Cortex Mori extracts induce apoptosis and inhibit tumor invasion via blockage of the PI3K/AKT signaling in melanoma cells

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Melanoma, the most aggressive and deadliest form of skin cancer, has attracted increased attention due to its increasing incidence worldwide. The Cortex Mori (CM) has long been used as a classical traditional Chinese medicine (TCM) to treat various diseases, including cancer. The bioactive components and underlying mechanisms, however, remain largely unknown. The current study aims to investigate the anti-melanoma effects of CM and potential mechanisms through combined network pharmacology and bioinformatic analyses, and validated by in vitro and in vivo experiments. We report here that CM has anti-melanoma activity both in vitro and in vivo. Furthermore, 25 bioactive compounds in CM were found to share 142 melanoma targets, and network pharmacology and enrichment analyses suggested that CM inhibits melanoma through multiple biological processes and signaling pathways, particularly the PI3K-AKT signaling inhibition and activation of apoptotic pathways, which were further confirmed by biochemical and histological examinations. Finally, partial CM-derived bioactive compounds were found to show anti-melanoma effects, validating the anti-melanoma potential of bioactive ingredients of CM. Taken together, these results reveal bioactive components and mechanisms of CM in inhibiting melanoma, providing them as potential anti-cancer natural products for the treatment of melanoma.

KEYWORDS
melanoma, network pharmacology, anti-cancer natural product, molecular docking, Cortex Mori
Introduction

Melanoma, derived from the malignant transformation of melanocytes, is a highly aggressive and drug-resistant malignancy, with a 5-year survival rate of merely 29.8% (Huang and Zappasodi, 2022; Li et al., 2022). Currently, surgical excision is the most common clinical treatment for melanoma, followed by postoperative adjuvant treatments such as radiation or chemotherapy (Carr et al., 2020). However, these treatments usually elicit low response rates and severe side effects (Tyagi et al., 2022). As a result, developing new drugs or adjuvants with high activity and low toxicity for melanoma treatment is critical.

TCM has been increasingly utilized in Asia for thousands of years to treat a variety of illnesses (Xiang et al., 2019). TCM has recently gained widespread acceptance as an adjuvant therapy option following surgery, chemotherapy, radiotherapy, or other types of therapy due to its beneficial therapeutic outcomes for cancer patients (Chen et al., 2008). Furthermore, natural compounds derived from herbs are considered suitable drugs for cancer therapies due to their advantages of multi-targeting, efficacy, and low toxicity (Sayanta et al., 2019). CM, derived from the Morus alba L. (a mulberry tree), is commonly used as TCM for the alleviation of various diseases, such as cough (Yang et al., 2014), diabetes (Wang et al., 2020), inflammation (Carson et al., 2002), and cancers (Nam et al., 2002; Park et al., 2012; Guo et al., 2022). Modern pharmacological studies have revealed that the anti-lung cancer effect of CM extracts can induce cell apoptosis by inhibiting microtubule assembly (Carson et al., 2002). CM extracts also showed anti-tumor activity in colon cancer, leukemia, and hepatic cancer (Huang et al., 2011; Chen et al., 2017). Besides, previous studies have confirmed CM extracts possess anti-melanogenic and cytotoxic effects against mouse skin melanoma B16-F10 cells (Choe, 2011; Wu et al., 2018). However, the specific effective bioactive compounds and the underlying molecular mechanisms of CM remain unclear.

The complexity of various components, multiple targets, and synergistic interactions makes it difficult to explore the underlying mechanism of TCM (Ma et al., 2015). Network pharmacology, on the other hand, would systematically discover the interaction of the complex, diverse relationships among targets, drugs, diseases, and pathways, thereby providing a systematic approach to evaluate the feasibility and compatibility of TCM by delineating the detailed “multi-gene-multi-target-complex disease” multi-level interaction networks (Hopkins, 2007; Liu et al., 2022; Liu et al., 2022). Since its initial presentation in 2007, network pharmacology has been used extensively as a promising approach to TCM research (Hopkins, 2007).

In the present study, we investigated the potential bioactive compounds in CM using pharmacological indexes, and the anti-melanoma mechanisms of CM were determined using network pharmacology molecular docking and other bioinformatic analyses. Importantly, in vitro and in vivo experiments were carried out to validate the anti-cancer effects and underlying mechanisms of CM-derived bioactive ingredients in melanoma inhibition.

