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Research Article

Integrative Network Pharmacology Unveils *Limonia acidissima* as a Potential Natural Product for Targeting Cancer

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Abstract

Cancer remains a formidable health challenge worldwide, with complex molecular mechanisms driving its initiation, progression, and therapeutic resistance. In this study, we employed bioinformatics analyses to elucidate the molecular underpinnings of cancer biology, focusing on Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. Our GO analysis revealed the enrichment of key biological processes such as protein phosphorylation, regulation of programmed cell death, and transmembrane receptor signaling pathways, underscoring the critical roles of signaling cascades and regulatory mechanisms in tumorigenesis. Similarly, molecular functions such as protein kinase activity and ATP binding were identified as significantly enriched, highlighting the importance of protein kinases and molecular interactions in cancer development and progression. The KEGG pathway analysis further delineated dysregulated signaling pathways associated with cancer, including the MAPK and PI3K-Akt signaling pathways, implicating these pathways as central regulators of cancer progression. These findings deepen our understanding of cancer biology and offer potential targets for therapeutic intervention. Integrating multi-omics data and systems biology approaches may provide deeper insights into the intricate networks underlying cancer pathogenesis, paving the way for developing more effective treatments for cancer patients.

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INTRODUCTION

Cancer represents a formidable global health challenge characterized by uncontrolled cell growth and proliferation[1,2]. Despite advancements in conventional treatments such as surgery, chemotherapy, and radiotherapy, cancer remains a complex disease with formidable resistance mechanisms[3]. In recent years, there has been a surge in innovative therapeutic strategies, including stem cell therapy, targeted therapy, ablation therapy, nanoparticles, natural antioxidants, radionics, chemodynamic therapy, and ferroptosis-based therapy[4]. Integrating traditional medicinal knowledge with modern scientific methodologies offers a promising avenue for developing novel and effective cancer treatments[5].

Limonia acidissima, a deciduous tree belonging to the Rutaceae family, is indigenous to the Indian subcontinent and Southeast Asia[6]. Recognized for its rich phytochemical profile, including alkaloids, flavonoids, tannins, and terpenoids, *L. acidissima* has been traditionally used for its medicinal properties[7]. Emerging evidence supports the anticancer potential of *L. acidissima* extracts, as demonstrated by their efficacy against various cancer cell lines[6].

Cancer remains a significant global health challenge despite advancements in therapeutic strategies. The heterogeneous nature of cancer cells, their ability to evade immune responses, and the emergence of drug resistance underscore the urgent

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need for innovative treatment approaches[8,9]. Network pharmacology, a systems biology-based approach, offers a comprehensive framework for understanding the intricate interplay between drugs and biological systems[10]. Its application in elucidating the mechanisms of action of natural products has gained momentum in recent years[11]. By employing computational tools, network pharmacology can unveil the complex interactions of multiple bioactive compounds within biological networks[12,13].

Limonia acidissima has demonstrated promising anticancer activity in preclinical studies[6,14]. However, the precise molecular mechanisms underlying its therapeutic efficacy remain elusive. This study aims to employ a network pharmacology approach to elucidate the potential molecular targets and signaling pathways modulated by *L. acidissima* constituents. By bridging the gap between experimental data and mechanistic understanding, this research seeks to contribute to the development of novel therapeutic strategies for cancer treatment.

MATERIALS AND METHODS

Materials

This study utilized several online resources for data retrieval and analysis. These included PubChem (https://pubchem.ncbi.nlm.nih.gov/), SwissTargetPrediction (http://swisstargetprediction.ch/), GeneCards (https://www.genecards.org/), Venny (https://bioinfogp.cnb.csic.es/tools/venny/), and STRING (https://string-db.org/). Cytoscape 3.10.1 (https://cytoscape.org/) was employed for network visualization and analysis. Additionally, the CytoHubba plugin 0.1 was utilized within Cytoscape for network centrality analysis[15]. A comprehensive literature review was conducted to identify secondary metabolite compounds commonly found in *L. acidissima*. The results of this review[14] are summarized in **Table I**.

