsorption and thermoregulation; The monoamine neurotransmitters DA, NE, and 5-HT, which exhibit antagonistic effects [20]; AChE, reflecting cholinergic signaling; And 17-OHCS [21], a marker of sympathetic-adrenal system activity. Additionally, heat syndrome markedly activated the hypothalamic-pituitary-thyroid axis (via the cascade of TSH, and thyroid hormones T3/T4) [22], leading to enhanced pituitary and thyroid function and further contributing to disruptions in thermoregulation and salt metabolism. Investigations into the mechanisms underlying gardenia's heat-resistant properties reveal that its iridoid and crocin components exert significant inhibitory effects on the excitatory state of the central nervous system and the hyperactive endocrine axis. While polysaccharide components also demonstrate some inhibitory effects, their action is relatively weaker.

The core mechanism by which *Gardeniae Fructus* counteracts heat syndrome involves its multi-target inhibition of the comprehensive metabolic hyperactivity that defines this condition. This state is characterized by accelerated hepatic glycogen breakdown through the upregulation of PYGL and the inhibition of glycogen synthesis by GSK-3 [23], enhanced aerobic oxidation driven by increased SDH activity, elevated glycolytic flux and lactate conversion indicated by higher LDH levels, accelerated ATP turnover prompted by elevated cAMP signaling, increased ATP consumption due to higher $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity, and enhanced mitochondrial biogenesis [24]. The cold property of *Gardeniae*

Fructus, mediated primarily by its iridoid, crocin, and polysaccharide fractions, counteracts this hypermetabolism by initiating a coordinated metabolic reprogramming. This reprogramming centers on the upregulation of P-mTOR to promote anabolic recovery and the concurrent downregulation of PGC-1 α to inhibit mitochondrial biogenesis and respiratory function [25]. This central signaling shift coherently downregulates downstream metabolic fluxes, including suppressed PDH and SDH activities which reduce pyruvate oxidation and TCA cycle turnover. It also attenuates glycogen breakdown through decreased PYGL and GSK-3 activity, while lower Na⁺-K⁺-ATPase activity and cAMP levels reflect a diminished cellular ionic workload and reduced adrenergic stimulation. Together, these actions cooperatively suppress glycogen breakdown, aerobic oxidation, and ATP turnover. In contrast, the aliphatic components show weaker effects. The synergistic effect of the total extract thus arises from the combined regulation of the mTOR/PGC-1 α pathway and its downstream effectors by multiple bioactive components. This aligns with the established general mechanism through which cold-natured Chinese medicines reduce heat by suppressing central nervous excitation, endocrine axis hyperactivity, and overall energy metabolism.

Heat syndrome promotes metabolic hyperactivity by altering the gut microbiota structure. Species composition analysis revealed that heat syndrome increased the proportion of the top 10 dominant bacteria, an effect significantly reversed by Gardeniae Fructus and its fractions (total extract, iridoid, and polysaccharide). Alpha diversity indicated that heat syndrome reduced microbial richness and diversity, with polysaccharides notably improving diversity and alleviating dysbiosis. Beta diversity confirmed that the microbial community structure in the model group deviated significantly from the control group, while the polysaccharide group helped restore it toward normal conditions. The observed shift in gut microbiota composition, specifically the reduction in Firmicutes and Lactobacillus and the increase in Bacteroidota, carries direct functional implications for host metabolism. Firmicutes are generally associated with enhanced energy harvest from the diet, while Bacteroidota are often linked to greater production of short-chain fatty acids such as propionate, which can inhibit hepatic gluconeogenesis and improve insulin sensitivity [26]. The decrease in Lactobacillus, a genus frequently elevated in pro-inflammatory and metabolic hyperfunction states, further aligns with the alleviation of heat syndrome. Moreover, PICRUST functional prediction indicated that these taxonomic changes corresponded to a downregulation of microbial pathways involved in carbohydrate metabolism, lipid biosynthesis, and energy production. Thus, the remodeling of the gut microbiota by Gardeniae Fructus likely contributes to its “cold” property not only structurally but also functionally, by collectively reducing microbial-driven energy extraction and systemic metabolic flux.