Materials and methods

Cell lines and reagents

Malignant melanoma cell lines A375 and MV3 were procured from the American Type Culture Collection (CRL-1619) and Beyotime (C6282), respectively. All cell lines were cultured in a humidified incubator with 5% CO2 at 37°C and maintained in high-glucose Dulbecco’s modified Eagle’s medium (DMEM; VivaCell, China) supplemented with 10% fetal bovine serum (FBS; BI, United States), 100 U/mL penicillin, and 100 μg/ml streptomycin. Sangenone H (HY-N2607), Moracin O (HY-N3244), Kaempferol (HY-14590), and Mulberoside C (HY-N0620) were obtained from MCE (China). Cortex Mori was purchased from Tianjiang Pharmaceutical (China). Antibodies against human Tubulin (AF1216) were obtained from Beyotime (China), and antibodies against phospho-Akt (Thr308, 13038), BAX (2774), Bcl-2 (15,071), phospho-PI3 Kinase p85 (p-PI3K Tyr458, 17366), Cleaved Caspase-9 (9507), and Cleaved PARP (5625) were purchased from Cell Signaling Technology (CST).

Characterization of bioactive ingredients in cortex mori

The bioactive compounds from CM were determined using the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP, https://old.tcmsp-e.com/tcmsp.php), which comprises pharmacokinetic properties for natural compounds involving oral bioavailability (OB), drug-likeness (DL), intestinal epithelial permeability, blood-brain-barrier, aqueous solubility, etc., (Ru et al., 2014). Based on screening conditions with OB ≥ 30% and DL ≥ 0.18, around 25 active compounds were obtained.

Target prediction of active ingredients in cortex mori and network construction

The protein targets of the active substances in CM were retrieved from the TCMSP database. GeneCard (https://www.genecards.org/), targets with relevance score ≥1 were screened and OMIM (Online Mendelian Inheritance in Man, https://omim.org/search/advanced/geneMap) were used to collect melanoma-related target genes. Finally, target genes of CM for the treatment of melanoma were acquired by the R package “Venn”.

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The cross-targets obtained above were entered into the search tool for the Retrieval of Interacting Genes (STRING) database (https://string-db.org) to construct a PPI network. 0.95 was taken as the minimum required interaction score, and the disconnected targets were removed (Abhimanyu et al., 2022; Pan et al., 2022). The PPI network was then analyzed and visualized with Cytoscape-3.9.0 for further screening with median values of Betweenness (BC), Closeness (CC), Degree (DC), and Eigenvector (EC) using a plug-in of Cytoscape, CytoNCA.

The compound-target network was established using Cytoscape-3.9.0 to further analyze the molecular mechanism of CM in the therapy of melanoma. Compounds, targets, CM, and melanoma were shown as nodes in the graphical network, while the interactions were represented as edges.

Enrichment analysis

The cross target gene names were transformed into entrezID by the R package “org.Hs.eg.db” and the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were conducted and visualized with the R packages “clusterProfiler”, “org.Hs.eg.db”, “enrichplot”, “ggplot2”, and “pathview” with adjusted p-values of <0.05.

Molecular docking analysis

To further verify the binding capacity of bioactive compounds and potential targets, the core targets were chosen as receptors for molecular docking. The 2D structures of bioactive compounds were obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov/) and imported into ChemBio3D 14.0 software to minimize energy and obtain 3D structures, which were further inputted into AutoDockTools-1.5.7 and saved in “pdbqt” format. The protein 3D structures were downloaded from the Protein Data Bank (PDB, http://www.rcsb.org/) and then imported into PyMOL-2.5.2 to remove organics, solvents, and ions. Subsequently, hydrogens were added by the AutoDockTools-1.5.7 software, and the resultant structure was saved in “pdbqt” format. The center grid box of the target protein was determined by AutoDockTools-1.5.7 based on the parameters as follows: 40 points in x-, y-, and z-dimension and 1.000 for spacing (angstrom). Finally, AutoDock Vinna-1.1.2 software was employed for molecular docking, and the results were visualized by PyMOL-2.5.2.

Colony formation assay

A375 and MV3 cells were seeded in a 24-well plate at a density of 500 cells per well. Cells were cultured overnight before treatment with DMSO or different bioactive substances for 7 days. To count the colonies, the cell monolayer was rinsed twice with 1× PBS, fixed with 4% paraformaldehyde (PFA), and stained with 1% crystal violet solution. The number of colonies in each well was counted.

EdU staining

Click-iT® EdU Imaging Kits (Invitrogen, United States) were used for cell proliferative ability assay as described previously (Li et al., 2021). Approximately 2 × 10^4 cells were seeded into 24-well cell culture plates and placed in an incubator overnight, and then incubated with DMSO or various bioactive compounds for 48 h. Then, the cells were incubated with 10 μM EdU (Sigma Aldrich, United States) for 40 min and fixed with 4% PFA for 15 min. The cells were then rinsed thrice with 1× PBS before being permeabilized for 2 hours with in 0.5% Triton X-100 in 5% bovine serum albumin (BSA). The cells were then incubated with Click-iT® reaction cocktails at room temperature protected from light for 40 min. The nuclei were stained with DAPI for 20 min at room temperature. The cells were imaged by an inverted fluorescent microscope, and the number of cells that were positive for EdU in each field was calculated (Ji et al., 2022).