Table I. Secondary metabolite compounds of *L. acidissima*[14].

No	Compounds	PubChem ID	Parts
1	2,6-dimethoxy benzoquinone	68262	Fruit
2	3-formylindole	10256	Stem
3	4-hydroxybenzoic acid	135	Fruit
4	4-methoxy-1-methyl-2-quinolone	182073	Stem
5	4-methoxy-2-quinolone	600167	Stem
6	5-(3-acetoxypropenyl)-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-2,3- dihydroxybenzofuran-3-ylmethyl acetate	73207012	Stem
7	5-hydroxy-2-(-hydroxyphenyl)-7-methoxy-6-(3-methylbut-2-enyl)chroman-4- one	146026481	Root
8	Acidissimin	6442730	Fruit, root
9	Acidissiminol	14506785	Fruit
10	Acidissiminol epoxide	5363185	Fruit
11	Aurapten	1550607	Root
12	Bergapten	2355	Fruit, stem, root, leaf
13	Columbianetin	92201	Stem
14	Demethylsuberosin	5316525	Fruit, stem
15	Dihydrosuberenol	14077805	Root
16	Dihydroxyacidissiminol	101676196	Fruit
17	Edulitine	826073	Stem
18	Gallic acid	370	Fruit
19	Gallocatechin	65084	Fruit
20	Hederatriol	44144287	Stem
21	Isopimpinellin	68079	Fruit, stem, root
22	Limodissimin A	163183899	Stem
23	Limonin	179651	Stem
24	Lupeol	259846	Stem
25	Marmesin	334704	Stem, root
26	N, N-dimethyltryptamine	6089	Stem
27	N-benzoyltyramine	577614	Fruit
28	Obacunone	119041	Stem
29	Orientin	5281675	Leaf
30	Osthenol	5320318	Fruit, stem, root
31	Osthol	10228	Root
32	Physcion	10639	Stem
33	Psoralen	6199	Fruit, stem, root
34	Rutaevin	441805	Stem

35	Saponarin	441381	Fruit, leaf
36	Seselin	68229	Stem
37	Stigmasterol	5280794	Stem, root, leaf
38	Suberenol	5375166	Stem
39	Syringaldehyde	8655	Stem
40	Syringaresinol	100067	Stem
41	Tanakamine	101413702	Stem
42	Tanakine	57357311	Stem
43	Tembamide	177583	Stem
44	Vitexin	5280441	Fruit, leaf
45	Xanthotoxin	4114	Fruit, stem, root
46	Yangambin	443028	Stem

Methods

The SMILES (Simplified Molecular Input Line Entry System) code for each identified secondary metabolite compound was retrieved from the PubChem database[16]. Subsequently, these SMILES codes were input into SwissTargetPrediction to predict potential protein targets[17]. Cancer-related proteins were identified using GeneCards[18]. The intersection of protein targets predicted by SwissTargetPrediction and those associated with cancer (from GeneCards) was determined using Venny[19]. Finally, the STRING database[20] was utilized to construct a pharmacological network illustrating potential protein-protein interactions among the identified proteins, providing insights into the potential mechanisms of action of secondary metabolites from *L. acidissima* in relation to cancer.

Data analysis

Protein-protein interaction data, encompassing predicted interactions between *L. acidissima* secondary metabolites and cancer-related proteins, were retrieved from the STRING database. To elucidate the functional implications of these interactions, Gene Ontology (GO) enrichment analysis for biological processes, molecular functions, and cellular components, as well as Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were conducted. The interaction network was visualized and analyzed using Cytoscape[15]. Key hub proteins within this network were identified using the CytoHubba plugin[21], specifically employing the Maximal Clique Centrality (MCC) algorithm to identify the most influential nodes.

