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Research article

Effectiveness and mechanism of the Chinese medicine Weiren Xiaoyou formula in improving palmoplantar warts

Xin Liu [a,1], Ruofan Xi [a,1], Linyan Cheng [a,1], Yi Wang [a], Yiwen Nie [b], Ge Yan [a], Dongjie Guo [a], Wanjun Guo [a], Ting Du [a], Hanzhi Lu [a], Peiyao Wang [a], Jianyong Zhu [b,*], Fulun Li [a,**]

[a] *Department of Dermatology, Yueyang Hospital of Integrated Traditional Chinese and Western Medicine, Shanghai University of Traditional Chinese Medicine, Shanghai, 200437, China*

[b] *Department of Pharmacy Research, Yueyang Hospital of Integrated Traditional Chinese and Western Medicine, Shanghai University of Traditional Chinese Medicine, Shanghai, 200437, China*

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ABSTRACT

Background: Palmoplantar warts (PWs) are a usual skin disease associated with human papillomavirus (HPV) that can affect patients' quality of life. The traditional Chinese medicine (TCM) Weiren Xiaoyou formula (WRXYF) is a relatively gentle and effective therapy that has achieved good therapeutic effects in clinical practice, but its mechanism has not yet been studied.

Methods: A meta-analysis was carried out to identify the potential advantages of topical TCM for PW treatment. Clinical cases suggested that WRXYF was an effective therapeutic agent against PWs. Network pharmacology was utilized to predict potential targets for the main bioactive compound, tanshinone IIA (Tan IIA), in WRXYF. High-performance liquid chromatography with electrospray mass spectrometry (HPLC/ESI-MS) was applied to detect major components. The bioactivity of Tan IIA against PWs was then validated with quantitative polymerase chain reaction (q-PCR), fluorescence *in situ* hybridization (FISH), electron microscopy and Western blotting.

Results: A meta-analysis was conducted on 10 randomized clinical trials (RCTs) involving 2260 participants suggested that topical TCM could more effectively treat PWs than conventional medications. Network pharmacology identified Tan IIA as a candidate agent from 17 major compounds assessed by HPLC/ESI-MS because of its stable binding with 10 PW targets. HPV2, HPV27, and HPV57 were the main infectious strains in tissues obtained from PW patients and in HPV-infected HaCaT cells. Tan IIA treatment effectively destroyed viral particles and reduced the viral copy numbers of the three HPV subtypes. The results shown that Tan IIA has the ability to halt the cell cycle of HPV-infected HaCaT cells specifically in the G_0/G_1 phase. A total of 6 cell cycle-related proteins were regulated after Tan IIA treatment, demonstrating the role of Tan IIA in inhibiting the cell cycle.

Conclusion: Tan IIA, the primary bioactive constituent in WRXYF, enhances PWs by halting the cell cycle in the G_0/G_1 phase via modulation of the p53 signaling pathway.

* Corresponding author.
** Corresponding author.
E-mail addresses: jyzhu@foxmail.com (J. Zhu), drlifulun@163.com (F. Li).
[1] Xin Liu, Ruofan Xi and Linyan Cheng contributed equally to this work.

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1. Introduction

Palmoplantar warts (PWs), also known as verruca vulgaris, constitute a chronic and benign skin disease caused by human papillomavirus (HPV) infection. Epidemiological surveys have shown that PWs affect approximately 7%–12 % of the population worldwide [1], with a higher incidence among children and adolescents than among adults and no significant sex difference [2]. PWs can usually heal spontaneously, and only a small proportion of adults experience long-term symptoms [3]. However, physical and psychosocial disorders caused by pain and impairment still prompt patients to seek professional treatment [4].

HPV can be present people who have innate, intact intrinsic, and acquired immunity, and HPV infection is usually asymptomatic, benign, and self-limiting. Studies have shown that the reproductive cycle of HPV is not connected to the proliferation cycle of keratinocytes, which allows inactivation of cytokines that induce an immune response. Additionally, the L1 shell is resistant to Langerhans cells, which can result in immune escape and long-term persistence of the virus in infected individuals [5]. Current treatment methods for palmoplantar warts include cryotherapy, laser treatment, and salicylic acid treatment [6]. However, these treatment methods are painful and scarring, which is a cruel experience especially for children. Therefore, there is an urgent need for a painless and non-invasive treatment method for palmoplantar warts.

Patient's with PWs have a decrease in the quantity of several types of lymphocytes found in the peripheral blood, such as $CD3^{+}$, $CD4^{+}$, $CD8^{+}$, and CD4+/CD8+ T cells. Furthermore, the reduction in $CD3^{-}CD16/56^{+}$ (natural killer) cells lead to inactivation of cytotoxic T lymphocytes, which are responsible for the nonspecific direct killing of virally infected target cells and are important factors in viral infection among patients with PWs [6]. Studies have revealed that the HPV oncogenes E7 and E6 disrupt the cell cycle by inducing degradation of the cell cycle regulatory factors p53 and Rb, creating a conducive environment for HPV replication and epithelial tumor development [7]. p53 has been demonstrated to target the C region of the E2 protein's DNA-binding domain, leading to inhibition of viral replication [8]. These findings suggest that p53 is a protein that targets and inhibits viral replication.

Evidence has suggested that some herbal medicines and TCM prescriptions exhibit remarkable antiviral bioactivity [9–11]. However, whether they can be used exclusively for the treatment of PWs in clinical practice remains unclear. WRXYF is a Chinese herbal prescription (Table 1) that has been applied for years and has been demonstrated to be clinically effective in our hospital, but its substance basis and underlying mechanism have not yet been elucidated. It shows great advantages in clinical use, especially for paediatric patients. Its painless and non-invasive treatment method is recommended by many parents. We therefore wished to understand more about the principle of action of WRXYF in the therapy of palmar warts. This study investigated the bioactive compounds and mechanism of WRXYF in the treatment of PWs to support its clinical application. This is the first integrative study to explore the mechanism of TCM in PW treatment.