Wound-healing assay

When the cells reach 100% confluency, linear wounds are carved using 200 µL pipette tips. After being washed thrice with 1× PBS, the cells were cultured in an FBS-free medium containing DMSO or various bioactive ingredients. After 30 h, the wounds were imaged and the closure rates were determined.

Measurement of cell viability

The MV3 and A375 cells were seeded in a 96-well plate at a density of 1× 10^4 cells per well. After 24 h, the cells were treated for another 48 h with DMSO or various doses of bioactive substances. The CCK8 assay was used to assess cell viability. The absorbance in each well was measured at 450 nm using a microplate reader (Thermo Fisher, United States).

Cell apoptosis analysis

Cell apoptosis was determined using the cell apoptosis kits procured from Beyotime (C1062M, China). After 48 h of treatment with DMSO or CM, cells were harvested and washed thrice with 1 X PBS. Then cells were incubated with Annexin V-FITC and propidium at room temperature for 15 min in the dark according to the manufacturer’s protocol.
Finally, the apoptotic cell ratio was detected using a flow cytometer and analyzed by FlowJo_v10.6.2 software.

**Western blotting**

Total protein was extracted using RIPA (Beyotime, P0013K) with phenylmethanesulfonyl fluoride (PMSF) and phosphatase inhibitor (Abcam, ab201112), and the protein concentration was detected using a bicinchoninic acid (BCA) kit (ThermoFisher Scientific, 23227). 30 µg of sample protein per lane was added, and the loading volume for each sample was normalized using deionized water. As described previously, the protein was separated on 8%–15% SDS-PAGE gels and then transferred to polyvinylidene fluoride (PVDF) membranes. After a 2-h incubation with 5% BSA, the membranes were incubated with primary antibodies at 4°C overnight and washed three times with 1× TBST (10 min), followed by incubation with second antibodies at room temperature for 2 h (Zhang et al., 2018; Li et al., 2020). The relative expression of each target protein was measured using β-Tubulin as the reference.

**Quantitative real-time PCR**

Total RNA was extracted and purified using RNAiso Plus (Takara, Japan) and was further used to synthesize the first-strand cDNA using the GoScript™ Reverse Transcription System (Promega, United States). qRT-PCR was performed on
a Roche LightCycler 96, using GoTaq® qPCR master mix (Promega, United States) with the following reaction mixture: 2 µl cDNA template, 10 µl 2 × GoTaq®qPCR master mix, 7 µl nuclease-free water, and 0.5 µl forward and reverse primers. All qRT-PCR primers were obtained from PrimerBank (https://pga.mgh.harvard.edu/primerbank/) and are listed in Supplementary Table S1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control. The 2-ΔΔCt method was used to determine the relative mRNA expression level (Zhang et al., 2017; Yang et al., 2020; Hu et al., 2021).

### Tumor xenograft model

BALB/c null mice were obtained from Gempharmatech Co., Ltd. (Chengdu, China) and raised in the specific pathogen-free (SPF) room. The Committee for Animal Protection and Utilization at Southwest University approved the study. Four-week-old mice were injected subcutaneously with A375 cells (5 × 10⁵) in the left flank. Mice were randomly allocated into the following two groups (n = 5) after a week: the control group (1× PBS) and the CM group. Each week, the tumor volume and the body weight of mice were measured. After that, mice were sacrificed, and tumors were collected for weight, H&E staining, and immunohistochemistry (IHC).

### Immunohistochemistry and H&E staining

Tumor samples were fixed, dehydrated, and embedded in paraffin for sections. The sections were stained with H&E or incubated in 0.3% H₂O₂ to block endogenous peroxidase for 20 min at room temperature. After blocking, the sections were incubated overnight with Ki67 or 4′,6-diamidino-2-phenylindole. Finally, the sections were incubated with a biotinylated anti-rabbit antibody and observed under the microscope (Lian et al., 2022).

### TUNEL staining

Cellular apoptosis in tumor tissues was detected using the DAB (SA-HRP) Tunel Cell Apoptosis Detection Kit (G1507,
Servicebio, China) by the manufacturer’s instructions. The sections were stained with Biotin-labeled dUTP (Biotin-dUTP) for 1 h at 37°C, then incubated with Streptavidin-HRP (SA-HRP) for 10 min at 37 °C followed by 3,3′-diaminobenzidine (DAB). The sections were observed and imaged using microscopy, and the nuclei of apoptotic cells were stained brown.