RESULTS AND DISCUSSION

Network pharmacology of secondary metabolite of L. acidissima

A comprehensive phytochemical analysis of *L. acidissima* identified 46 secondary metabolites distributed across its various plant parts (fruit, stems, roots, and leaves) (**Table I**)[14]. To predict potential protein targets for these metabolites, SwissTargetPrediction was employed, with a probability value threshold of >0 for further analysis.[22] GeneCards was utilized to identify cancer-related proteins, revealing 629 potential targets for *L. acidissima* metabolites among a broader set of 9,894 cancer-associated proteins. A Venn diagram analysis (**Figure 1**) identified a subset of 449 proteins implicated in cancer that also exhibited potential interactions with *L. acidissima* metabolites.

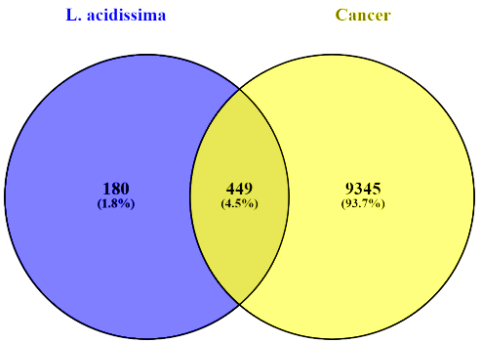

Figure 1. The Venn diagram intersection between proteins predicted to be able to interact with secondary metabolites of *L. acidissima* and proteins associated with cancer.

A pharmacological network analysis was conducted utilizing STRING (**Figure 2**), a comprehensive database containing over nine million proteins from diverse sources[22]. This analysis enabled the prediction of protein-protein interactions within the context of the 449 identified proteins[23]. The resulting network elucidates the interconnectivity between the selected target proteins and associated biological pathways.