2. Materials and methods

2.1. Meta-analysis

2.1.1. Literature research

Chinese scientific journals, PubMed, Embase, Cochrane, Wanfang, and the Chinese Biomedicine Literature Service System were searched for relevant literature. The articles that were considered were published between March 31, 2022, and the earliest date that could be found. Following are the search parameters used by PubMed: "1#external therapy of Chinese medicine OR acupuncture OR manipulation OR electroacupuncture OR Acupoint OR Cupping OR moxibustion OR abdominal acupuncture OR auricular point, 2# warts OR verruca, 3#1#and2#." Other literary databases and search engines also utilized similar but more suitable search phrases. papers that were not found in the aforementioned electronic databases were evaluated manually, and the reference libraries of all listed publications were searched for additional relevant citations. The authors of the studies that were considered for inclusion were contacted if more information was needed [12]].

2.1.2. Criteria for considering research

The criteria for inclusion: 1) all participants were diagnosed with PWs in accordance with the relevant guidelines; 2) patients were randomly assigned to either external TCM treatment in conjunction with Western medicine or external treatment alone; 3) there were at least 10 patients in each group; 4) each group had a minimum of two weeks of follow-up; and 5) the endpoint indicators included the

Table 1
The composition of WRXYF.

Chinese name	Latin name	Source plants	Weight (g)
Weilingxian	Clematidis radix et rhizoma	*Clematis chinensis* Osbeck. (Ranunculaceae)	60
Yiyiren	Coicis semen	*Coix lacryma-jobi* L. *var. maj/uew* (Roman.) Stapf (Gramineae)	30
Danshen	Salviae miltiorrhizae radix et rhizoma	*Salvia miltiorrhiza* Bge. (Labiatae)	30
Huangqi	Astragali radix	*Astragalus membranaceus* (Fisch.) Bge. (Fabaceae)	30
Baizhu	Atractylodis macrocephalae rhizoma	*Atractylodes macrocephala* Koidz (Compositae)	30
Baihuasheshecao	Hedyotis diffusae herba	*Hedyotis diffusa* Willd. (Rubiaceae)	30
Machixian	Portulacae herba	*Portulaca oleracea* L. (Portulacaceae)	20
Ezhu	Curcumae rhizoma	*Curcuma phaeocaulis* VaL. (Zingiberaceae)	30

WRXYF: Weiren Xiaoyou formula.

percentage of regression of verruca vulgaris or other pertinent test indicators.

The exclusion criteria were as follows: 1) there was no randomization or blinding in the study; 2) the patients who participated did not have a definitive diagnosis; 3) the researchers compared various traditional Chinese medicine medications; 4) The trial simply reported improvements in symptoms and did not include objective laboratory data; and 5) the methodological quality was inadequate, with a Jadad score of less than 2.

2.1.3. Study selection

Available citations were collected and selected by two of the evaluators (LYC and RFX) independently and in duplicate. Subsequently, data extraction was carried out, and the methodological quality of the trials was evaluated. Any discrepancies were resolved either through discussion among the researchers or by seeking input from a third evaluator.

2.2. Case reports of WRXYF for the treatment of PWs

WRXYF is a commonly used topical formula for the treatment of PWs in clinical practice in our hospital and has a good therapeutic effect. Representative images of six patients showed the efficacy of WRXYF against PWs. The research followed the *Declaration of Helsinki* and was also reviewed, authorized, and supervised by the Ethics Committee of Yueyang Hospital (2022-008). All patients provided informed consent prior to participation.

2.3. Network pharmacology

2.3.1. Collecting data

WRXYF ingredient data was obtained from TCMSP (http://tcmspw.com/tcmsp.php). Our criteria included setting compound OB to $\geq$30 % and compound DL to $\geq$0.18, with additional selection of ingredients showing significant pharmacological activity despite not meeting these criteria. Targets of verruca vulgaris were retrieved from the GEO database using dataset GSE72140. Differentially expressed genes (DEGs) with $|\log2\ FC| > 1$ and $P < 0.05$ were selected. Subsequently, we identified intersections between WRXYF and the targets using the Venn tool (http://www.interactivenn.net/).

2.3.2. Protein–protein interaction network

STRING (https://string-db.org/cgi/input.pl) was used to create a network of gene-protein interactions (PPIs). The PPI network was obtained by downloading the file in the "string_interactions.tsv" format. For the gene list, prevalent targets of Cordyceps in tachyarrhythmia and bradyarrhythmia were utilized. The PPI network was constructed using Cytoscape 3.7.2.

2.3.3. Gene set enrichment analysis

To delve into the molecular signaling pathways implicated in PWs, the gene set enrichment analysis (GSEA) program was used. The c2.cp.kegg.v7.3.symbols.gmt gene sets were obtained from the official site and used for pathway enrichment assessments. A false discovery rate (FDR) $P < 0.01$ was considered indicative of significance.

2.3.4. Functional and pathways enriched analysis

We used Metascape (http://www.Metascape.org/) to conduct Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses will be used to physiologically and functionally characterize the genes in each herb-disease-target network and PPI network. Nominal p-values with a 0.05 threshold were used to rank functional activities and pathways.

2.4. Molecular docking

Structural information in three dimensions was acquired from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). Candidate targets were identified from the Protein Data Bank database (http://www.rcsb.org/) through searches on the UniProt database (https://www.uniprot.org/). Autodock tool 1.5.7 flexed its muscles for tasks like water clearance, hydrogenation, Gasteiger charge calculations, and target prep. Bioactive component data strutted in, donning their "pdbqt" attire. PyMOL 2.4.0 took the stage for a captivating visualization of binding conformations.

2.5. HPLC/ESI-MS analysis

An Agilent 1100 HPLC system was used for separation (Agilent Technologies, MA, USA). Samples were separated using a GS-120-5-C18-BIO chromatographic column (5 μm, 250 × 4.6 mm) at 35 °C. A linear gradient elution was performed using a mixture of 0.1 % formic acid in water (A) and acetonitrile (B) using the following gradient procedure: at 0 min, 5 % B was utilized, and at 60 min, the concentration of B was increased to 40 % (v/v). The rate of flow was 1.0 mL/min, and the amount injected was 10 μL. The DAD was activated, and the target wavelength was concurrently adjusted to 210 nm. The mass spectrometer had a split ratio of 1:3. The negative ion mode acquisition settings included the use of collision gas, ultrahigh-purity helium (He); nebulizer gas (N_2) at a pressure of 35 psi, at a flow rate of 10 L/min; drying temperature set at 350 °C; high voltage (HV) at 3500 V; mass scan ranges from m/z 100 to 2200; target mass at m/z 500; compound stability at 100 %; and trap drive level at 100 % [13]. The ChemStation software (Agilent Technologies, MA, USA) then stepped up to analyze the data.