Statistical analysis

The data from all three biologically independent replicates are presented as mean ± SD. GraphPad Prism 8.0 was used to determine the significant differences between groups using the Student’s t-test. The asterisks indicate statistically significant differences (*p < 0.05 and **p < 0.01).

Results

CM exhibits anti-melanoma activity in vitro and in vivo

In the present study, in vitro experiments were first conducted to validate the anti-melanoma effects of CM. The A375 and MV3 cells exposed to CM changed their morphology and cell numbers dramatically (Figure 1A). The CCK8 assay revealed a significant dose-dependent decrease viability (Figure 1B). As shown in Figure 1C, CM treatment resulted in significant cell apoptosis revealed by flow cytometry. Furthermore, nude mice xenografted subcutaneously with A375 cells were used to test the anti-cancer effects of CM in vivo. CM treatment significantly reduced tumor growth compared to the control group (Figure 1D,E). It is worth
mentioning that CM treatment had no significant effect on the body weight of tested mice (Figure 1F).

Identification of bioactive compounds in cortex mori

To understand the underlying mechanism of CM against melanoma, bioactive compounds in CM were identified. A total of 194 ingredients were collected from TCMSP, of which 25 bioactive compounds were obtained under screening conditions with OB $\geq$ 30% and DL $\geq$ 0.18. These compounds are mostly flavonoids and phenols according to structural classification, and their detailed information is provided in Table 1. These bioactive ingredients were chosen for further investigation.

Targets identification and PPI network establishment

After removing duplicates, 169 target genes of the above 25 bioactive compounds were acquired from the following table:

### Table 2: Potential target genes of bioactive compounds in Mori Cortex for melanoma.

| NO. | Symbol | Entrez ID | NO. | Symbol | Entrez ID | NO. | Symbol | Entrez ID | NO. | Symbol | Entrez ID |
|-----|--------|-----------|-----|--------|-----------|-----|--------|-----------|-----|--------|-----------|
| 1   | AR     | 367       | 37  | AKR1B1 | 231       | 73  | CYPIA4 | 1576      | 109 | NQO1   | 1728      |
| 2   | PTGS2  | 5743      | 38  | MMP3   | 4314      | 74  | CYPIA2 | 1544      | 110 | PARP1  | 142       |
| 3   | CA2    | 760       | 39  | RELA   | 5970      | 75  | CAV1   | 857       | 111 | AHR    | 196       |
| 4   | ADRB2  | 154       | 40  | EGFR   | 1956      | 76  | MYC    | 4609      | 112 | SLC2A4 | 6517      |
| 5   | DPP4   | 1803      | 41  | AKT1   | 207       | 77  | F3     | 2152      | 113 | CXCL11 | 6373      |
| 6   | LTA4H  | 4048      | 42  | VEGFA  | 7422      | 78  | GJA1   | 2697      | 114 | CXCL2  | 2920      |
| 7   | SCNSA  | 6331      | 43  | CCND1  | 595       | 79  | CYPIA1 | 1543      | 115 | CHEK2  | 11200     |
| 8   | NCOA2  | 10499     | 44  | BCL2L1 | 598       | 80  | ICAM1  | 3383      | 116 | CLDN4  | 1364      |
| 9   | HSP90AA1 | 3320    | 45  | FOS    | 2353      | 81  | IL1B   | 3553      | 117 | PPARA  | 5465      |
| 10  | ESR1   | 2099      | 46  | CDKN1A | 1026      | 82  | CCL2   | 6347      | 118 | PPARD  | 5467      |
| 11  | PRSS1  | 6544      | 47  | EIF6   | 3692      | 83  | SELE   | 6401      | 119 | HSFI   | 3297      |
| 12  | CDK2   | 1017      | 48  | PLAU   | 5328      | 84  | VCAM1  | 7412      | 120 | CXCL10 | 3627      |
| 13  | CCNA2  | 890       | 49  | MMP2   | 4313      | 85  | PTGER3 | 5733      | 121 | CHUK   | 1147      |
| 14  | NOS2   | 4843      | 50  | MMP9   | 4318      | 86  | CXCL8  | 3576      | 122 | SPP1   | 6696      |
| 15  | PTGS1  | 5742      | 51  | MAPK1  | 5594      | 87  | PRKCB  | 5579      | 123 | RUNX2  | 860       |
| 16  | F7     | 2155      | 52  | IL10RA | 3587      | 88  | BIRC5  | 332       | 124 | RASSF1 | 11186     |
| 17  | KDR    | 3791      | 53  | EGF    | 1950      | 89  | NOS3   | 4846      | 125 | E2F1   | 1869      |
| 18  | ESR2   | 2100      | 54  | RBI    | 5925      | 90  | HSPB1  | 3315      | 126 | E2F2   | 1870      |
| 19  | NCOA1  | 8648      | 55  | TNF    | 7124      | 91  | IL2RA  | 3559      | 127 | ACP3   | 55        |
| 20  | KCHN2  | 3757      | 56  | ILE6   | 3570      | 92  | NR1H2  | 8856      | 128 | CTSD   | 1509      |
| 21  | RXRA   | 6256      | 57  | TP53   | 7157      | 93  | CYPI1H | 1545      | 129 | IGBP3  | 3486      |
| 22  | PGR    | 5241      | 58  | ELK1   | 2002      | 94  | CCNB1  | 891       | 130 | IGFI   | 3481      |
| 23  | SLC6A4 | 6532      | 59  | NFKBIA | 4792      | 95  | PLAT   | 5327      | 131 | CD40LG | 959       |
| 24  | OPRA1  | 4988      | 60  | ODC1   | 4953      | 96  | THBD   | 7056      | 132 | IFR1   | 3659      |
| 25  | BCL2   | 596       | 61  | TOP1   | 7150      | 97  | SERPINE1 | 5054  | 133 | ERBB3  | 2065      |
| 26  | BAX    | 581       | 62  | RAF1   | 5894      | 98  | COL1A1 | 1277      | 134 | HK2    | 3099      |
| 27  | CASP9  | 842       | 63  | SOD1   | 6647      | 99  | IFNG   | 3458      | 135 | RASA1  | 5921      |
| 28  | CASP3  | 836       | 64  | MMP1   | 4312      | 100 | ALOX5  | 240       | 136 | GSTM1  | 2944      |
| 29  | CASP8  | 841       | 65  | HIF1A  | 3091      | 101 | IL1A   | 3552      | 137 | NRS2C2 | 4306      |
| 30  | PRKCA  | 5578      | 66  | STAT1  | 6772      | 102 | MPO    | 4353      | 138 | NRS2C1 | 2908      |
| 31  | MAP2   | 4133      | 67  | RUNX1T1| 862       | 103 | TOP2A  | 7153      | 139 | IKBKB  | 3551      |
| 32  | PPARG  | 5468      | 68  | CDK1   | 983       | 104 | NCF1   | 653361    | 140 | MAPK8  | 5599      |
| 33  | MAPK14 | 1432      | 69  | HSPA5  | 3309      | 105 | ABCG2  | 9429      | 141 | PPS5CA | 5530      |
| 34  | GSK3B  | 2932      | 70  | ERBB2  | 2064      | 106 | HAS2   | 3037      | 142 | AKR1C3 | 8644      |
| 35  | CHEK1  | 1111      | 71  | ACACA  | 31        | 107 | GSTP1  | 2950      |       |         |            |
| 36  | ACHE   | 43        | 72  | HMOX1  | 3162      | 108 | NFE2L2 | 4780      |       |         |            |

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source of DrugBank on TCMSP. In contrast, 4065 unique melanoma-related targets were obtained from the GeneCard and OMIM databases. 142 potential targets of CM for the treatment of melanoma were obtained (Figure 2A), which were listed in Table 2.

Subsequently, the PPI network was constructed by the STRING database and visualized by Cytoscape based on these 142 potential targets, which include 114 nodes and 367 edges (Figure 2B). Thereafter, the CytoNCA screen resulted in a network with 33 nodes and 136 edges based on median values of BC, DC, CC, and EC (Figure 2C). Finally, based on further above-mentioned screening, the core network with 11 nodes and 36 edges was acquired (Figure 2D). These hub genes include MAPK1, MAPK14, AKT1, TNF, HSP90AA1, TP53, RELA, MYC, ESR1, FOS, and HIF1A, and most of them have a degree in the PPI network no less than 15 (Figure 2E).