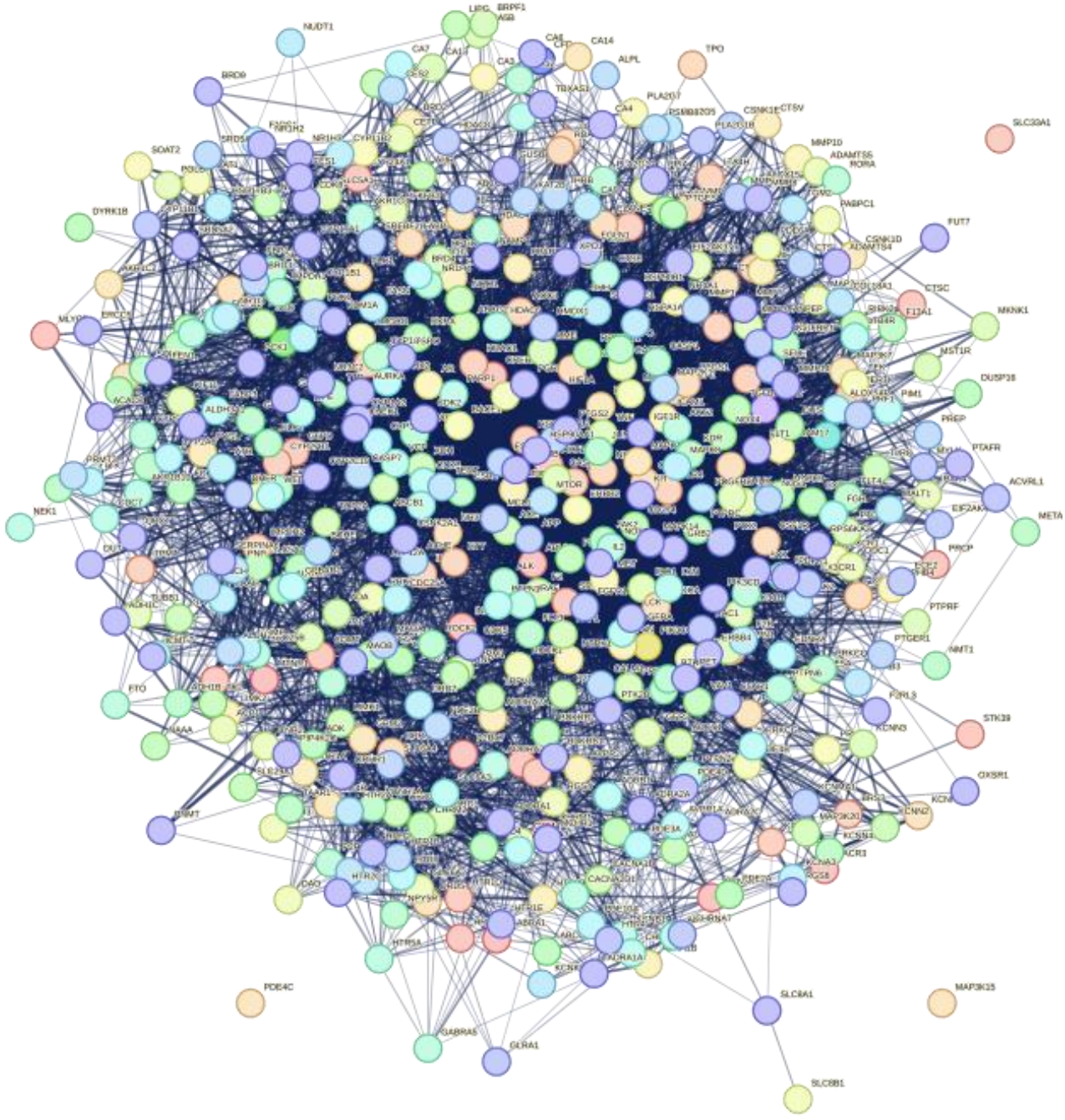

Figure 2. The network pharmacology using STRING.

Gene ontology and KEGG enrichment analysis

To elucidate the biological mechanisms underlying the identified gene sets, GO and KEGG enrichment analyses were conducted. GO categorizes genes into three ontologies: Biological Process (BP), Molecular Function (MF), and Cellular Component (CC)[24]. The KEGG provides manually curated pathways representing molecular interactions and reactions[25]. The significance of enrichment results was assessed using the False Discovery Rate (FDR) value, which represents the

expected proportion of false positives among identified gene sets. A lower FDR value indicates a higher degree of confidence in the results[26]. In this study, FDR values are expressed as -log(p-values). A higher -log(p-value) corresponds to a lower probability of error (**Figure 3**).

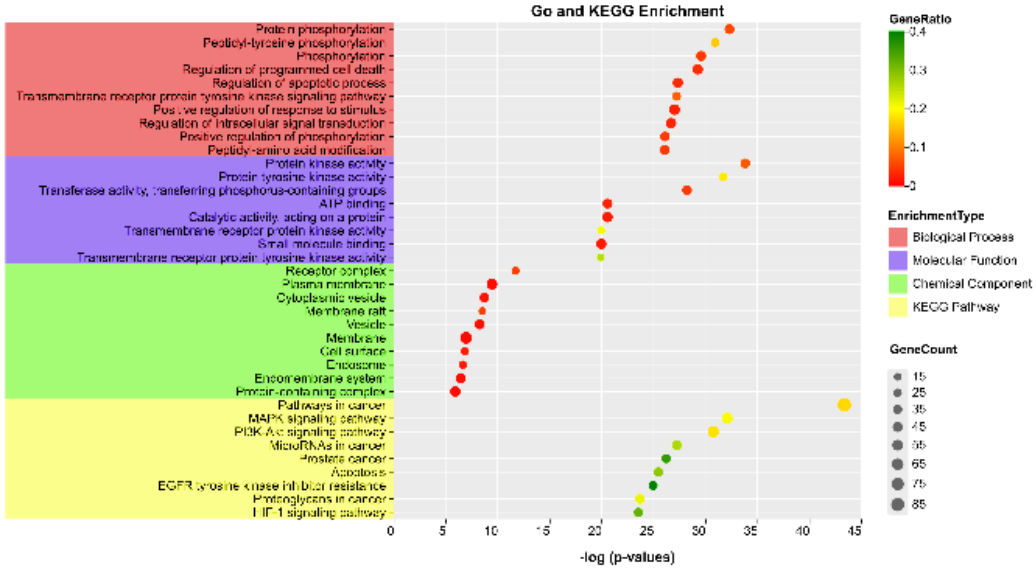

Figure 3. Gene Ontology and KEGG enrichment analysis.