2.6. Cell culture

HaCaT cells, sourced from the China Center for Type Culture Collection (CCTCC, Wuhan, China), were nurtured in DMEM supplemented with 10 % fetal bovine serum and 1 % penicillin/streptomycin from Wisent, QC, Canada. These cells, housed in 10-mm Petri dishes, underwent two passages post-thawing. For human palmoplantar warts tissue, it underwent a process of mincing and washing with a 1 % penicillin/streptomycin solution. Following this, a 30-min trypsin digestion with 25 mL of trypsin was performed, stopped by equal volumes of culture medium. After centrifugation, the supernatant was collected by filtering the remaining mixture through a 70-μm filter.

2.7. FISH assay

HPV2, HPV27 and HPV57 probe kits were used (Servicebio, Wuhan, China), and the probes are presented in Table S1. Paraffin-embedded sections were incubated at 56 °C overnight. Following overnight incubation, slides were subjected to routine dewaxing and underwent three rounds of 10 min in xylene and three rounds of 10 min in 100 % ethanol. Tissue specimens were then coated with coverslips and secured with rubber cement before placement in the hybridizer. Denaturation at 73 °C for 5 min, afterward overnight hybridization at 37 °C. The next day, specimens were retrieved, and rubber cement was removed. Subsequently, specimens underwent a 2 min wash in eluent (2 × SSC/0.3 % NP40) at 73 °C, followed by a 5 s wash in eluent at room temperature, and air-dried naturally. Contrast staining was achieved using diamidinophenylindole, and samples were observed under a fluorescence microscope.

2.8. Transmission electron microscopy

The materials were immobilized and incorporated as previously described [14]. In summary, samples of tissue were immersed in a 1 % solution of glutaraldehyde (Sigma–Aldrich, MA, USA) for a duration of 4 h, followed by immersion in a 1 % solution of osmium acid (Sigma–Aldrich, MA, USA) for a duration of 1 h. Following the process of dehydration using acetone, the specimens were then immersed in resin 12 (Ted Pella, USA) for embedding. A transmission electron microscope (JEOL, Japan) was used to inspect and capture thin slices (70 nm thick) that had been treated with 1 % uranyl acetate and 1 % lead citrate (Sigma-Aldrich, MA, USA).

2.9. Specimen collection and real-time quantitative PCR

Human PW tissues were procured from our hospital, with informed consent and approval obtained from the Ethics Committee of Yueyang Hospital (2022-008). HPV was extracted from verruca vulgaris tissue-infected HaCaT cells. The extraction of total RNA was performed using TRIzol reagent (Invitrogen, MA, USA). The synthesis of cDNA was performed using the PrimeScript RT Reagent Kit from TaKaRa, in Beijing, China. Subsequently, qPCR was performed using a SYBR Prime Script RT–PCR kit (TaKaRa, Beijing, China) following the instructions. PCR primers utilized are listed in Table S2.

2.10. Cell cycle analysis

Cells were initially seeded at a density of 1.5×10^6 cells in 6-cm diameter culture dishes and allowed to proliferate for 24 h. Subsequently, the growth medium was replaced with serum-free medium to induce cell cycle arrest. Following 16–18 h of serum starvation, the cells reached the G_0 phase of the cell cycle. Various experimental reagents were then introduced into the serum-free medium. Following the designated treatments, cells (1×10^6) were collected using trypsin digestion, washed twice with cold PBS, and fixed in 70 % alcohol on ice for 30 min. Following three further PBS washes, the cells were resuspended in 1 mL of Krishan staining solution and incubated overnight at 4 °C.

The following day, the cell suspension was filtered through a 96-μm pore size nylon mesh, and 10,000 stained cell nuclei were analyzed using flow cytometry (BD Biosciences, CA, USA). DNA histograms were constructed, and ModFit analysis software (BD Biosciences, CA, USA) was employed to fit the M phase of the G_0/G_1, S, and G_2 cell cycle fractions of cells in the G phase. Each experimental condition was meticulously replicated in triplicate to ensure the robustness and reproducibility of the findings.

2.11. Western blotting analysis

The proteins were obtained by extracting them using RIPA150 lysis solution, which was supplemented with 1 × protease inhibitor from Sigma-Aldrich, MO, USA. Afterward, the proteins were separated using sodium dodecyl sulfate–polyacrylamide electrophoresis gels and then transported onto polyvinylidene fluoride membranes (Bio-Rad, CA, USA). Next, the membranes were subjected to an overnight incubation at 4 °C, during which they were exposed to primary antibodies followed by appropriate secondary antibodies [15]. Specifically, rabbit monoclonal antibodies targeting crucial proteins were utilized: p53 (GB111740), CCNA1 (GB111132), CCNE1 (GB11305), CCND1 (GB111372), CDK4 (GB111602), CDK6 (GB111606), and CDK2 (GB112129). These antibodies were procured from Servicebio (Wuhan, China).

2.12. Statistical analysis

For this data synthesis, the R program "meta" was used. The MD with a 95 % CI is used to represent continuous variables, whereas