Potential anti-melanoma mechanisms of *cortex mori* revealed by network pharmacology and enrichment analysis

The CM–Active Ingredients–Targets–Melanoma network was constructed by Cytoscape, which has 169 nodes and 488 edges, demonstrating that the multi-component, multi-target, and multi-channel therapeutic properties of TCM (Figure 3A). After converting gene symbols into entrezIDs, 142 potential targets of 25 bioactive ingredients in CM for melanoma therapy were collected for GO enrichment and KEGG pathway enrichment analysis (Table 2). Go analysis revealed that the biological processes (BP) such as response to oxygen levels, response to hypoxia, and response to decreased oxygen levels, cellular components (CC) like serine/threonine protein kinase complex, protein kinase complex, and cyclin-dependent protein kinase holoenzyme complex, and...
molecular function (MF) such as DNA-binding transcription factor binding, ubiquitin-like protein ligase binding, and ubiquitin protein ligase binding may be potential anti-cancer mechanisms for CM for treatment of melanoma (Figures 3B–D). The KEGG pathway enrichment analysis ($p < 0.05$) demonstrated that CM may affect various signaling pathways closely related to carcinogenesis, including the PI3K-AKT signaling pathway, MAPK signaling pathway, TNF signaling pathway, apoptosis, and others (Figure 3E). Additionally, the PI3K-AKT signaling pathway was selected for annotation due to the significance of the enrichment, where many key factors are targets of bioactive ingredients in CM (Supplementary Figure S1).

### Table 3 The results of molecular docking.

| Chem        | Mol ID       | PDB   | Receptor    | Best affinity (kcal/mol) |
|-------------|--------------|-------|-------------|--------------------------|
| Moracin D   | MOL003858    | 6QE1  | MAPK14      | −9.7                     |
| quercetin   | MOL000098    | 1A02  | FOS         | −9.5                     |
| Glabrone    | MOL004912    | 6QE1  | MAPK14      | −9.5                     |
| quercetin   | MOL000098    | 5WBL  | AKT1        | −8.8                     |
| quercetin   | MOL000098    | 1A8M  | TNF         | −8.7                     |
| mulberroside C | MOL012735 | 1BYQ  | HSP90AA1    | −8.7                     |
| kaempferol  | MOL000422    | 5WBL  | AKT1        | −8.6                     |
| sanggenone F | MOL012753   | 1BYQ  | HSP90AA1    | −8.6                     |
| sanggenone H | MOL012755   | 1BYQ  | HSP90AA1    | −8.4                     |
| kaempferol  | MOL000098    | 1A8M  | TNF         | −8.3                     |
| quercetin   | MOL000098    | 6WQX  | TP53        | −8.3                     |
| Moracin D   | MOL003858    | 1A52  | ESR1        | −8.2                     |
| Moracin D   | MOL003858    | 1BYQ  | HSP90AA1    | −8.2                     |
| mulberroside C | MOL012735 | 1A52  | ESR1        | −8                       |
| sanggenone F | MOL012753   | 1A52  | ESR1        | −8                       |
| quercetin   | MOL000098    | 1H2K  | HIF1A       | −8                       |
| moracin O   | MOL012719    | 1A52  | ESR1        | −7.9                     |
| moracin O   | MOL012719    | 1BYQ  | HSP90AA1    | −7.8                     |
| quercetin   | MOL000098    | 7E75  | MAPK1       | −7.8                     |
| Moracin C   | MOL003857    | 1BYQ  | HSP90AA1    | −7.7                     |
| Moracin F   | MOL012753    | 1BYQ  | HSP90AA1    | −7.7                     |
| Iristectorigenin (9CI) | MOL003758 | 6QE1  | MAPK14      | −7.7                     |
| Moracin A   | MOL012714    | 1BYQ  | HSP90AA1    | −7.6                     |
| Sexangularetin | MOL002514 | 1BYQ  | HSP90AA1    | −7.6                     |
| sanggenone H | MOL012755   | 1A52  | ESR1        | −7.5                     |
| Moracin B   | MOL003856    | 1BYQ  | HSP90AA1    | −7.5                     |
| quercetin   | MOL000098    | 1BYQ  | HSP90AA1    | −7.5                     |
| Glabrone    | MOL004912    | 1A52  | ESR1        | −7.4                     |
| sanggenone M | MOL012760   | 1A52  | ESR1        | −7.4                     |
| Moracin C   | MOL003857    | 1A52  | ESR1        | −7.3                     |
| Iristectorigenin (9CI) | MOL003758 | 1BYQ  | HSP90AA1    | −7.3                     |
| pelargonidin | MOL001004    | 1BYQ  | HSP90AA1    | −7.2                     |
| kaempferol  | MOL000422    | 1BYQ  | HSP90AA1    | −7.2                     |
| Iristectorigenin (9CI) | MOL003758 | 1A52  | ESR1        | −7.1                     |
| Moracin B   | MOL003856    | 1A52  | ESR1        | −7.1                     |
| beta-sitosterol | MOL000358 | 1BYQ  | HSP90AA1    | −6.8                     |
| kaempferol  | MOL000422    | 5XNX  | RELA        | −6.3                     |
| quercetin   | MOL000098    | 5XNX  | RELA        | −6.1                     |
| quercetin   | MOL000098    | 1A93  | MYC         | −5.6                     |
Active components of *cortex mori* target key genes in melanoma

