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Interpreting the Pharmacological Mechanisms of Sho-saiko-to on Thyroid Carcinoma through Combining Network Pharmacology and Experimental Evaluation

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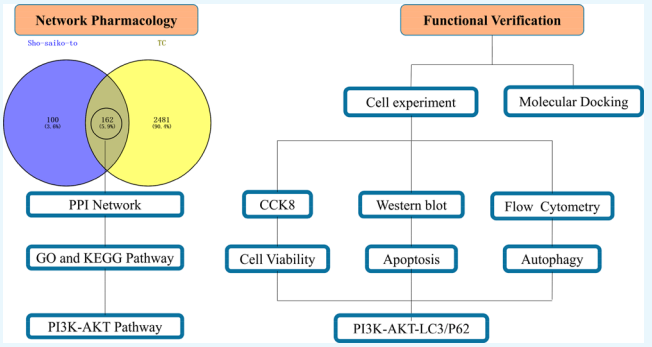
ABSTRACT: Sho-saiko-to is a well-known traditional Chinese medicine compound and is considered to have therapeutic effects against many diseases, including thyroid cancer (TC). However, the mechanisms and therapeutic targets of Sho-saiko-to against TC remain unclear. In this study, network pharmacology, molecular docking, and cell experiments were combined to predict and verify the targets and mechanisms of the active ingredients of Sho-saiko-to against TC. The results demonstrated that the main chemical ingredients of Sho-saiko-to could suppress the viability and proliferation of TC cells, promote apoptosis through the caspase3 pathway, and induce autophagy through the PI3K-AKT pathway. In addition, Sho-saiko-to could also induce the redifferentiation of anaplastic thyroid cancer. Our study provides a novel approach for treating differentiated thyroid cancer (DTC) or radioactive iodine refractory differentiated thyroid cancer (RAIR-DTC).



1. INTRODUCTION

Thyroid cancer (TC) is the most common endocrine malignancy in clinical practice, accounting for approximately 3% of all malignant cancers.[1,2] Furthermore, the morbidity and mortality rates of TC have shown a steep increase over the past few decades. Thyroid cancer can be classified mainly into three types, namely, differentiated thyroid cancer (DTC), anaplastic thyroid cancer (ATC), and medullary thyroid cancer (MTC), among which DTC is the main type accounting for more than 90% of all malignancies.[3] The traditional therapeutic approaches mainly include surgery, radioactive iodine therapy and hormone treatment and molecular targeted therapy in clinical practice.[4] Of these, surgery including near total (NT) thyroidectomy and total thyroidectomy (TT) are the important therapeutic approaches for DTC or ATC, but the median months of Disease-Specific Survival (DSS) is only 6−10 for patients who received surgical management.[5] Besides, selective postoperative ^{131}I is also considered as a standard practice in DTC to reduce recurrence and metastasis. However, approximately 30% of all cases may become radioactive iodine-refractory differentiated thyroid cancer (RAIR-DTC) with a 10-year survival rate of less than 10% for the malignant progression.[6,7] Molecular targeted therapy, such as sorafenib, lenvatinib, and so on, has the limited application due to the serious side effects and incomplete clinical evidence.[8] Therefore, exploring the mechanisms underlying TC and proposing more reasonable and novel therapeutic strategies will be very urgent for the clinical treatment of TC.

Sho-saiko-to, also named the Xiao Chaihu Decoction, was first described in the "Shanghanzabinglun", which was an extremely famous medical book written by Zhang Zhongjing in ancient China. It is composed of Rhizoma Pinelliae (banxia), Radix Bupleuri (chaihu), Radix Ginseng (renshen), Radix Scutellariae (huangqin), Rhizoma Zingiberis Recens (shengjiang), Radix Glycyrrhizae (gancao), and Fructus Jujubae (dazhao), and the proportion of Sho-saiko-to is shown in Table 1.[9−11] Recently, several studies have shown that Sho-saiko-to could achieve substantial curative effects in many diseases, such as depression,[12] hepatitis,[13] tumors,[9,14] and so on. Of these, the therapeutic benefits on cancers have increasingly attracted the attention of many clinicians. The work of Kim et al.[15] showed that Sho-saiko-to could efficiently inhibit the cell viability and proliferation of nasopharyngeal carcinoma cells *in vivo* and *in vitro*. The Rhizoma Pinelliae (an herbal component of Sho-saiko-to) could inhibit the viability of papillary thyroid cancer by downregulating nuclear factor erythroid 2-related factor 2 (Nrf2) and inducing autophagy.[16]

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Table 1. Components of Sho-saiko-to

herbs	herbs (Chinese name)	weight (g)
Radix Bupleuri	chaihu	7.0
Rhizoma Pinelliae	banxia	5.0
Radix Ginseng	renshen	3.0
Fructus Jujubae	dazhao	3.0
Radix Scutellariae	huangqin	3.0
Radix Glycyrrhizae	gancao	2.0
Rhizoma Zingiberis Recens	shengjiang	1.0

Although previous studies have verified the therapeutic benefits of Sho-saiko-to in TC, the related mechanisms remain unclear.

Network pharmacology, established by Hopkinsal in 2007, refers to the methodology that combines pharmacology, electronic technology, bioinformatics, and molecular biology to construct the network relationships among chemical components, related targets of traditional Chinese medicine (TCM), and diseases pathways.[17,18] Through network pharmacological analysis, we can predict more effective targets of drug activity, better explore the mechanism of drugs, and better understand the relationship between drugs and diseases. With the development and combination of network pharmacology and TCM, the therapeutic strategy of "multi-targets, multicomponents" has gradually emerged, which is congruent with the multietiology and multichannel pathogenesis of cancers. At present, network pharmacology has become the primary mean to understand the multitarget, multicomponent therapy strategy of TCM.

In this study, network pharmacology, molecular docking, and experimental verification were employed to explore the therapeutic mechanism of Sho-saiko-to in TC, which will lay the foundations for the application of Sho-saiko-to against TC (see Figure 1).

2. RESULTS

2.1. Screening of Target Genes for Sho-saiko-to and TC. To study the therapeutic mechanism of Sho-saiko-to, we first determined the active ingredients of Sho-saiko-to. By following the criteria of DL ≥ 0.18 and OB ≥ 30% in the TCMSP database,[19,20] we obtained 193 active ingredients (Radix Bupleuri: 17, Rhizoma Pinelliae: 11, Radix Ginseng: 19, Radix Scutellariae: 32, Rhizoma Zingiberis Recens: 3, Fructus Jujubae: 24, Radix Glycyrrhizae: 87) (Table S1). Subsequently, the target proteins were filtered based on the active components and transferred into 262 target genes through the Uniprot database (Table S2). Figure S1 showed that different herbs had many repetitive targets, which provided the basis for the synergism of different herbs and components. Finally, we used Cytoscape v3.7.2 to build a systematic herb-component-target network (H-C-T) to visualize the replication relationship between them (Figure 3), then we obtained the top five active components of Sho-saiko-to by the Degree value of every node in the H-C-T network and the top five active components were regarded as the main active components of Sho-saiko-to in this study.