The microbial biomarkers identified by LEfSe analysis provide specific insights into the functional remodeling of the gut ecosystem under heat syndrome and its correction by Gardeniae Fructus. The enrichment of *Lactobacillus* and *Lactobacillales* in the model group is consistent with a state of metabolic and inflammatory stress, as certain *Lactobacillus* species thrive in and can further promote a pro-inflammatory milieu and energy-harvesting efficiency. Their reduction by the total extract, iridoid, and polysaccharide fractions aligns with the alleviation of heat syndrome symptoms. Conversely, the enrichment of *Bacteroidales*/*Bacteroidota* in the iridoid group, and of *Oscillospirales* in the polysaccharide group, points toward a restoration of microbial balance. *Bacteroidota* are associated with increased production of short-chain fatty acids like propionate, which can modulate host metabolism and immunity, while *Oscillospirales* are often linked to gut barrier integrity and anti-inflammatory effects. The increase in *Lachnospiraceae* and Clostridia in the aliphatic group, and in *Erysipelotrichaceae* in the total extract group, may reflect compensatory shifts in microbial community structure, though their net contribution to the cold property appears limited given the weaker activity of the aliphatic fraction. Thus, the LEfSe biomarkers

not only distinguish treatment groups but also highlight functionally coherent microbial shifts that support the systemic anti-hypermetabolic effects of *Gardeniae Fructus*.

Chemical analysis reveals that the cold property of *Gardeniae Fructus* is not evenly distributed across all components: the aliphatic fraction, rich in fatty acids and sterols, exhibits weak activity, while the iridoid and crocin fractions (containing iridoid glycosides such as geniposide and gardenoside, as well as carotenoids like crocins) represent the primary material basis of the cold property, demonstrating significant antipyretic, neuroendocrine-modulating, and metabolic-suppressing effects. The polysaccharide fraction, though not fully characterized at the monomer level, also contributes to cooling by regulating gut microbiota and energy metabolism. Overall, the cold property intensity follows the order: iridoids > crocins > polysaccharides > aliphatic components. Iridoids exert the strongest inhibitory effects on neural excitation, endocrine hyperactivity, and energy metabolism via the mTOR/PGC-1 α pathway, yet excessive use may lead to reduced gut microbial diversity and diarrhea, reflecting an “overly cold” nature. Crocins show similar metabolic inhibition, while polysaccharides act more mildly. The core anti-heat mechanism involves modulating gut microbiota structure (reducing *Firmicutes/Lactobacillus* and increasing *Bacteroidota*) and inhibiting energy/carbohydrate/lipid metabolism pathways, with iridoids being the most potent regulators, followed by polysaccharides. These findings provide important insights for the precise dosing and side-effect management of cold-nature Chinese medicines.

While this study offers preliminary insights into the material basis and multi-system intervention characteristics of the cold property of *Gardeniae Fructus* in a heat syndrome model, several limitations should be noted. The levothyroxine-induced hyperthyroidism model, while useful, primarily reflects thyroid axis-driven “heat manifestations.” This means our findings are most directly relevant to hormonal/metabolic hyperthermia, and may not fully generalize to heat syndromes arising from other TCM etiologies. Consequently, the identified cold mechanisms should be interpreted within this specific metabolic context. While four main fractions were obtained, the specific active compounds within them remain unidentified. This limits our ability to pinpoint exact molecular entities responsible for the cold property, and the synergistic or antagonistic relationships among components within active fractions require further investigation. Thus, the reported fraction-level activities represent a collective effect, not a definitive compound-activity mapping. Although associations with central nervous, endocrine, metabolic, and gut microbiota systems were observed, the causal and interactive network underlying the holistic cold property is not fully elucidated. Therefore, the proposed multi-tiered model, while integrative, remains correlative and hypothesis-generating; the temporal and directional relationships between systems await experimental validation.