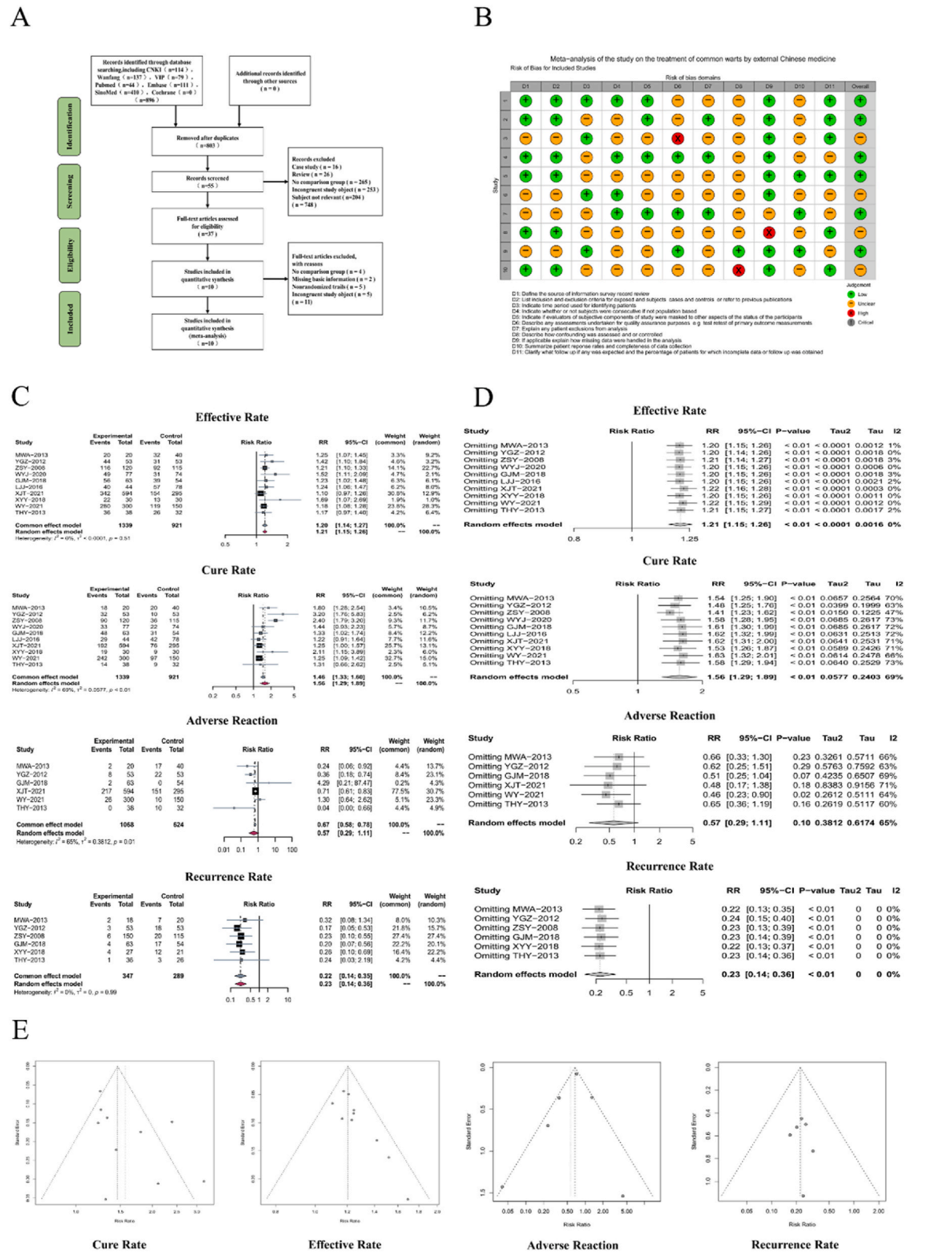

Fig. 1. Meta-analysis evaluating the efficacy of topical traditional Chinese medicine for PWs. (A). Flowchart of literature screening. **(B).** Quality assessment of included studies. **(C).** Effectiveness rate, cure rate, adverse reactions and recurrence rate. **(D).** Sensitivity analysis. **(E).** Publication bias analysis. PW: Palmoplantar wart.

the OR with a 95 % CI is used for dichotomous variables. For statistical significance, a two-tailed $P < 0.05$ was used. To assess heterogeneity among trials, Chi-square and I2 tests were conducted. Additionally, a reliable number was used to assess the extent of publishing bias. The experiments were replicated across four independent trials. Data analysis was performed using GraphPad Prism 8.0, and the results are depicted as the mean ± standard deviation (SD). Multiple sets of independent data were compared using one-way analysis of variance with Bonferroni post hoc correction to determine any differences. A significance level of $P < 0.05$ was deemed to indicate statistical significance.

3. Results

3.1. The efficacy of TCM in treating PWs

Ten randomized controlled trials (RCTs) were incorporated, all published in Chinese (Fig. 1A). Eight trials reported the use of acupuncture to treat PWs, while the remaining two reported the use of topical TCM. Table 2 outlines the 10 eligible RCTs. The sample sizes varied from 60 to 890 participants. In total, these 10 RCTs recruited 2260 participants, 1339 of whom received external TCM treatment as the intervention, while 921 participants received conventional therapy as the control. The period of therapy ranged from three weeks to two years, and the main outcome of all studies was the effectiveness of the treatment. Six out of the eight studies revealed drug-related side effects, such as skin allergies and pain. Moreover, six studies reported recurrence. Using the AHRQ cross-sectional study quality evaluation tool, the contained literature was deemed qualified. Risk of bias analysis is detailed in Fig. 1B. Heterogeneity tests revealed no statistical heterogeneity in group comparisons for efficacy ($P < 0.01$, $I2 = 62.4$ %), prompting the use of a random-effects model. Results showed a variation between groups (RR = 1.21, 95 % CI [1.15, 1.26], $Z = 7.93$, $P < 0.01$), indicating improved efficiency with external TCM treatment for common warts. Similarly, for cure rate, no statistical heterogeneity was observed ($P < 0.01$, $I2 = 69.2$ %), with vital differences between groups (RR = 1.56, 95 % CI [1.30, 1.90], $Z = 4.63$, $P < 0.001$), suggesting enhanced cure rates with TCM treatment. Regarding adverse reactions, statistical heterogeneity was present ($P < 0.01$, $I2 = 64.6$ %). However, no major difference was found between groups (RR = 0.57, 95 % CI [0.29, 1.11], $Z = -1.65$, $P = 0.098$), indicating comparable adverse effects between TCM and conventional treatments. For recurrence rate, statistical heterogeneity was observed ($P < 0.01$, $I2 = 74.6$ %), with a valuable difference between groups (RR = 0.27, 95 % CI [0.14, 0.35], $Z = -6.32$, $P < 0.01$), suggesting a reduction in recurrence with external TCM treatment.Overall, external TCM treatment demonstrated improvements in efficacy and cure rate while reducing adverse reactions and recurrence rates in palmoplantar wart treatment (Fig. 1C). No studies exhibited significant sensitivity (Fig. 1D). However, asymmetry was observed in the literature concerning cure rate and adverse reaction rate, indicating potential publication bias. Conversely, symmetry in efficacy rate and recurrence rate graphs suggested no publication bias (Fig. 1E). Detailed meta-analysis results are provided in Table S3.