In addition, we obtained 1478, 637, and 1175 TC related genes from Genecard database, OMIM database, and DisGeNET database respectively, and then 2643 different genes were obtained by deleting the repeated genes.

2.2. Protein−Protein Interaction Network Analysis. As shown in Figure 2, by defining the interaction between the 262 target genes associated with Sho-saiko-to and the 2643 target genes associated with TC, we got 162 different genes, which

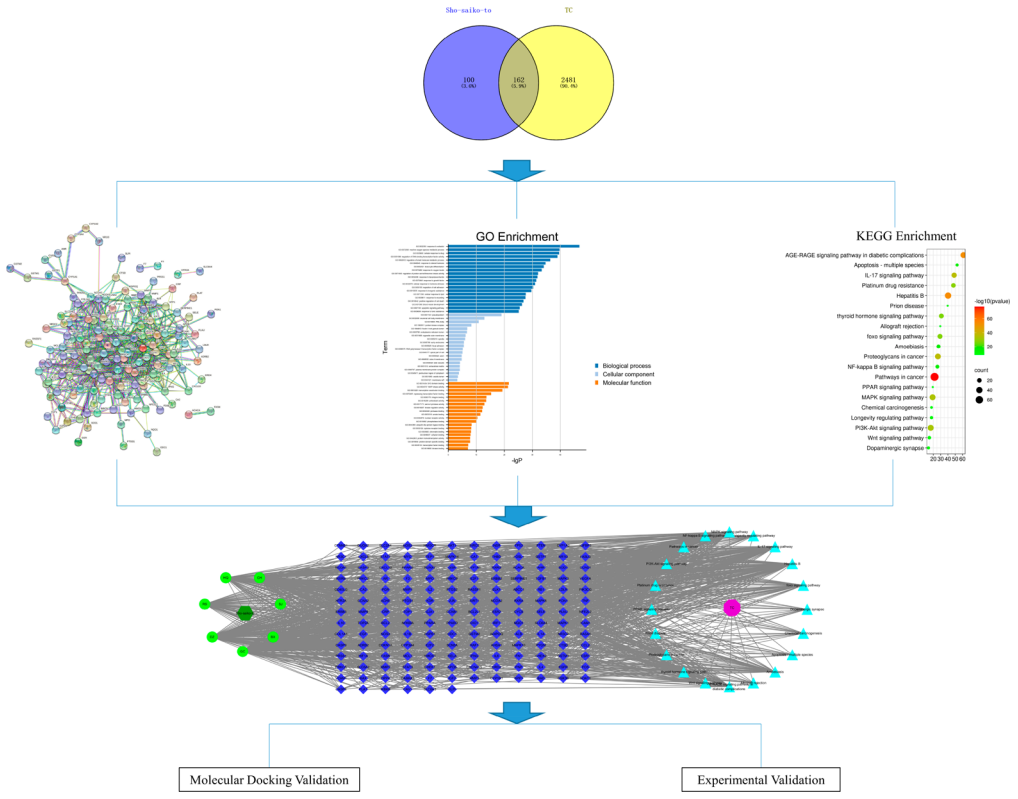

Figure 1. Conceptual framework of this study.

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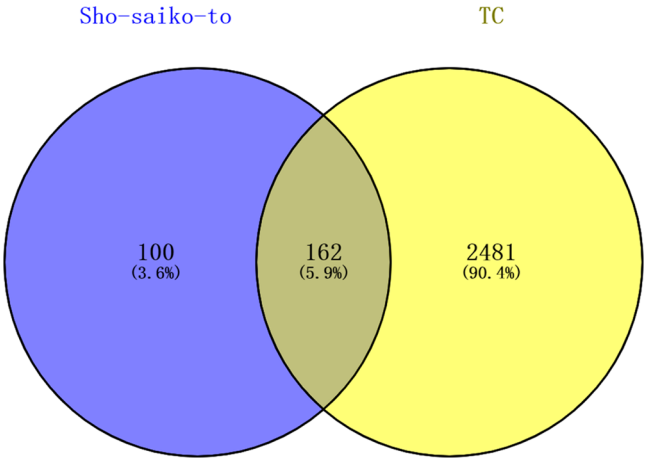

Figure 2. Venn diagram showing the numbers of the overlapping genes between Sho-saiko-to and thyroid cancers.

were considered as the key targets in the treatment of thyroid cancer. We then input these genes into the STRING database to build a PPI network containing 146 nodes and 790 edges (Figure 4A). By analyzing the topological characteristics of the protein—protein interaction (PPI) network in Cytoscape v3.7.2 software, we selected the top 20 target genes according to the degree value (AKT1, MAPK3, STAT3, MAPK1, JUN, TP53, MAPK14, FOS, EGFR, RELA, IL6, ESR1, VEGFA, CTNNB1, MYC, NR3C1, MAPK8, RXRA, TNF, EGF, NCOA1) as the core genes (Figure 4B, Table S3), and built a PPI core network with 21 nodes and 120 edges (Figure S2). AKT1 was then selected as the most important target of the PPI network according to the degree value (degree value: 43, betweenness: 0.1248589, closeness: 0.53667954).

2.3. Gene Ontology Functional Enrichment Analyses. In order to reveal the biological characteristics of 162 intersecting target genes, we performed gene ontology (GO) enrichment analysis using the Metascape tool and selected enrichment results under the conditions of $P < 0.01$, minimum enrichment >1.5, and minimum overlap of 3. Figure 5A,B shows the top 20 terms that are significantly enriched in terms of biological processes (BP), molecular function (MF), and cellular component categories (CC).

We identified some biophysical processes (BP) in the top 20 terms, such as the apoptotic signaling pathway, blood vessel development, positive regulation of cell death, cellular response to hormone stimulus, reactive oxygen species metabolic process, which were all closely associated with the occurrence and development of TC.[21,22] Therefore, we speculated that Sho-saiko-to exerted an anticancer effect through the above biological processes. The GO−CC enrichment of target genes included the membrane raft, vesicle lumen, extracellular matrix, perinuclear region of cytoplasm, and so on; the GO-MF enrichment for target genes included transcription factor binding, nuclear receptor activity, and kinase binding and so on.