4. Materials and Methods

4.1. Materials and Reagents

The *Gardeniae Fructus* material was purchased from Jiangxi Jiangzhong Chinese Herbal Pieces Co., Ltd. (Batch No.: 220901, Nanchang, China). It was identified by Professor Xiaomei Fu of Jiangxi University of Chinese Medicine as the dried ripe fruit of *Gardenia jasminoides* Ellis (Rubiaceae). A voucher specimen has been deposited at the Department of Identification of Chinese Materia Medica, Jiangxi University of Chinese Medicine. Sodium-levothyroxine was received from Merck KGaA (Darmstadt, Germany). SDH, LDH enzymes and Na⁺-K⁺-ATPase assay kits were purchased from Nanjing Jiancheng Biological Co., Ltd. (Nanjing, China). AMPK, cAMP, cGMP, GSK-3, PYGL, TSH, T3, T4, 17-OHCS, AchE, NE, DA, 5-HT, AVP and PDH ELISA kits were obtained from Shanghai mlbio Biotechnology Co., Ltd. (Shanghai, China). Anti-Phospho-mTOR and anti-mTOR antibodies were all obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-PGC1 α and alpaca

anti-rabbit IgG-HRP antibodies were purchased from HUABIO (Hangzhou, China). β -actin polyclonal antibody was purchased from Proteintech Group (Wuhan, China).

4.2. Pretreatment and Sequential Extraction

The extraction and fractionation procedure is summarized in Figure A1. All general chemicals were from Merck KGaA (Darmstadt, Germany), and chromatography resins (HP-20, polyamide) were from Cytiva (Chicago, IL, USA). Starting with 2 kg of *Gardeniae Fructus*, the aliphatic fraction was obtained by double reflux extraction with petroleum ether (12 *v/w*, 1 h each). The dried marc was then sequentially extracted with 95% ethanol (12 *v/w*, after 2 h soaking) and 75% ethanol (10 *v/w*) under reflux. These ethanol extracts were combined, concentrated, and adsorbed onto pre-treated HP-20 resin packed in a column (2.5 cm I.D. $\times$ 20 cm, $\sim$ 100 mL bed volume). Concurrently, the aqueous extract was concentrated, precipitated with ethanol (75%), and the precipitate was washed. The combined material from the ethanol precipitation step was loaded onto the same HP-20 column at a sample-to-resin ratio of $\sim$ 1:10 (dry weight). The column was eluted stepwise with water, 25% ethanol, 40% ethanol, and 95% ethanol at 4 mL/min. The water-eluted fraction, combined with the dried alcohol precipitate, yielded the polysaccharide fraction. The 40% ethanol fraction was further separated on a polyamide column (eluted with 15% and 75% ethanol). The 25% ethanol (HP-20) and 15% ethanol (polyamide) fractions were combined to give the iridoid fraction, while the 95% ethanol (HP-20) and 75% ethanol (polyamide) fractions were combined to give the crocin fraction.

4.3. GC-MS and UPLC-MS Analysis

The aliphatic fraction was analyzed using qualitative analysis on a GC-MS system (Nexis GC-2030, GC-MS-TQ 8050NX) equipped with a DB-5MS column (0.25 mm $\times$ 30 m, 0.25 μ m) after methyl esterification. The carrier gas helium was used at a flow rate of 1.4 mL/min, split ratio 3:1. The column oven was programmed as follows: After 3 min at 80 $^{\circ}$ C, T was raised at 315 $^{\circ}$ C/min to 200 $^{\circ}$ C (hold 20 min), then at 7 $^{\circ}$ C/min to 300 $^{\circ}$ C (hold 10 min). Injector, transfer line, and ion source temperatures were set at 280 $^{\circ}$ C, 280 $^{\circ}$ C, and 250 $^{\circ}$ C, respectively. The full scan mode covered *m/z* 50–500. That GC-MS response factors are compound-dependent and that the percentages represent relative MS response.

The iridoid and crocin fractions were qualitatively analyzed on a UPLC-MS system (Waters, UPLC, Acquity) equipped with a Waters HPLC column (ACQUITY UPLC BEH C18, 2.1 mm $\times$ 100 mm, 1.7 μ m). The operation parameters were set as follows: injection volume, 1 μ L; flow rate, 0.3 mL/min; column temperature, 40 $^{\circ}$ C. The mobile phases consisted of acetonitrile (A) and water (B) (containing 0.1% formic acid (*v/v*)). A gradient program was used: 0–50 min (5–85% A) and reflux to initial conditions. The MS data were collected in the range of 100–1200 *m/z* in the continuous mode. The conditions in the negative ion mode (and positive ion mode) for ESI were as follows: capillary voltages 3 kV; cone voltage 40 V; desolvation temperature 350 $^{\circ}$ C; scanning time 0.4 s. Accordingly, the model established by thyroxine administration effectively replicates heat syndrome manifestations.