Our analysis of the gene expression data revealed several biological processes associated with cancer. Notably, protein phosphorylation emerged as a central mechanism implicated in these processes[27]. Phosphorylation, a key regulatory mechanism in cells, involves the addition of phosphate groups to proteins, influencing a wide range of cellular functions, including signaling, proliferation, differentiation, and apoptosis[28]. Among the enriched biological processes, peptidyl-tyrosine phosphorylation and the transmembrane receptor protein tyrosine kinase signaling pathway stood out[29]. Tyrosine phosphorylation of proteins, especially within the context of RTKs, plays a critical role in regulating cell growth, survival, and migration[30]. Aberrant protein phosphorylation is a common hallmark of cancer[31,32]. Understanding the molecular mechanisms underlying peptidyl-tyrosine phosphorylation and RTK signaling pathways could potentially lead to the identification of novel therapeutic targets for precision cancer therapy[33]. By targeting these pathways, researchers may be able to develop more effective and targeted treatments for cancer patients.

Apoptosis, a programmed cell death mechanism, is a critical regulator of cell survival in cancer. Dysregulation of apoptosis pathways allows cancer cells to evade cell death and proliferate uncontrollably[34,35]. Intracellular signaling cascades play a pivotal role in transmitting information from the extracellular environment to the cell, influencing various cellular processes including proliferation, differentiation, and survival[36]. Aberrant signaling pathways can contribute to cancer development by promoting uncontrolled cell growth and survival[37]. Cancer cells often exhibit heightened sensitivity to external stimuli, enabling them to adapt to the complex tumor microenvironment and facilitate tumor progression and metastasis[38]. This enhanced responsiveness reflects the dynamic nature of cellular responses to diverse environmental cues and stress signals[39].

Molecular function analysis revealed that the identified proteins are enriched in functions associated with cancer, particularly those related to protein kinases. Protein kinases play pivotal roles in cellular signaling by catalyzing the phosphorylation of target proteins, making them attractive targets for cancer therapy[40]. Several kinase inhibitors, especially those targeting tyrosine kinases, have been approved for clinical use or are under investigation[41]. Transferase enzymes, responsible for transferring functional groups between molecules, including phosphate-containing groups, further highlight the significance of phosphorylation in cellular signaling and regulation[42]. The enrichment of transferase activity, particularly those involving phosphorus-containing groups, emphasizes the crucial role of phosphorylation events in cancer-related processes[43].

ATP binding is a pivotal molecular process underpinning diverse cellular functions, including energy metabolism, signal transduction, and protein synthesis[44]. Kinases, a class of enzymes involved in phosphorylation reactions, utilize ATP as a phosphate donor[45]. Targeting ATP-binding sites in key regulatory proteins, particularly receptor tyrosine kinases, offers a promising therapeutic strategy for cancer. These proteins play a critical role in transmitting extracellular signals into intracellular responses[47,48]. Aberrant activation of receptor tyrosine kinases, such as EGFR and VEGFR, drives tumor growth, angiogenesis, and metastasis[49]. Targeting these receptors and their downstream signaling pathways has yielded effective cancer treatments, with several receptor tyrosine kinase inhibitors approved for clinical use[50].

Small molecule binding encompasses a range of molecular interactions between ligands and their binding partners[51]. Small molecules, including drugs and endogenous metabolites, can modulate protein function and activity by binding to specific sites[52]. Leveraging small molecule-protein interactions is a powerful approach to drug discovery and therapeutic intervention in cancer. Targeted therapies that selectively inhibit oncogenic signaling pathways while minimizing off-target effects can be developed through this strategy[53].