2.4. KEGG Pathway Enrichment Analysis. To further study the role of 162 intersecting target genes in TC, KEGG pathway enrichment was analyzed by using KEGG database with $P < 0.01$ as the threshold value. As shown in Figure 6A,B, The top 20 KEGG pathways related to TC were as follows: pathways in cancer, IL-17 signaling pathway, PI3K-AKT signaling pathway, FOXO signaling pathway, MAPK signaling pathway, thyroid hormone signaling pathway, and so on. Furthermore, based on these 20 significant KEGG pathways, the "drug-target-pathway" network (D-T-P) was constructed (Figure 7), which revealed the characteristics of multiple components, multiple targets, and multiple pathways of Sho-saiko-to in the treatment of TC.

2.5. Molecular Docking Results and Analysis. The molecular docking between the five main components and the five key target proteins was demonstrated based on the analysis of the PPI network and KEGG enrichment. The Docking affinity score was computed and displayed in Figure 8A. In general, the binding affinity lower than −5.0 kal/mol indicates that the bindings have good interactions with lower numbers indicating stronger binding.[17] Beta-sitosterol (MOL000358) and stigmasterol (MOL000449) strongly interacted with AKT1, and quercetin (MOL000098), baicalein (MOL002714), and kaempferol (MOL000422) strongly interacted with PI3KCG. These interactions demonstrated

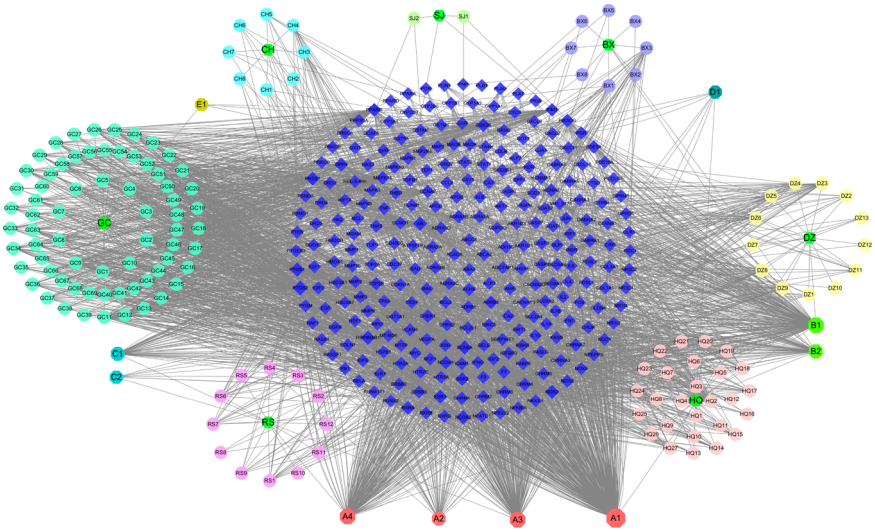

Figure 3. Herb-component-target (H-C-T) network of Sho-saiko-to, the circle nodes are the chemical compounds of Sho-saiko-to, the hexagon nodes are the herds of Sho-saiko-to, and the rhombus nodes are target genes.

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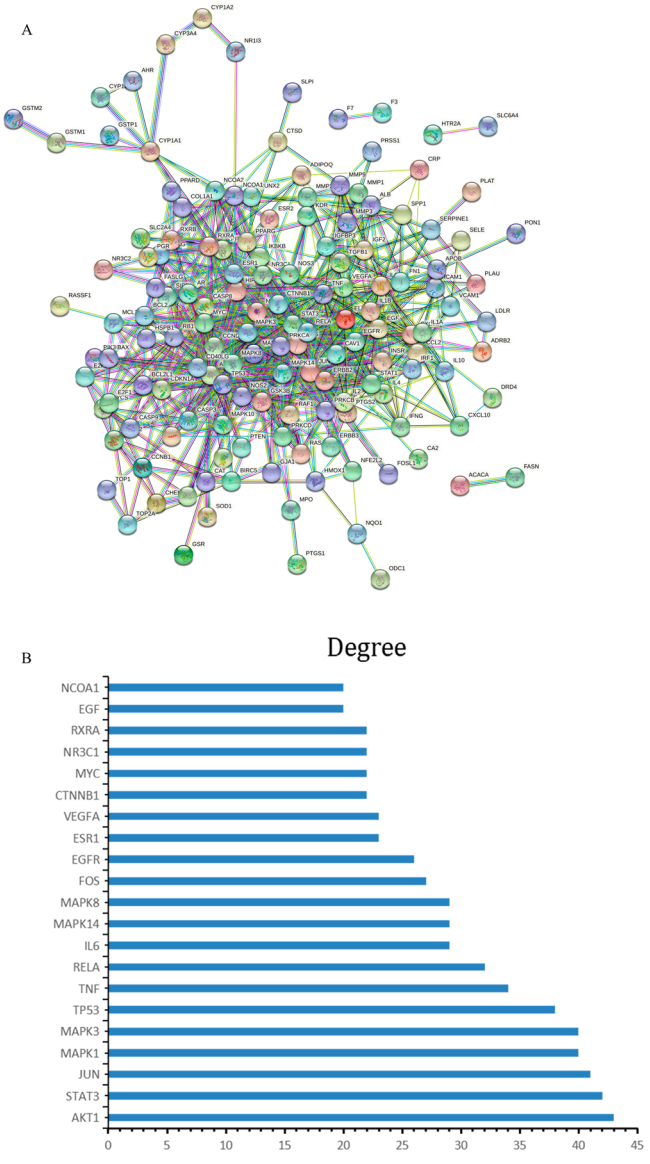

Figure 4. Interaction network of the overlapping targets. (A) The PPI network of the overlapping targets. (B) Bar plot of the number of hub gene links.

that the therapeutic benefit of the five components (baicalein, quercetin, stigmasterol, beta-sitosterol, kaempferol) could have been achieved by AKT1, CASPASE3, TP53, MYC and PI3KCG, and the PI3K-AKT pathway might be the most important therapeutic pathway. The conformations of the key chemical ingredients and the main target proteins were shown in Figure 8B−F.

2.6. The Five Active Components Inhibited the Viability of TC Cells. To verify the effects of Sho-saiko-to, the top five components (baicalein, quercetin, stigmasterol, beta-sitosterol, kaempferol) were cultured with the FTC-133 and 8505C cell lines. The results showed that the main components could prohibit TC cells viability in a concentration-dependent manner. The 24h IC50 values of baicalein, quercetin, stigmasterol, beta-sitosterol, kaempferol were 64.33, 147.4, 124.7, 119.8, 162.1 μM for FTC-133 (Figure 9A−E) and 77.67, 121.8, 92.89, 94.99, and 172.6 μM for 8505C, respectively (Figure 9F−J).

2.7. The Five Active Components Induced Apoptosis of TC Cells. The five active components were added to the FTC-133 and 8505C cell lines at 24h IC50 and cultivated for 24h. FTC-133 (Figure 10A,C) and 8505C cells (Figure 10E,G) showed an increase of apoptosis rates, compared to the control group, which indicated that the main components of Sho-saiko-to could induce apoptosis of TC cells. The result of Western blotting also verified that the active components could activate the caspase 3 to cause the occurrence of apoptosis in FTC-133 (Figure 10B,D) and 8505C cells (Figure 10F,H).