4.4. Component Identification of Split Fractions from *Gardeniae Fructus*

GC-MS data were analyzed using the Automated Mass Spectral Deconvolution and Identification System (AMDIS) software (version 2.73, NIST). Compounds were identified by comparison of the observed mass spectra to authentic compounds found in the NIST Library. UPLC-MS data were based on the molecular ion peak information from primary mass spectrometry, the fragment assignment from secondary tandem mass spectrometry, and the relative retention time of the compounds, combined with existing literature on

the chemical composition of *Gardeniae Fructus*, the chemical constituents of the iridoid components and crocin components were identified.

4.5. Animal Experimental Design

Male SD rats (200 ± 20 g) were purchased from Hunan SJA Laboratory Animal Co., Ltd. (Hunan, China). and acclimatized for 7 days in the Jiangxi University of Chinese Medicine animal facility. The rat models of heat syndrome were established through gastric administration of levothyroxine sodium at a dose of $120 \mu\text{g}/\text{kg}$ every day. Treatment with *Gardeniae Fructus* total extract or its fractions began concurrently with levothyroxine administration. All treatments (levothyroxine and herbal materials) were administered once daily. At the end of the 15-day treatment period, rats were fasted overnight, anesthetized, and euthanized for sample collection. Blood samples were collected from the abdominal aorta, and serum/plasma was separated by centrifugation. Brain and liver tissues were promptly excised, weighed, and either processed immediately for homogenization or snap-frozen in liquid nitrogen. Fecal samples were collected freshly prior to euthanasia for gut microbiota analysis. According to the 2020 edition of the Chinese Pharmacopoeia, the recommended daily dosage of *Gardeniae Fructus* for adults is 6–10 g, which converts to a rat crude drug dosage of $0.54\text{--}0.9 \text{ g}\cdot\text{kg}^{-1}$. Preliminary experiments in this study used a daily crude drug dosage of $2.1 \text{ g}\cdot\text{kg}^{-1}$ administered twice daily, which resulted in significant cold-related adverse effects. Therefore, this dosage was selected for the total extract group. The dosages for each chemically separated fraction were calculated based on the proportion of *Gardenia* extract obtained from each elution segment and administered to the experimental rats accordingly. Specifically, the total extract group received $993.9 \text{ mg}\cdot\text{kg}^{-1}$ per dose, the iridoid group received $174.7 \text{ mg}\cdot\text{kg}^{-1}$, the aliphatic group received $311.6 \text{ mg}\cdot\text{kg}^{-1}$, the crocin group received $81.9 \text{ mg}\cdot\text{kg}^{-1}$, and the polysaccharides group received $324.7 \text{ mg}\cdot\text{kg}^{-1}$. Investigations using experimental animals was conducted in accordance with the European Community guidelines (EEC Directive of 1986; 86/609/EEC). All animal experiments were approved by the Animal Ethics Committee of Jiangxi University of Chinese Medicine (Ethics No.: JZLLSC20230594).

4.6. General Status Observation

Daily changes in body weight and anal temperature were recorded for each group. Anal temperature was measured three times prior to modeling and administration, and the average value was taken. Mental state, fur condition, urination, and defecation including color and shape, were also observed and documented.

4.7. Indicator Detection

Blood samples were taken from the abdominal aorta and centrifuged at 3500 rpm for 10 min. The brain and hepatic tissue (100 mg) were homogenized in a 900 μL pre-cooled saline solution and then centrifuged at 3500 rpm for 10 min. Those supernatants were collected to assess the status of the central nervous and endocrine systems, as well as energy metabolism, in each group of rats. NE, DA, AVP, and 5-HT in brain tissue, as well as 17-OHCS and AChE in plasma, were measured to evaluate the central nervous system. The plasma levels of TSH, T3, and T4 were detected as characteristics of the endocrine system. Those were measured using ELISA detection kits, as directed by the manufacturer. The levels of PDH, SDH, and LDH enzymes, GSK-3, PYGL and cAMP in plasma, as well as AMPK and $\text{Na}^+\text{-K}^+\text{-ATPase}$ in liver tissue, were measured to evaluate the energy metabolism. Those were measured using an assay kit.