3.2. Cases of successful treatment of PW by applying WRXYF

We used WRXYF in clinical practice at our hospital, which achieved good therapeutic effects. For patients who are unwilling to undergo laser or cryotherapy treatments, we recommend WRXYF for topical bathing therapy. The information of the 15 patients is presented in Table 3.

Photos of six cases are shown in Fig. 2. All patients experienced complete shedding of their warts within 3–4 weeks and reported no pain or discomfort after using the medication. Most of the patients achieved an elimination rate of greater than 80 % and reported no pain or discomfort during the follow-up, indicating the effectiveness of WRXYF in treating PWs.

3.3. Prediction of bioactive agents and targets by integrative network pharmacology

In the TCMSP database, 105 significant WRXYF components and 268 associated targets were determined. A total of 5434 DEGs were acquired. The junction of the WRXYF targets with the DEGs yielded 109 DEGs, comprising 44 upregulated and 65 downregulated

Table 2
Basic characteristics of the literature included in the meta-analysis: ① effective rate, ② cure rate, ③ recurrence rate, and ④ adverse reactions.

Study	Sample size		Randomization	Main outcomes
	TCM	C		
XJT–2021 [16]	594	295	Central randomized method	①②④
WY–2021 [17]	300	150	Convenience sampling method	①②④
WYJ–2020 [18]	77	74	N/A	①②
GJM–2018 [19]	63	54	Random number table	①②③④
XYY–2018 [20]	30	30	Random number table	①②③
LJJ–2016 [21]	44	78	N/A	①②
MWA–2013 [22]	20	40	N/A	①②③④
THY–2013 [23]	38	32	Random number table	①②③④
YGZ–2012 [24]	53	53	N/A	①②③④
ZSY–2008 [25]	120	115	Random number table	①②③

TCM: traditional Chinese medicine; C: conventional therapy; N/A: not applicable.

Table 3
Clinical cases of WRXYF treatment for PWs.

Patient	Sex	Age	Course of disease (months)	Location of warts	Previous treatment	Wart remission rate	Treatment period (weeks)
S01	Female	15	12	Fingers	Freezing	95 %	2
S02	Male	9	2	Feet	N/A	91 %	3
S03	Male	9	3	Face/fingers	CO_2 laser	75 %	4
S04	Female	1	1	Fingers	N/A	92 %	12
S05	Female	9	1	Feet	N/A	98 %	4
S06	Male	10	3	Fingers	Polymyxin injection	88 %	1.5
S07	Male	7	2	Fingers/feet	N/A	93 %	3
S08	Female	12	5	Fingers	CO_2 laser	94 %	4
S09	Male	24	13	Fingers	Freezing	90 %	6
S10	Female	56	38	Feet/fingers	Freezing/imiquimod/cidofovir/ polymyxin injection	81 %	2
S11	Male	39	26	Feet	Salicylic acid/fluorouracil/freezing	84 %	4
S12	Female	10	2	Feet	CO_2 laser	80 %	5
S13	Female	14	5	Feet	CO_2 laser/polymyxin injection	76 %	6
S14	Female	21	16	Neck	N/A	93 %	10
S15	Male	26	10	Feet	Imiquimod/polymyxin injection	86 %	12

WRXYF: Weiren Xiaoyou formula; PW: palmoplantar wart; N/A: not applicable.

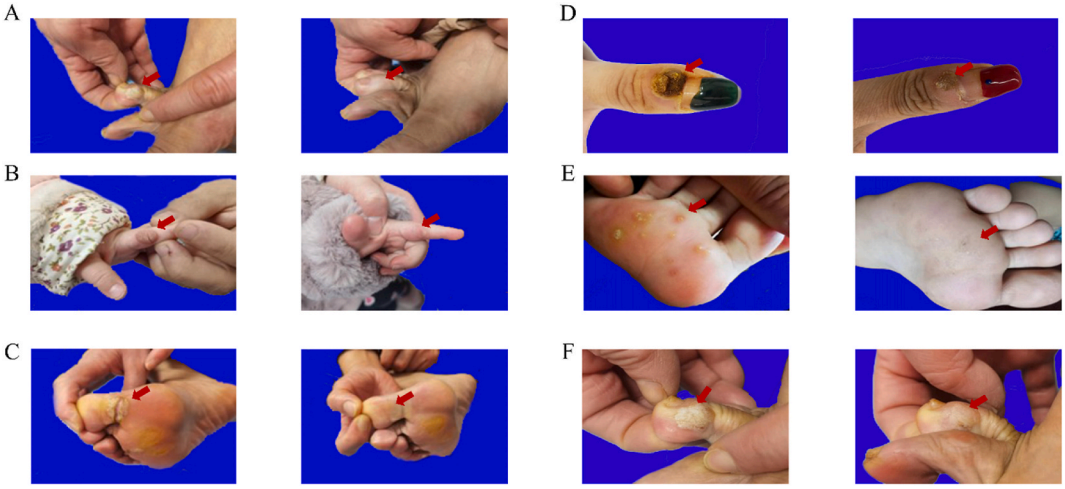

Fig. 2. Cases A–F represented the effect of WRXYF on PW. WRXYF: Weiren Xiaoyou formula; PW: Palmoplantar wart.