2.8. The Five Active Components Induced Autophagy of TC Cells. On the basis of the results of KEGG enrichment analysis and molecular docking, we focused on the effects on the PI3K-AKT pathway caused by the active components of Sho-saiko-to. As shown in Figure 11A, the five active components could inhibit the phosphorylation of PI3K and AKT1 in FTC-133, especially baicalein, stigmasterol, and kaempferol. Furthermore, quercetin, stigmasterol, beta-sitosterol, and kaempferol could increase the hallmark proteins of autophagy (P62, LC3) in FTC-133 cells. Besides, the phosphorylation of PI3K and AKT1 was inhibited and autophagy proteins were promoted in 8505C exposed to the five active components of Sho-saiko-to (Figure 11B). PI3K and AKT were the common upstream regulator of autophagy, therefore we concluded that the main active components of Sho-saiko-to could induce autophagy through the PI3K-AKT pathway.

2.9. The Five Active Components Induced the Redifferentiation of ATC Cells. We tested the expression of differentiation-associated proteins (TTF-1 and PAX8) and iodine metabolism-related proteins (NIS, TPO, TSHR) to clarify the effects on the redifferentiation of anaplastic thyroid cancer cells. As shown in Figure 12, compared with the control group, the five active components could significantly increase the protein levels of TTF-1, PAX8, TPO, and TSHR, meaning that the main active ingredients of Sho-saiko-to could promote the redifferentiation of ATC and be possibly applied to treat RAIR-DTC.

3. DISCUSSION

In this study, we predicted and verified the targets and mechanism of Sho-saiko-to in the treatment of TC by combining network pharmacology, molecular docking, and *in vitro* experiments. The results showed that the main active components of Sho-saiko-to could inhibit the cell viability and proliferation of thyroid cancer cells, promote apoptosis through the caspase3 pathway, induce autophagy through the PI3K-AKT pathway, and moreover, Sho-saiko-to could also promote the redifferentiation of ATC, which provided a new rationale for the treatment of DTC or RAIR-DTC.

Network pharmacology has been an important method of detecting and developing drugs in recent years, especially in the area of exploring the molecular activity of TCM. Network pharmacology provides a network model of multicomponents, multitargets, and multipathways, which echoed with the functional characteristics of TCM and the inherent characteristics of the occurrence and development of diseases. In this study, 193 active components were obtained from the TCMSP database, and 262 target genes were screened out by the Uniprot database. Then, 2643 genes closely related to TC were obtained from the Genecard database, OMIM database, and DisGeNET database. Through the intersection of target genes between TC and Sho-saiko-to, we obtained 162 overlapping

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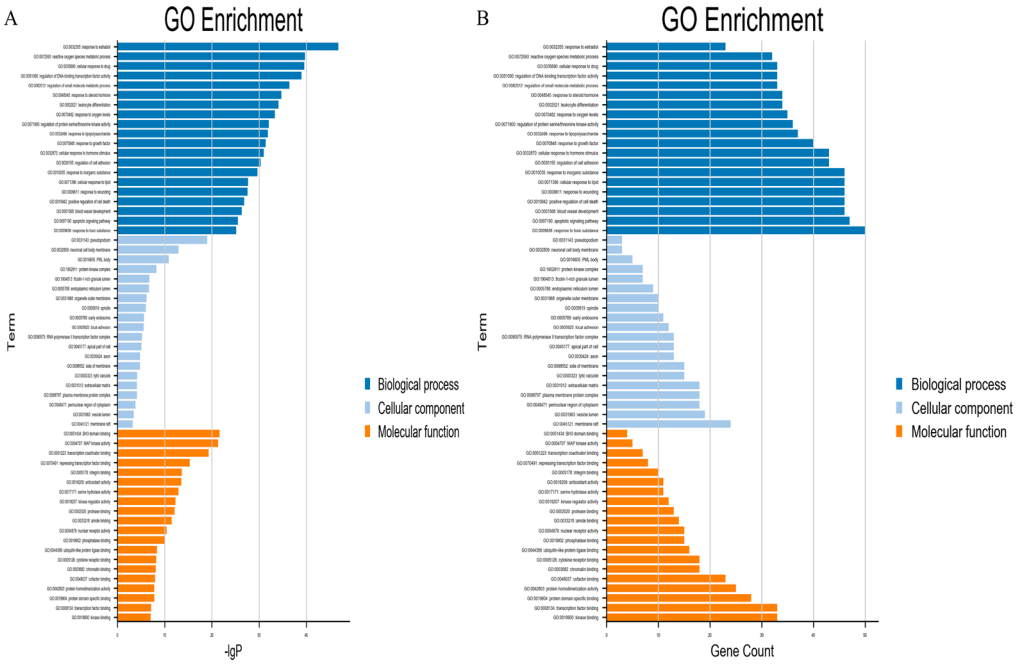

Figure 5. GO enrichment analysis of the overlapping targets. (A,B) Biological process, cellular component and molecular function for the overlapping targets ($P < 0.01$).

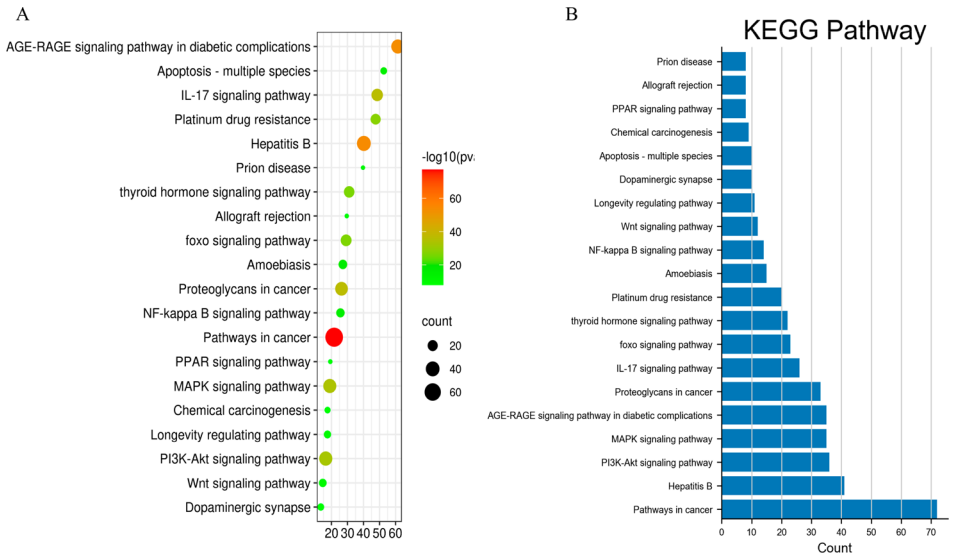

Figure 6. KEGG enrichment analysis of the overlapping targets. (A,B) KEGG pathways enrichment for the overlapping targets ($P < 0.01$).