To reveal the candidate ingredients targeting melanoma-related proteins, 11 hub targets in the core PPI network were selected for molecular docking with corresponding active components in CM (Figure 2D). Lower binding energy (affinity) suggests a more potent and stable interaction between the compound and receptor, while binding energy of $< -7.0 \text{ kcal/mol}$ indicates better binding activity (Saraswati et al., 2019). According to docking results, three pairs share binding energy less than $-9.0 \text{ kcal/mol}$, and the majority of molecular docking results have binding affinity less than $-7.0 \text{ kcal/mol}$, demonstrating that the active components of CM have good binding capacity toward melanoma target proteins (Table 3). The docking diagram of eight pairs of bioactive components and hub targets with higher affinity is shown in Figure 4, and the bioactive components include Moracin D, Quercetin, Glabrone, Mulberrieside C, Kaempferol, Sanggenone F, and Sanggenone H.

*Cortex mori* induce the PI3K/AKT pathway inhibition and apoptosis

To further verify the underlying anti-melanoma mechanism of CM discovered through network pharmacology, protein and mRNA expression levels related to the PI3K/AKT pathway and apoptosis were investigated. The western blotting results showed that CM inhibited the protein expression of phospho-PI3K and phospho-AKT, two critical nodes in the PI3K/AKT pathway. Furthermore, both pro-apoptotic protein proteins (Cleaved caspase 9, Cleaved PARP, Bax) and mRNA (Caspase 9, Bax) levels were significantly increased in a dose-dependent manner in melanoma cells, whereas anti-apoptotic protein and mRNA level of Bcl2 was decreased sharply (Figure 5A–C). Meanwhile, the Ki67 staining results revealed that the CM-treated group had lower Ki67 protein levels than the control group (Figure 5D). TUNEL staining showed that CM treatment induced cell apoptosis in tumor tissues (Figure 5E). Furthermore, histological changes were observed by H&E staining, and CM treatment...
significantly reduced the number of nuclei in each field of view (Figure 5F).

**Cortex mori-derived bioactive ingredients inhibited melanoma cell growth in Vitro**

In the present study, four bioactive compounds derived from CM were selected to validate the anti-melanoma efficacy of bioactive ingredients in CM. A375 and MV3 cells exhibited significantly sliced colony formation ability when exposed to these bioactive compounds (Figure 6A–B). Furthermore, in an EdU staining assay, these bioactive substances significantly decreased the percentage of EdU-positive cells as compared to the control (Figure 6C,D). In addition, A375 and MV3 cells treated with bioactive compounds extracted from CM showed a significant impairment in migratory abilities (Figure 6E,F).

**Discussion**

Melanoma, a highly metastatic tumor, is the leading cause of death from skin cancer (Tong and Young, 2014). Melanoma is largely fatal, though surgical resection of early-stage melanoma is frequently curative (Sykes et al., 2016; Du et al., 2020). Therefore, the discovery of effective natural compounds against melanoma may have important public health implications. Meanwhile, various signaling pathways and many molecular targets are characterized to be involved in melanoma progression (Li et al., 2022). Network pharmacology emphasizes multiple signaling pathway modulation, which increases drug therapeutic impact while decreasing toxic and side effects, making it a powerful method for the mechanistic study of complicated TCM against melanoma (Zuo et al., 2018). The main purpose of this study is to decipher the mechanism of CM for melanoma treatment through the application of network pharmacology and experimental verification.

In the present study, 25 bioactive compounds were identified from CM (Table 1). They were considered pharmacokinetically active under screen conditions with OB ≥ 30% and DL ≥ 0.18, indicating that they possessed essential pharmacokinetic properties for compounds to reach systemic circulation (Xu et al., 2012; Tao et al., 2013). Previous studies have shown that mulberry extracts have good anti-
tumor activities. For instance, Sanggenon C has been reported to inhibit colon cancer cell proliferation and induce apoptosis by increasing reactive oxygen species, decreasing nitric oxide levels, and activating the mitochondrial apoptosis pathway (Chen et al., 2017). Anti-tumor properties of Sanggenon C have also been described in leukemia by inducing cell cycle arrest and cell death (Huang et al., 2011). Quercetin has anti-tumor activity in a variety of cancers, including non-small cell lung cancer, colorectal cancer, and others (Li et al., 2022; Liu et al., 2022). Kaempferol also has anti-tumor properties due to its modulation of the Akt/mTOR signaling and FAK pathways (Hung et al., 2017; Wang et al., 2021). In this study, four bioactive compounds derived from CM significantly inhibit melanoma cell proliferation and migration (Figure 6). These results suggest that CM-derived bioactive compounds, such as Moracin B, moracin C, Moracin D, Sanganenone F, Sanganenone H, and Sanganenone M, may have good anti-tumor properties that merit further investigations.