Our CC analysis identified several proteins associated with cancer, emphasizing the significance of cell-surface receptors in cellular communication and signaling[54]. Receptor complexes function as molecular hubs, facilitating the binding of extracellular ligands and initiating intracellular signaling cascades that govern crucial cellular processes, including growth, differentiation, and survival[55]. Dysregulation of these receptor signaling pathways is a hallmark of cancer, with aberrant activation of growth factor receptors contributing to tumor progression and metastasis[56]. Targeting these receptor complexes and their downstream signaling pathways represents a promising therapeutic approach for disrupting oncogenic signaling networks and inhibiting tumor growth[57].

The plasma membrane and membrane rafts serve as critical platforms for cell-cell interactions, signal transduction, and molecular trafficking[58]. These membrane microdomains, enriched in cholesterol and sphingolipids, facilitate the clustering and organization of signaling molecules, including receptors and kinases, thereby modulating their activity and downstream signaling pathways[59]. Alterations in the composition and organization of the plasma membrane have been implicated in cancer development and metastasis, underscoring the importance of membrane-associated processes in tumor biology[60]. Dysregulation of the endomembrane system has been implicated in various pathological conditions, including cancer[61,62]. The endomembrane system encompasses a network of membrane-bound organelles, including the endoplasmic reticulum, Golgi apparatus, and vesicles, responsible for crucial cellular functions like protein trafficking, folding, and sorting[63-66]. Disruptions in this system can lead to abnormal receptor turnover, signaling, and drug sensitivity in cancer cells, suggesting its potential as a therapeutic target[67].

Pathway analysis, such as the "Pathways in Cancer" database, provides valuable insights into the molecular mechanisms underlying tumorigenesis[68]. Key signaling pathways implicated in cancer include the MAPK and PI3K-Akt pathways, which regulate cell proliferation, survival, and metastasis[69,70]. Additionally, dysregulated apoptotic pathways contribute to tumor cell survival and therapy resistance, emphasizing the need for therapeutic interventions targeting these pathways[71]. MicroRNAs also play a pivotal role in cancer biology, influencing gene expression and tumor progression. Targeting dysregulated miRNAs or their downstream targets represents a promising therapeutic strategy[72].

Resistance to targeted therapies, as exemplified by EGFR tyrosine kinase inhibitor resistance, remains a major obstacle in cancer treatment. Developing novel therapeutic strategies to circumvent these resistance mechanisms is imperative[73]. The tumor microenvironment significantly influences cancer progression, with proteoglycans playing a pivotal role in regulating tumor cell behavior, angiogenesis, and immune evasion[74]. Targeting proteoglycan signaling pathways or their downstream effectors within the tumor microenvironment presents promising therapeutic avenues[75]. Furthermore, hypoxia-induced signaling through the HIF-1 pathway promotes tumor growth and metastasis, highlighting another potential target for therapeutic intervention[76]. By elucidating dysregulated pathways through comprehensive pathway analysis, we can identify promising therapeutic targets and develop effective strategies to improve cancer treatment outcomes[77].

MCC analysis

To identify the most influential proteins within the pharmacological network, a hub network analysis was conducted using the MCC method. This approach identifies groups of proteins forming tightly interconnected sub-networks, known as

maximal cliques. The centrality of proteins within these cliques, which reflects their importance in maintaining or influencing network connections, was then assessed[21].

MCC enrichment analysis using CytoHubba revealed several key hub genes that may play pivotal roles in cancer pathogenesis and progression. These top-ranking hub genes included BCL2, SRC, ESR1, HIF1A, MDM2, MTOR, BCL2L1, AKT1, CD274, and CASP3 (**Figure 4**). The BCL2 family of proteins, including BCL2 and BCL2L1, plays a crucial role in regulating apoptosis[78]. SRC and AKT1 are protein kinases involved in signaling pathways associated with cell proliferation, survival, and metastasis[79]. Estrogen receptor alpha (ESR1) is a well-established biomarker and therapeutic target in hormone receptor-positive breast cancer[80]. Hypoxia-inducible factor 1 alpha (HIF1A) is a master regulator of cellular responses to hypoxia, a common feature of the tumor microenvironment[81]. Activation of HIF1A promotes angiogenesis and metastasis, facilitating tumor adaptation to low-oxygen conditions[82]. MDM2 and MTOR are key regulators of cell growth and proliferation, with critical roles in cancer development and progression[83]. CD274 (programmed death-ligand 1) and CASP3 (caspase 3) are intriguing hub genes implicated in immune evasion and apoptotic pathways, respectively[84].