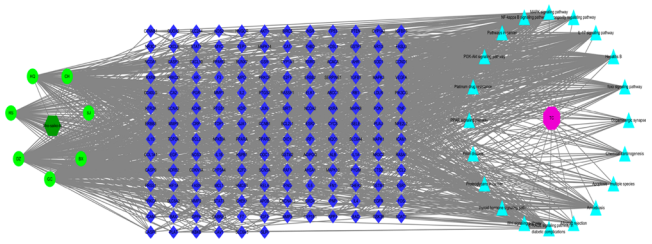

Figure 7. D-T-P: the dark green node is Sho-saiko-to, the green nodes are herds of Sho-saiko-to, the blue nodes are target genes, and the light green nodes are pathways; the pink node is thyroid cancer.

genes, which were regarded as the key targets of Sho-saiko-to against TC. On the basis of these overlapping genes, we constructed the herb-component-target network and defined the main active components of Sho-saiko-to (baicalein, quercetin, stigmasterol, beta-sitosterol, kaempferol). Of these, baicalein, quercetin, and kaempferol were all flavonoids, which have a wide variety of antitumor activity, including against TC. Wang et al.[23] found that baicalein could induce autophagy and apoptosis of ATC cells (FRO cell line) though the ERK-PI3K-AKT pathway. Furthermore, quercetin could also down-regulate the expression of Hsp90 and inhibit chymotrypsin-like proteasome activity, inducing the inhibition of cell viability and proliferation and caspase-dependent apoptotic in papillary thyroid cancer cells.[24] Interestingly, quercetin could enhance the inhibitory effects of sorafenib on the growth and migration of thyroid cancer cells,[25] which meat that synergistic treatment of Sho-saiko-to and other chemotherapy regimens might has broader application prospects.

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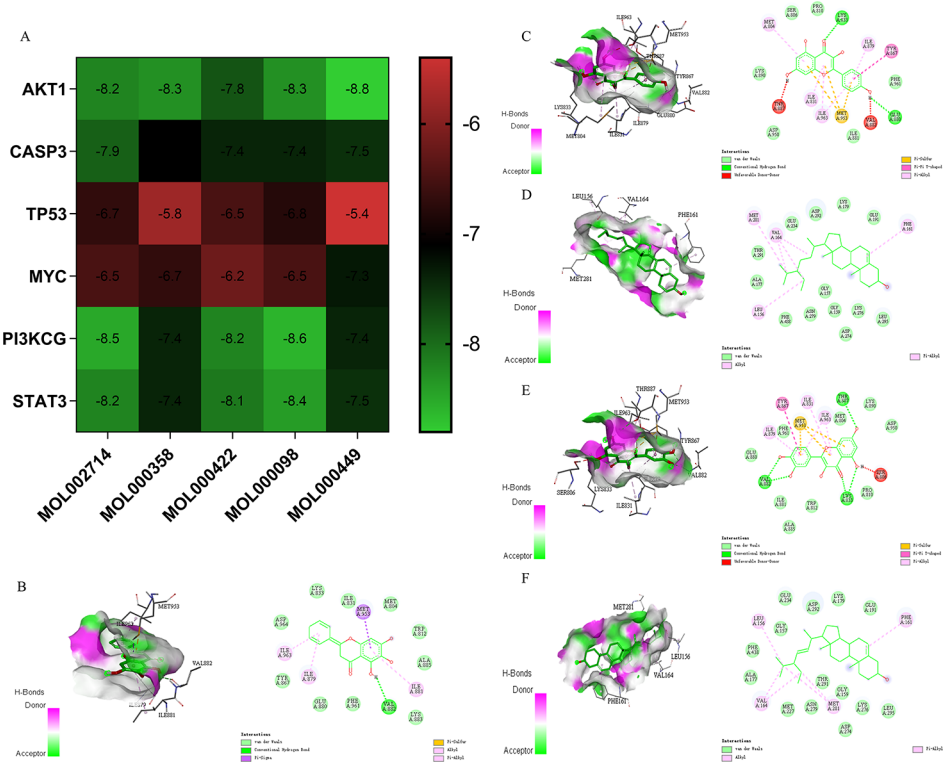

Figure 8. Result of molecular docking. (A) The heat map of the docking score. (B–F) The represented results for the action mode of active compounds with five targets protein using molecular docking. (B) Action mode of Baicalein (MOL002714) with target PI3KCG (PDB ID: 4FUL). (C) Action mode of Kaempferol (MOL000422) with target PI3KCG (PDB ID: 4FUL). (D) Action mode of beta-sitosterol (MOL000358) with target AKT1 (PDB ID: 4GV1). (E) Action mode of Quercetin (MOL000098) with target PI3KCG (PDB ID: 4FUL). (F) Action mode of Stigmasterol (MOL000449) with target AKT1 (PDB ID: 4GV1).

In addition, we also built a PPI network and obtained the top 20 genes of Sho-saiko-to against thyroid cancer (AKT1, MAPK3, STAT3, MAPK1, JUN, TP53, MAPK14, FOS, EGFR, RELA, IL6, ESR1, VEGFA, CTNNB1, MYC, NR3C1, MAPK8, RXRA, TNF, EGF, NCOA1) by degree value. According to the 162 core target genes, the GO enrichment analysis demonstrated that the apoptosis signal pathway (count: 47) might be the most important pathway of Sho-saiko-to in the pathogenicity of TC. Subsequently, the KEGG enrichment analysis was also performed, which demonstrated that Sho-saiko-to could interfere with a variety of TC signal pathways, especially the PI3K-AKT signaling pathway, apoptosis-multiple species, the thyroid hormone signaling pathway, and so on. The PI3K-AKT pathway, as a fundamental intracellular signaling pathway, is involved not only in normal cell physiology (cell growth,[26] proliferation, and survival[27]) but also in cancers (autophagy and cell metabolism[28]). The function of the PI3K-AKT pathway in TC, particularly follicular thyroid cancer (FTC) and ATC, has been extensively explored. Point mutations in PI3K have been found to be more frequent in aggressive TC, particularly in ATC where they have been found in up to 23% of cases.[29,30] Meanwhile, PI3K or AKT inhibitors, such as GDC-094, MK2206, and LY294002, have been found to inhibit the aggression of thyroid cancer *in vivo* and *in vitro*.[31−34]

To validate the reliability of the data analysis, we selected the main active components (baicalein, quercetin, Stigmasterol, beta-sitosterol, kaempferol) and the key genes of TC (TP53, PIK3CG, STAT3, Caspase3, MYC, and AKT1) for molecular docking based on the results of the PPI network, GO, and KEGG enrichment analysis. The results demonstrated that the main components and the target genes all had a high affinity. To verify the precision of the data analysis, cell experiments indicated that the main active compounds of Sho-saiko-to could suppress the TC cells proliferation in a concentration-dependent manner and induce apoptosis by the caspase3 pathway. In addition, we found that the main active components of Sho-saiko-to could promote the expression of autophagy-related genes (LC3 and P62) in TC cells through PI3K-AKT pathway. P62 is a scaffold protein and a stress-inducible protein with multiple domains,[35] which could interact with LC3 to promote the formation of autophagosome.[36] Han et al.[37] found that baicalein could induce apoptosis in thyroid cancer cells (FRO cells) mainly through activation of the ERK/p38 MAPK signaling and partially through PI3K signaling pathways.