genes (Fig. 3A). To investigate the involvement of the targets in the illness, the intersecting genes were submitted to the STRING database, and a PPI structure with 109 sites and 1778 relationships was built, with a mean score value of 16.3. The 109 intersecting genes were subjected to GO enrichment and KEGG pathway analysis (Fig. 3B). Most DEGs in the GO BP category were engaged in processes linked to oxygen levels (GO:0070482), hypoxia (GO:0001666), reduced oxygen levels (GO:0036293), nutrition levels (GO:0031667), and steroid hormone (GO:0048545). In the GO CC category, membrane raft, membrane microdomain, membrane area, collagen-containing extracellular matrix, and transferase complex and phosphorus-containing groups were significantly enriched. The primary DEGs in the GO MF category were enriched in Ubiquitin-like protein ligase binding (GO:0044389), Endopeptidase activity (GO:0004175), G protein-coupled amine receptor activity (GO:0008227), Serine hydrolase activity (GO:0017171), and Serine-type endopeptidase activity (GO:0004252). KEGG pathway enrichment analysis revealed significant involvement in pathways such as the p53, Cellular senescence, HIF-1, TNF, IL-17, PI3K-Akt, VEGF, MAPK, Toll-like receptor, and NOD-like receptor signaling pathway. Subsequently, GSEA highlighted enrichment of DEGs in the mTOR, RIG-I-like receptor, and p53 signaling pathways (Fig. 3C). Additionally, a network depicting the interactions among WRXYF, chemicals, and targets was constructed (Fig. 3D). Through network analysis, seven active ingredients (Arachidonic acid, Beta-Sitosterol, Isorhamnetin, Kaempferol, Quercetin, Luteolin, and Tanshinone IIA) and ten potential target genes (ALB, CCL2, CXCL8, EGF, HIF1A, IL1B, MMP9, PTGS2, TP53, and VEGFA) were selected based on high nodal values and assurance. Utilizing AutoDockTools-1.5.7, molecular docking experiments identified associations between active WRXYF components and putative target genes. Molecular docking suggested that Tan IIA had docking scores ≤ -5.0 kJ/mol with targets, indicating strong binding interactions between Tan IIA and key genes in PWs (Fig. 3E).

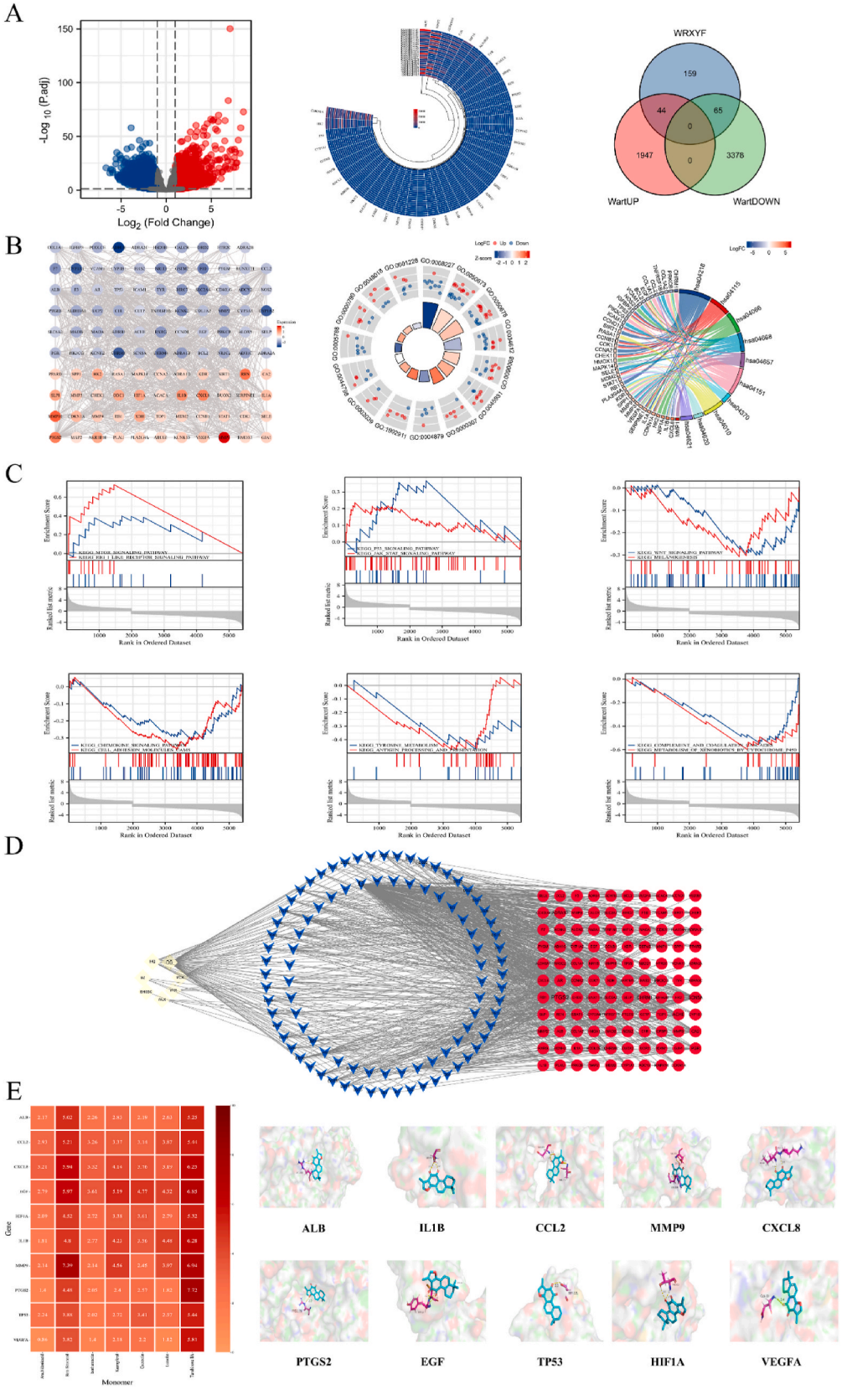

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Fig. 3. Effects of WRXYF on PW predicted by network pharmacology analysis. (A). Volcano map, circumferential heatmap and Venn diagram of DEGs. **(B).** PPI network, GO and KEGG analysis of DEGs. **(C).** Evolving signaling pathways predicted by GSEA analysis. **(D).** WRXYF-chemical composition-target network. **(E).** Molecular docking heat map of candidate compounds and docking relationship between Tan IIA and targets of PW. WRXYF: Weiren Xiaoyou formula; PW: Palmoplantar wart; PPI: protein-protein interaction; GO: gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; DEG: differentially expressed gene; GSEA: Gene Set Enrichment Analysis; Tan IIA: tanshinone IIA.