4.8. Western Blot (WB) Analysis

Hepatic tissues were collected and extracted protein was extracted. The protein sample of each group was separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to the polyvinylidene difluoride membrane, then blocked by immersion in protein free rapid blocking buffer for 30 min at room temperature and incubated with respective primary antibodies overnight at 4 °C, including mTOR, P-mTOR, PGC-1 α (1:1000), and β -actin (1:5000) was used as the loading control. After three washes with TBST buffer for 10 min each, membranes were incubated in a secondary antibody (1: 50,000) for 2 h at room temperature and again washed with TBST four times. Bands were visualized with an ECL system, and the density of each band was quantified using Image Lab software (version 6.1.0).

4.9. 16S rRNA Gene Sequencing

Fecal microbial genome DNA extracted using TruSeq™ DNA Sample Prep Kit (Illumina, San Diego, CA, USA) with confirmed DNA isolation via agarose gels. The genome DNA was amplified by PCR and quantified by the QuantiFluor™-ST Blue Fluorescence Quantification System (Promega Corporation, Madison, WI, USA), and then sequenced using Illumina. The α -diversity, β -diversity, Venn diagram, species composition, linear discriminant analysis effect size (LEfSe), and PICRUST were used to analyze the structure of the gut microbiota.

4.10. Statistical Analysis

Data were analyzed with IBM SPASS Statistics 20 (IBM Corp, Armonk, NY, USA). All values are presented as the mean $\pm$ standard deviation (SD). For the analysis of experimental data, using one-way ANOVA for data with normal distribution, the nonparametric test was performed for non-normally distributed data. All figures were produced using GraphPad Prism 9.0.0 (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was set at $p < 0.05$ and $p < 0.01$.

5. Conclusions

In summary, the cold property of Gardeniae Fructus can be understood as a systems-level intervention that restores homeostasis in heat syndrome through a coordinated, multi-target network. At the core, its active fractions, namely iridoids, crocins, and polysaccharides, converge on the mTOR/PGC-1 α axis to reprogram energy metabolism, suppressing mitochondrial biogenesis, aerobic oxidation, and glycogenolysis. This metabolic deceleration is reinforced through upstream modulation of the neuro-endocrine system: reduced sympathetic outflow and HPT-axis activity decrease central drivers of thermogenesis and catabolism. Simultaneously, the reshaping of gut microbiota attenuates microbial energy harvest and lowers systemic metabolic flux. Crucially, these systems do not act in isolation: the neuro-endocrine changes likely influence gut motility and secretion, thereby altering the microbiota environment; conversely, microbiota-derived metabolites can signal to host energy and immune pathways. Thus, the cold property emerges from the synergistic interaction of these three tiers: neuro-endocrine regulation, metabolic reprogramming, and microbial remodeling, with iridoids as the most potent component across all tiers. This integrated model moves beyond descriptive correlation to propose a testable, multi-system framework for understanding how cold-natured herbs mitigate heat syndrome.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms27041679/s1>.

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Abbreviations

TCM	traditional Chinese medicine
TIC	total ion chromatogram
SDH	succinate dehydrogenase
LDH	lactate dehydrogenase
AMPK	Adenosine monophosphate-activated protein kinase
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CPK	creatine phosphokinase
GSK-3	glycogen synthase kinase-3
PYGL	glycogen phosphorylase
TSH	thyroid-stimulating hormone
T3	triiodothyronine
T4	tetraiodothyronine
PGE2	prostaglandin E2
17-OHCS	17-hydroxycorticosteroids
AchE	acetylcholinesterase
NE	Norepinephrine
DA	dopamine
5-HT	5-hydroxytryptamine
AVP	arginine vasopressin
PDH	pyruvate dehydrogenase
ELISA	enzyme-linked immunosorbent assay
LEfSe	linear discriminant analysis effect size