Finally, we found that Sho-saiko-to could increase the expression of differentiation-associated protein (TTF-1 and PAX8) and iodine metabolism-related proteins (NIS, TPO, and TSHR). As thyroid-specific transcription factors, TTF-1 and PAX8 can participate in a series of physiological processes of thyroid follicular cells, such as proliferation and differentiation.[38] Furthermore, Pax8 and TTF-1 can regulate the expression of these functional thyroid genes by binding to the promoter and enhancer of the TSHR gene. The recovery of these genes expression means that ATC cells have achieved the redifferentiation, which lays the foundation of radioactive iodine treatment.

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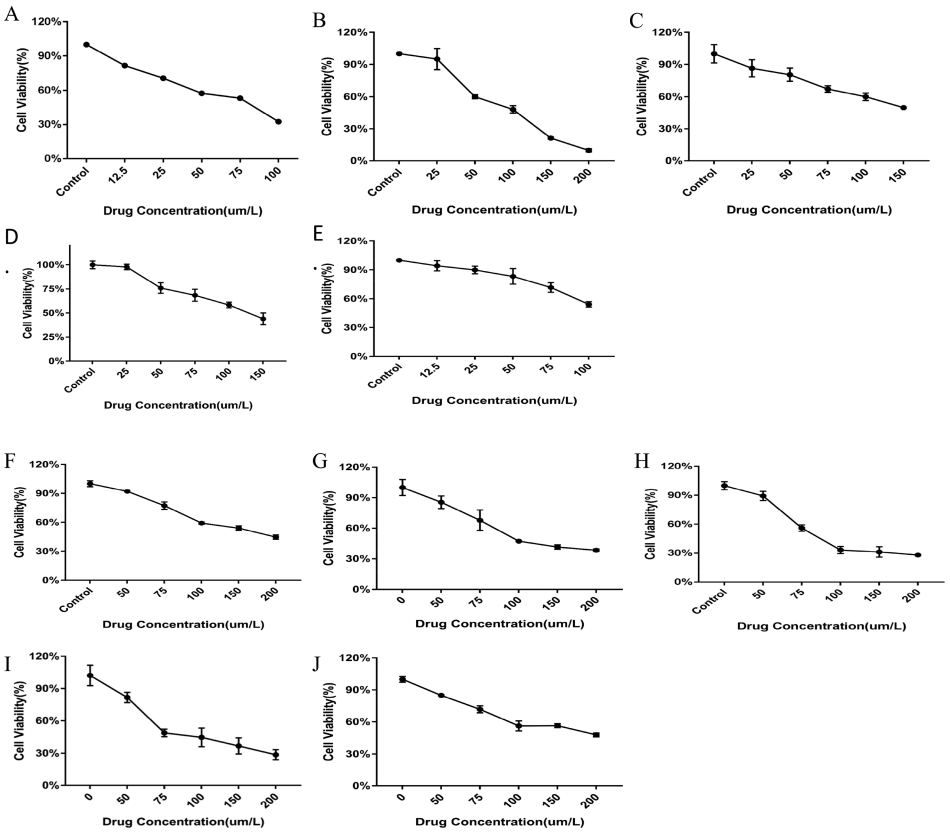

Figure 9. Cell viability inhibitory effects of five active compounds of Sho-saiko-to, including baicalein (A), quercetin (B), stigmasterol (C), β-sitosterol (D), and kaempferol (E) on FTC-133 cells. Panels F−J show the cell viability inhibitory effects of five active compounds of Sho-saiko-to, including baicalein (F), quercetin (G), stigmasterol (H), β-sitosterol (I), and kaempferol (J) on 8505C cells. Drug concentration-cell viability curves were generated based on the cell viability assay. The 24h IC50 values of baicalein, quercetin, stigmasterol, beta-sitosterol, and kaempferol were 64.33, 147.4, 124.7, 119.8, and 162.1 μM for FTC-133 (panels A-E) and 77.67, 121.8, 92.89, 94.99, and 172.6 μM for 8505C, respectively (panels F−J). All data were expressed as mean ± SD (n = 5).

4. CONCLUSION

In this study, network pharmacology, molecular docking and *in vitro* experiments were combined to predict and verify the targets and mechanisms of Sho-saiko-to against TC. In detail, the ingredients of Sho-saiko-to could suppress the cell viability of TC cells, promote apoptosis through the caspase3 pathway, induce autophagy through the PI3K-AKT pathway, and moreover Sho-saiko-to could also recover the redifferentiated of undifferentiated TC. Therefore, Sho-saiko-to may be considered as a potential drug for the effective treatment of DTC or RAIR-DTC.

5. MATERIALS AND METHODS

5.1. Compound Collection and Screening. Traditional Chinese Medicine Systems Pharmacology (TCMSP), a network pharmacology online platform based on Chinese herbal medicines, contains the relationships among diseases, targets, and drugs.[39] All herbs components of Sho-saiko-to were searched in the TCMSP database, and the active molecules with drug-likeness (DL) ≥ 0.18 and oral bioavailability (OB) ≥ 30% were defined to have better pharmacological activities and preserved,[19,20,40] and then the corresponding target names of the molecules were filtered. Subsequently, the targets protein names were converted to corresponding gene names in the UniProt database[41] (https://www.uniprot.org/) by filtering for "Popular organisms" as "Human". Finally, an UpSet plot and an herb-component-target (H-C-T) network was built by using an online stool (http://www.bioinformatics.com.cn) and Cytoscape v3.7.2,[42,43] respectively.

5.2. Identification of Associated Molecular Targets of TC. TC-related genes were identified by searching with the keywords "Thyroid cancer" or "Thyroid carcinoma" in GeneCards (http://www.genecards.org), Online Mendelian Inheritance in Man (http://omim.org/), DisGeNET database (http://www.disgenet.org/web/DisGeNET/). The Venn diagram was generated to identify the interaction genes between the target genes of Sho-saiko-to and the TC-related genes by the online tool Venny 2.1 (http://bioinfogp.cnb.csic.es/tools/venny/).