3.4. Validation of Tan IIA as the main bioactive agent in WRXYF

FISH was performed on normal and PW human samples, which revealed that the predominant HPV types involved were HPV2 and HPV27, with a relatively small proportion of HPV57 (Fig. 4A). The MeOH extract of WRXYF was measured by HPLC/ESI-MS in ion modes (Fig. 4B). Compounds from WRXYF have the retention time at 10.8 min, 13.9 min, 16.0 min, 17.1 min, 26.1 min, 28.1 min, 31.6 min, 2.1 min, 5.8 min, 8.7 min, 13.6 min, 14.2 min, 15.1 min, 16.9 min, 18.0 min, 18.8 min, and 21.8 min were identified as curcumol (**1**), lupenone (**2**), atractylenolide II (**3**), atractylenolide I (**4**), curdione (**5**), cryptotanshinone (**6**), tanshinone IIA (**7**), gallic acid (**8**), protocatechualdehyde (**9**), caffeic acid (**10**), jaranol (**11**), gingerone (**12**), astraisoflavan (**13**), rosmarinic acid (**14**), salvianolic acid B (**15**), salvianolic acid N (**16**) and miltirone (**17**) based on observation of the pseudomolecular ion peaks at *m/z* 235.1630 $[M+H]^+$ (**1**), *m/z* 447.1253 $[M+Na]^+$ (**2**), *m/z* 233.1439 $[M+H]^+$ (**3**), *m/z* 231.1356 $[M+H]^+$ (**4**), *m/z* 237.1861 $[M+H]^+$ (**5**), *m/z* 297.1531 $[M+H]^+$ (**6**), *m/z* 295.1315 $[M+H]^+$ (**7**), *m/z* 169.0149 $[M-H]^-$ (**8**), *m/z* 137.0299 $[M-H]^-$ (**9**), *m/z* 179.0377 $[M-H]^-$ (**10**), *m/z* 313.0720 $[M-H]^-$ (**11**), *m/z* 193.0504 $[M-H]^-$ (**12**), *m/z* 463.0908 $[M-H]^-$ (**13**), *m/z* 359.0824 $[M-H]^-$ (**14**), *m/z* 717.1534 $[M-H]^-$ (**15**), *m/z* 493.1188 $[M-H]^-$ (**16**), and *m/z* 281.1420 $[M-H]^-$ (**17**) in HPLC/ESI-MS. All 17 compounds were identified, as seen in Fig. S1 and in Table 4.

The copy numbers of the HPV subtypes 24 h after HPV infection in HaCaT cells are shown in Fig. 4C. Tan IIA significantly decreased the copy numbers of the three subtypes (Fig. 4D). In addition, electron microscopy revealed no HPV viral particles in HaCaT cells, but clear HPV viral particles were observed in HaCaT cells cocultured with HPV-infected primary cells (Fig. 4E). After treatment with Tan IIA, damage to HPV viral particles was evident, indicating that Tan IIA can disrupt the viral particles of the three HPV subtypes. FISH analysis revealed that Tan IIA reduced the copy numbers of HPV-2, HPV-27 and HPV-57 (Fig. 4F).

3.5. Tan IIA arrested the cell cycle in the HPV-infected cell model to treat PWs

Examination of cell cycle changes in the WT, HPV and Tan IIA groups ($n = 3$) revealed G_0/G_1 cell cycle arrest in the Tan IIA group, as shown in Fig. 5A, which confirmed the findings of the network pharmacology analysis and related reports [26].

RT–qPCR confirmed that the transcription amount of the p53 related genes previously identified through network pharmacology analysis and proteins associated with the G_0/G_1 stage of the cell cycle were significantly downregulated in the Tan IIA group (Fig. 5B). Tan IIA therapy increased levels of protein expression associated with cell cycle, such as CCNA1, CCNE1, CCND1, CDK2, CDK4 and CDK6 were significantly inhibited, while those of the p53 protein increased (Fig. 5C). These results collectively indicated that Tan IIA can regulate cell cycle proteins through the p53 signaling pathway, leading to cell cycle halt and cell proliferation inhibition in HPV-infected HaCaT cells, thereby exerting therapeutic effects on PWs.

4. Discussion

PWs constitute a chronic benign proliferative skin disease caused by infection with HPV, which is a small, envelopeless virus with circular DNA genome included in a coliseal structure. PWs exhibit a strong host specificity and display a pronounced affinity for skin or intricate mucosal epithelium [27]. In the presence of a compromised skin barrier, HPV targets cells of the infected epithelial basal layer [28]. The virus's life cycle is strongly related to host cell biology. HPV can exist in humans with inherent and acquired immunity, typically without symptoms or with benign symptoms [29]. TCM has certain advantages in the treatment of PWs, and topical treatment, including acupuncture, moxibustion, and TCM fumigation, is commonly used. In clinical practice, we have observed that topical WRXYF exhibits good therapeutic effects. Then, we investigated its therapeutic effects, bioactive components, and mechanism in treating PW.

The results of the meta-analysis summarise the clinical benefit and recurrence rates of external Chinese medicine methods for the therapy of palmopalmoplantar warts until 2022. This result demonstrates for the first time that traditional external Chinese medicine is a worthy means of clinical adoption, with the advantages of a low recurrence rate and low side effects. The cases also demonstrated the effectiveness of topical WRXYF use in treating PWs. To further investigate the mechanism of WRXYF in treating PWs, we subsequently screened 105 main bioactive components from WRXYF according to the TCMID database. In the network pharmacology analysis, 5435 DEGs in PWs and 109 targets were identified. Molecular docking further revealed seven bioactive components strongly associated with the top 10 targets in PWs, and tanshinone IIA was selected as a candidate agent. In the established HPV-infected cells, hyperexpression of three subtypes (HPV2, HPV27 and HPV57) was determined via RT–qPCR, but the copy number of the malignant subtype was not determined. *In vitro* studies conducted in established HPV-infected cells showed that Tan IIA could reduce the copy numbers and disrupt the viral particles of the three HPV subtypes. Further investigation revealed that Tan IIA inhibited the cell cycle at the G_0/G_1 phase.