The identification of BCL2, SRC, ESR1, HIF1A, MDM2, MTOR, BCL2L1, AKT1, CD274, and CASP3 as hub genes through MCC enrichment analysis highlights their potential significance in cancer pathogenesis and offers promising avenues for therapeutic intervention. Further experimental validation and functional studies are warranted to elucidate the specific mechanisms by which these hub genes contribute to tumorigenesis and to explore their potential as druggable targets in cancer therapy.

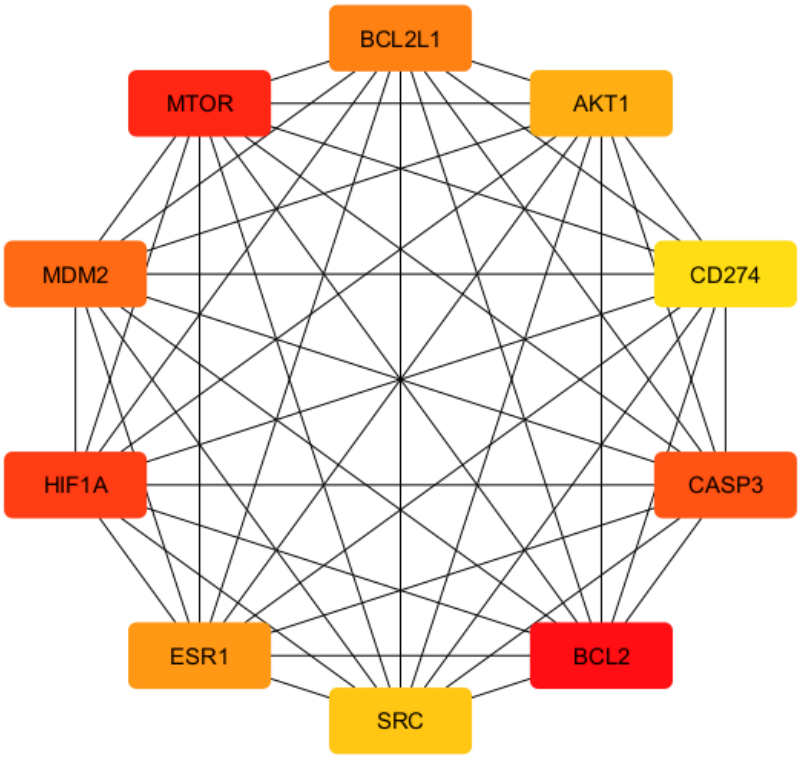

Figure 4. The top 10 proteins predicted to interact with secondary metabolites of *L. acidissima* and be associated with hypertension using the MCC method.

CONCLUSION

This study employed Gene Ontology and KEGG pathway analysis to elucidate the molecular mechanisms underpinning cancer. Our findings reveal significant enrichment in biological processes and molecular functions associated with cancer, emphasizing the pivotal role of signaling cascades and regulatory mechanisms in tumorigenesis. Notably, the MAPK and PI3K-Akt signaling pathways emerged as key regulators of cancer progression. These insights advance our understanding of cancer biology and offer potential therapeutic targets for the development of more effective treatments.

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AUTHORS' CONTRIBUTION

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Resources: -
Software: -
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Writing - review & editing: Reni Sri Wahyuni, M. Artabah Muchlisin, Ahmad Shobrun Jamil, Engrid Juni Astuti, Agustin Rafikayanti

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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