5.3. Construction of a Protein−Protein Interaction (PPI) Network. The interaction genes between Sho-saiko-to and TC were regarded as the core genes and were analyzed to build the PPI network using the STRING online tool[44] (https://string-db.org), where the confidence score >0.9, the species was "Homo sapiens". On the basis of the TSV format file from the STRING database,[45] the key topological parameters, such as degree and betweenness centrality, were demonstrated through CytoNCA plug-in in Cytoscape v3.7.2, and top 20 nodes were selected as core targets according to the degree value.

5.4. Gene Ontology (GO) and KEGG Pathway Enrichment Analyses. GO and KEGG enrichment analysis was conducted by importing the overlapping genes into Metascape

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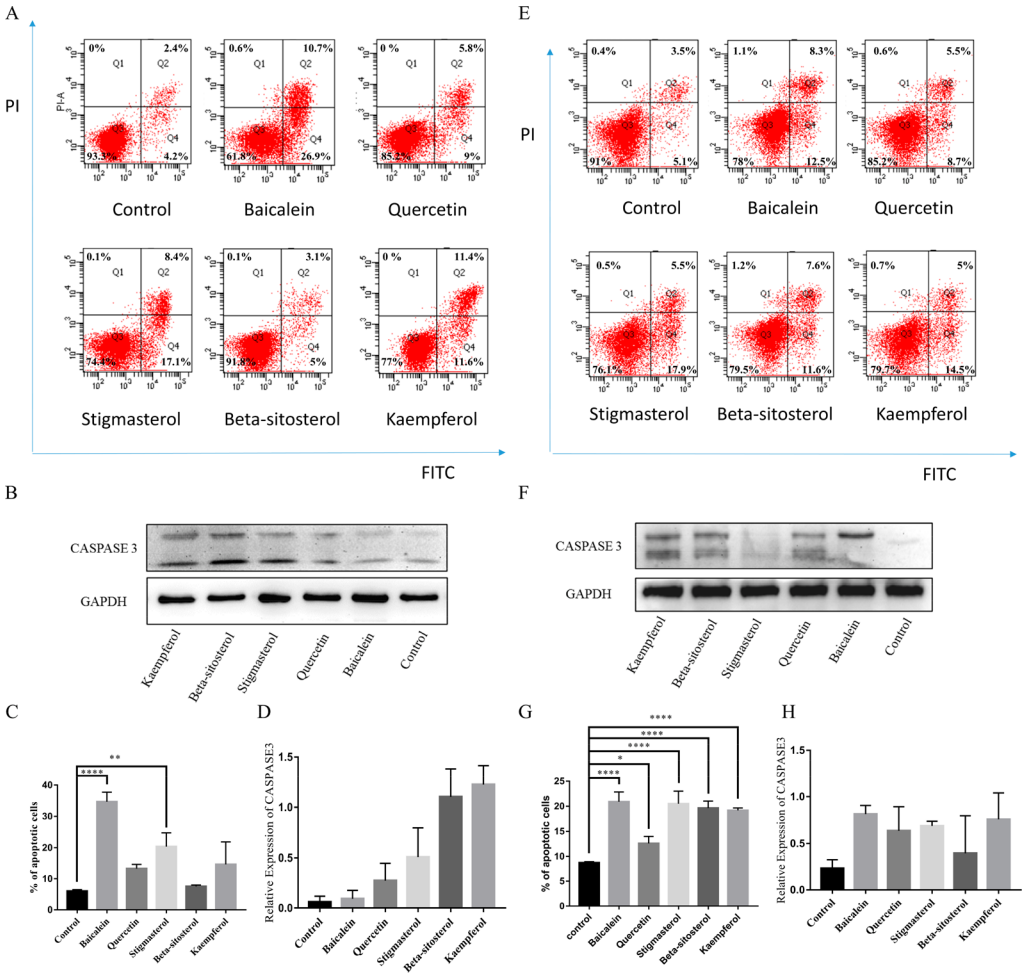

Figure 10. Representative profiles showing apoptosis of treated with the main ingredients of Sho-saiko-to alone. As determined by annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) staining, the ingredients alone induced apoptosis of FTC-133 (A) and 8505C cells (E). The quantified result of panels A and E is shown in panels C and G, respectively (n = 3). The main ingredients could increase the expression of CASPASE3 in FTC-133 (B) and 8505C cells (F). The protein expression levels were detected and evaluated in FTC-133 (D) and 8505C (H) by ImageJ software (n = 3).

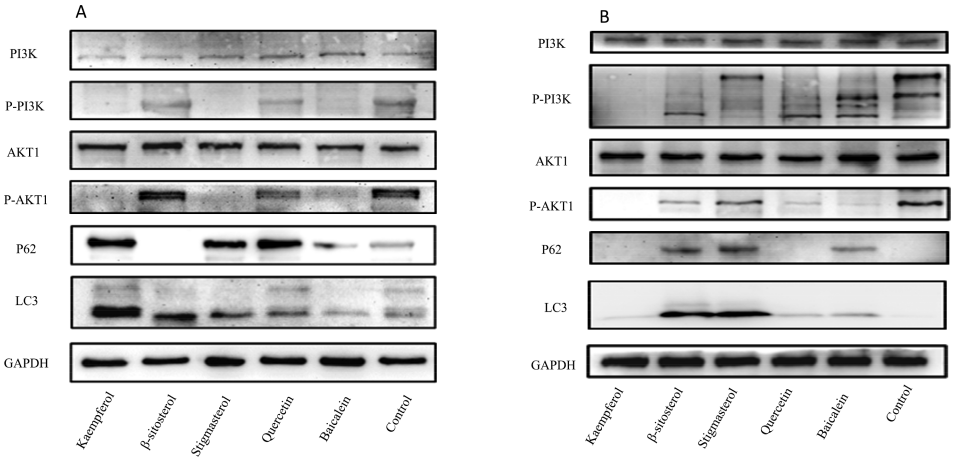

Figure 11. Autophagy induced by the main ingredients of Sho-saiko-to in FTC-133 (A) and 8505C (B).

(https://metascape.org/).[46,47] The enrichment terms with p < 0.05 were collected, and those with p < 0.01 were considered as the critical value of significant pathways and functions. Finally, the top 20 biological processes (BP), cellular components (CC), and molecular functions (MF) were defined as the terms with p < 0.01 and the pathways were identified based on p < 0.05. Then, with the help of Cytoscape v3.7.2 software the compound-target-pathway (C-T-P) was constructed by linking the core active constituents, predicted targets, and pathways. In the network, the nodes were

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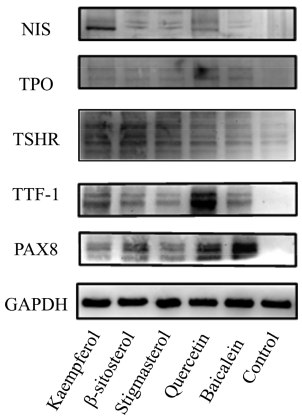

Figure 12. Expression of redifferention-related genes in 8505C cells.

representative of the active ingredients, signaling pathways, or potential targets, while the edges identified their interactions.