To determine the mechanism underlying the therapeutic effect of CM on melanoma, network pharmacology was used in this study, which revealed a core network containing 11 proteins (Figure 2). These proteins are well-known to be therapeutic targets for melanoma. AKT1, for instance, plays a key role in regulating cell survival, insulin signaling, angiogenesis, and tumor formation (Ronnstrand, 2004; Jia et al., 2021). Moreover, it has been shown that...
AKT1 promotes melanoma brain metastasis. Therefore, it could be a promising therapeutic target (Kircher et al., 2019). MYC and MAPK1 are involved in cell cycle progression, apoptosis, and cellular transformation, and their over-expression is common in melanoma (Savoia et al., 2019; Adam et al., 2020; Yang et al., 2022). The results of molecular docking verification revealed that CM-derived bioactive compounds had a high affinity for these targets (Figure 4; Table 3). These results suggest that CM could be applied in the alleviation and treatment of melanoma through the regulation of multiple targets by its bioactive ingredients.

GO and KEGG analysis showed that multiple molecular functions, biological processes, cellular components, and pathways were implicated in the potential treatment targets of CM for melanoma, embodying the TCM with characteristics of multiple targets and multiple pathways (Fu et al., 2022). Interestingly, multiple pieces of evidence in this study indicate that the HIF-1 signaling pathway, a master regulator of oxygen homeostasis, may be an important mechanism for CM in melanoma treatment. According to GO analysis, multiple molecular functions, such as response to oxygen levels, response to hypoxia, and response to decreased oxygen levels, pointed to oxygen levels (Figure 3). Furthermore, HIF1A was also discovered in the core network (Figure 2D), and quercetin exhibited excellent targeting ability to HIF1A (Saraswati et al., 2019), with the best affinity equal to -8.0 kcal/mol (Table 3). These findings are backed up by KEGG analysis (Figure 4).

Additionally, when exposed to CM, melanoma cells displayed significant proliferation inhibition and apoptosis induction, and expression levels related to apoptosis and the PI3K/AKT pathway were markedly affected (Figure 1; Figure 5). Previous studies have shown that the PI3K/AKT signaling pathway is an essential node in cancer cells that controls cell growth, migration, proliferation, and metabolism, and targeting the oncogenic PI3K/AKT signaling pathway is currently thought to be an extremely promising strategy for melanoma intervention (Shen et al., 2022; Vasan and Cantley, 2022). Apoptosis is crucial in tumorigenesis, development, and drug resistance (Braicu et al., 2022). Suppression of the PI3K/AKT pathway induces cell apoptosis via a variety of mechanisms, including regulation of Bcl-2 family members’ activities and activation of members of the caspase family of proteases (Kircher et al., 2019). Our results suggest that CM may inhibit melanoma cell proliferation and migration while inducing apoptosis via inhibition of the PI3K/Akt signaling pathway, providing a mechanistic foundation for exploiting CM and CM-derived bioactive components in melanoma treatment.

Conclusion

The potential mechanisms of CM on melanoma were systematically investigated using a network pharmacology approach and experimental validation. A total of 25 bioactive compounds were determined in CM, sharing 142 melanoma targets. GO and KEGG pathway enrichment analysis were used to investigate biological functions and signaling pathways. Molecular docking confirmed the ability of bioactive compounds to bind to core targets. In vivo experiments revealed that bioactive compounds derived from partial CM-derived bioactive compounds had excellent anti-cancer effects on proliferation and migration. In addition, our results show that CM may inhibit the growth and induce apoptosis of melanoma cells through the PI3K/Akt pathway, supporting the results obtained from network pharmacology. These findings provide the anti-tumor components and mechanisms of CM on melanoma. Our results also suggest that the bioactive ingredients in CM may have anti-cancer properties that warrant further investigation. Meanwhile, the precise mechanisms and side effects of these active ingredients need to be investigated further.

Data availability statements

The original contributions presented in the study are included in the article/Supplementary Materials, further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of Southwest University.

Author contributions

Study design: XH, KZ; Data collection: XH, KZ, and GP; Analysis and interpretation: XH, KZ; Statistical analysis: YW, YS, and CP; Drafting manuscript: XH, KZ, GP, and LD; Revision manuscript: XH, LD, YW, and HC. All authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.1007279/full#supplementary-material

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