Appendix A

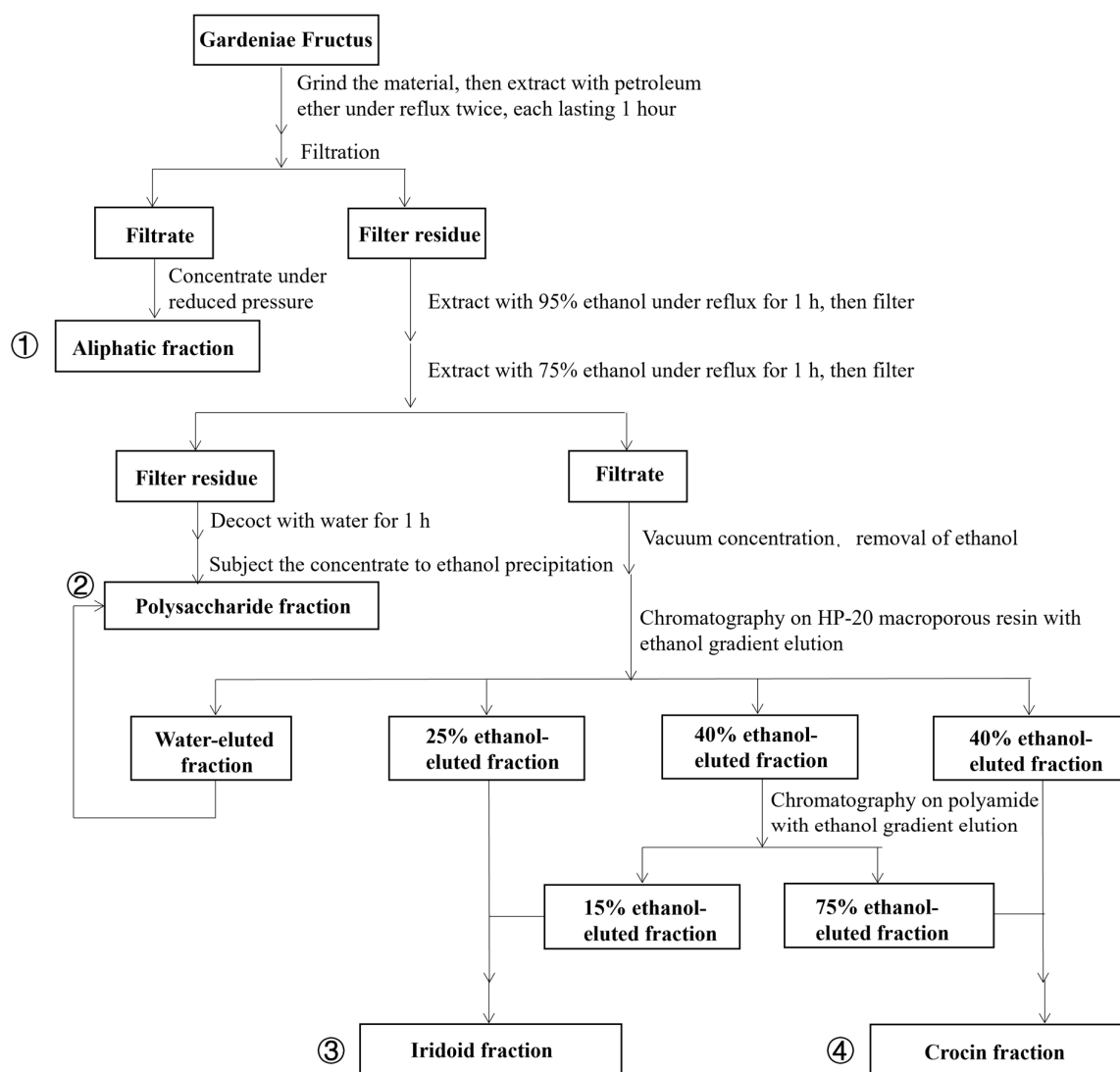


Figure A1. Methods for preparing fractions of Gardenia fructus.

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