5.5. Validation. *5.5.1. Molecular Docking.* Molecular docking is a kind of computer technology that could reveal the relationship between the chemical components and the target proteins in the treatment of diseases.[48] Therefore, the molecular docking was used to clarify the chemical ingredients of Sho-saiko-to and related targets against TC, which could explain the action mechanism and binding affinity of target proteins and active components to some extent. First, the compound structure in the Pubchem platform (https://pubchem.ncbi.nlm.nih.gov/) was downloaded and transformed to mol2 format file through Chem 3D software and then was downloaded the crystal structure of protein targets (PI3KCG, AKT1, CASPASE3, TP53, MYC, STAT3) from RCSB platform (https://www.rcsb.org/). The water molecules and ligands were deleted and hydrogen was added through the PyMOL 2.3.4 software and AutoDock 1.5.6 software. Finally, the molecular docking and docking conformation was visually analyzed by using Autodock Vina 1.1.2 and Discovery Studio 2020, respectively.

5.5.2. Cell Lines and Cell Culture. The human differential thyroid cancer cell line (FTC-133) and anaplastic thyroid cancer cell line (8505C) were kindly gifted by Prof. Hui Wang (Shanghai Jiao Tong University). The two cell lines were cultured in RPMI 1640 (Gibco, Invitrogen, Carlsbad, CA, U.S.A.) with 10 mg/mL of streptomycin, 10 000 units of penicillin (New Cell & Molecular Biotech, Suzhou, China), and 10% fetal bovine serum (FBS, Gibco, Invitrogen, Carlsbad, CA, U.S.A.). Cells were cultured at 5% CO_2 and 37 °C.

5.5.3. Cell Viability Assay. The CCK-8 assay (New Cell & Molecular Biotech, Suzhou, China) was used to evaluate the effects of the main active components of Sho-saiko-to on the cell viability of TC cells. FTC-133 and 8505C (4000 cells/well) were plated in 96-well plates and cultured for 24 h and then treated with the main active components (baicalein, quercetin, stigmasterol, beta-sitosterol, kaempferol) in different concentrations for another 24 h. The main active components were purchased from Yuanye Bio-Technology (Yuanye Bio-Technology, Shanghai, China). After that, the cell viability was determined by the CCK-8 assay.

5.5.4. Flow Cytometry for Analysis of Cell Apoptosis. Cell apoptosis was detected by an Annexin V-FITC apoptosis kit (Salarbio, Beijing, China).[49,50] FTC-133 and 8505C cells were plated in 24-well plates at a density of 10 000 and 8000, respectively, and cultured for 24 h. Then, they were treated with the main active components (baicalein, quercetin, stigmasterol, beta-sitosterol, kaempferol) with 24h IC50 for another 24 h (24h IC50, concentration causing 50% reduction growth in 24 h). After 24 h, cells were collected and washed with PBS and then washed with the 1xBinding Buffer. Thereafter, 5 μL Annexin V/FITC was added to cells and incubated at room temperature for 5 min under dark conditions. After 5 min, 5 μL of propidium iodide solution (PI) and 400 μL of PBS was added and incubated for 10 min. Cell apoptosis should be detected by Gallios Flow cytometer (Beckman Coulter, CA, U.S.A.) within 1 h.

5.5.5. Western Blot. FTC-133 and 8505C cells (2.5 × 106 cells/well) were cultured in 6-well plates for 24 h. Then the main active components (baicalein, quercetin, stigmasterol, beta-sitosterol, kaempferol) were added to the medium at the corresponding IC50, respectively and treated for 24 h. Total protein of FTC-133 and 8505C cells was gained using RIPA buffer (Beyotime Biotechnology, Shanghai, China). Each lane of a 10% SDS-PAGE (New Cell & Molecular Biotech, Suzhou, China) was added with equal amounts of protein (40 μg) and separated. Then protein-free rapid blocking buffer (EpiZyme, Shanghai, China) was used to block PVDF membranes (Millipore, New York, U.S.A.) for 2 h following protein transfer to the membranes. Then they were incubated with primary antibodies for 14 h at 4 °C. The primary antibodies are as follows: AKT1 (1:1500, Abclonal, Wuhan, China), and P-AKT1 (1:500, Proteintech, Wuhan, China), PI3KCG (1:1000, Proteintech, Wuhan, China), P-PI3KCG (1:1000, Proteintech, Wuhan, China), P62 (1:5000, Proteintech, Wuhan, China), LC3 (1:1500, Proteintech, Wuhan, China), CASPASE3 (1:500, Abclonal, Wuhan, China), GAPDH (1:1000, Abclonal, Wuhan, China), NIS (1:500, Abcam, Cambridge, U.K.), TTF-1 (1:1500, Proteintech, Wuhan, China), TPO (1:500, Abcam, Cambridge, U.K.), PAX-8 (1:2000, Proteintech, Wuhan, China), TSHR (1:500, Abcam, Cambridge, U.K.). The membranes were immersed in HRP-coupled secondary antibody solution (rabbit antimouse or goat antirabbit IgG, 1:10 000, Boster, Wuhan, China) for 2 h, incubated with ECL kit (Biosharp, Anhui, China) for 4 min, and imaged on a Visionwork system.

5.6. Statistical Analysis. All values were presented as the mean ± standard deviation (SD). The results were analyzed using GraphPad Prism 7.0. The significance level was set at 0.05.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c07335.

Active ingredients of Sho-saiko-to; targets genes of Sho-saiko-to; data of topological parameters of a PPI network; upset blot for the targets genes of Sho-saiko-to; PPI core network of the top 20 target genes according to degree value (PDF)

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Author Contributions

W.K. proposed the ideas, designed the experiments, and wrote the manuscript. W.C. and D.Y. conducted experimental operation, Q.R. and L.H. analyzed the data, G.Z. revised the whole manuscript.

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Notes

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■ ABBREVIATIONS

TC, thyroid cancer; RAIR-DTC, radioactive iodine refractory differentiated thyroid cancer; DTC, differentiated thyroid cancer; ATC, anaplastic thyroid cancer; MTC, medullary thyroid cancer; TCM, traditional Chinese medicine; Nrf2, Nuclear factor erythroid 2-related factor 2; DL, drug-likeness; OB, oral bioavailability; BP, biological processes; CC, cellular components; MF, molecular functions; FTC, follicular thyroid cancer; NT, near total thyroidectomy; TT, total thyroidectomy

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