Previous studies have shown that the HPV oncogenes E7 and E6 disrupt the cell cycle by mediating the degradation of the cell cycle regulatory factors p53 and Rb, providing a favorable environment for HPV replication and promoting the occurrence of epithelial

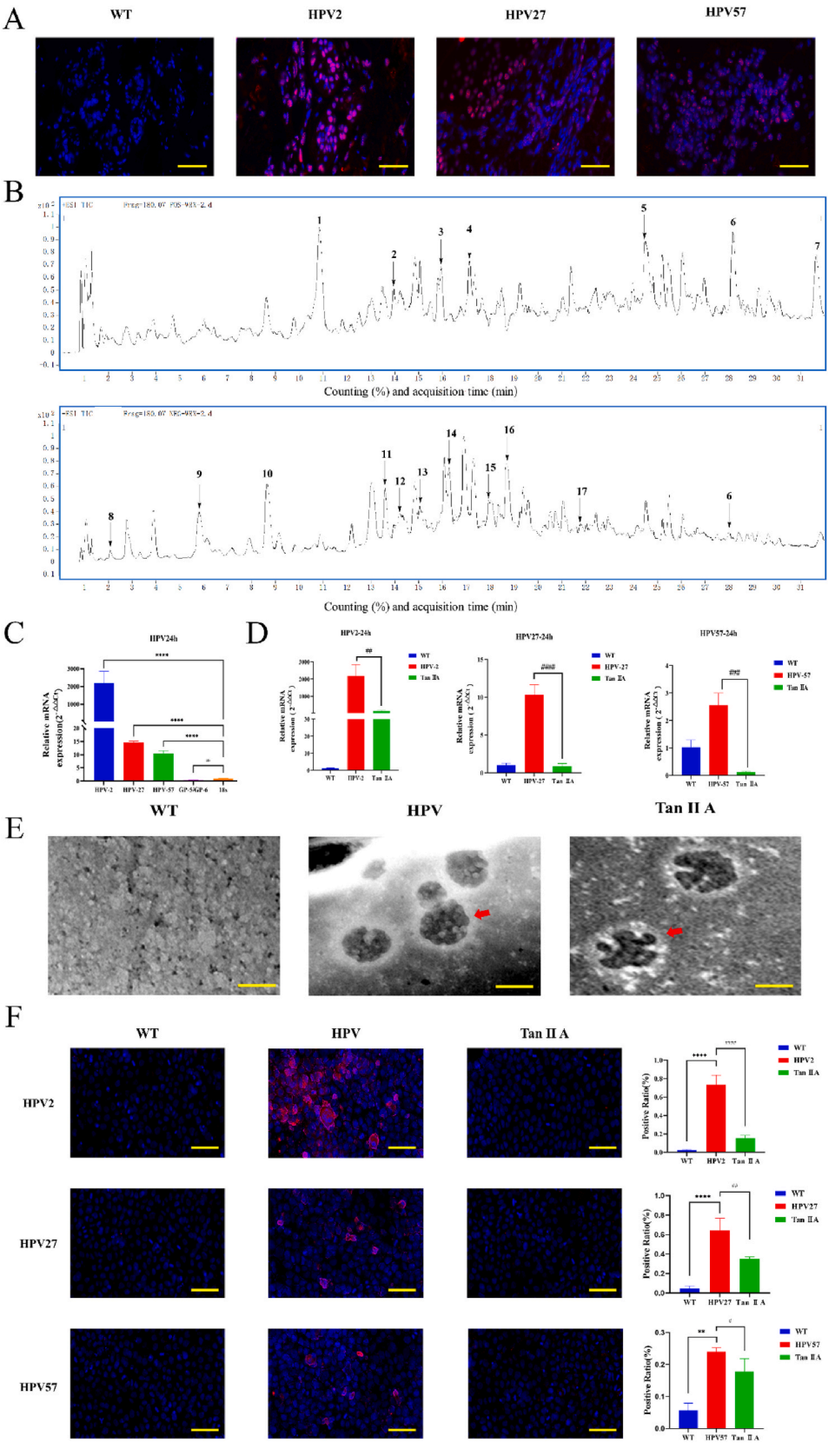

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Fig. 4. Screening of bioactive components against PW in HPV-infected HaCaT cells. (A). Results In situ hybridization for three HPV subtypes in human PW samples (magnification 400×, scale bar = 30 μm). **(B).** Identification of compounds in the water extract of WRXYF: curcumol (1), lupenone (2), atractylenolide II (3), atractylenolide I (4), curdione (5), cryptotanshinone (6), tanshinone IIA (7), gallic acid (8), protocatechualdehyde (9), caffeic acid (10), jaranol (11), gingerone (12), astraisoflavan (13), rosmarinic acid (14), salvianolic acid B (15), salvianolic acid N (16) and miltirone (17). **(C).** Copy numbers of HPV subtypes in the collected human PW tissue after 24 h. ns: no significance, ****P < 0.0001. **(D).** Tan IIA inhibited the copy numbers of three HPV subtypes determined by RT-qPCR. ##P < 0.01, ###P < 0.001, #####P < 0.0001. **(E).** Effect of Tan IIA on HPV viral particles observed under electron microscopy (magnification: 1500×, scale bar = 30 nm). **(F).** Inhibitory effect of Tan IIA on three HPV subtypes validated by FISH essay (magnification: 400×, scale bar = 30 μm), and the statistics were presented as mean ± SD (n = 3). WT vs. HPV groups: **P < 0.01, ****P < 0.0001. WT vs. Tan IIA groups: #P < 0.05, ##P < 0.01, ####P < 0.0001. PW: Palmoplantar wart; WT: wild-type; HPV: human papillomavirus; Tan IIA: Tanshinone IIA.

tumors [30]. p53 can also target the C-segment of the DNA-binding field of the E2 protein and thus inhibit viral replication [8]. The above studies showed that p53 is a protein that targets and inhibits viral replication. Moreover, tanshinone IIA was reported to induce ferroptosis in human gastric cancer cells by p53-mediated SLC7A11 downregulation [31]. It has the ability to suppress the growth and trigger programmed cell death in human nasal congestion cancer cells by the p53-cyclin B1/CDC2 pathway [32]. Therefore, we hypothesize that Tan IIA exerts its inhibitory effect on HPV and improves PWs by adjusting the p53 pathway.

The results of our study indeed corroborated this mechanism. The p53 signaling pathway protein TP53 and the cell cycle-related proteins CCND1, CDK1 and CCNB1 were significantly regulated. Moreover, the cell cycle-related kinases CDK2, CDK4, CDK6, CCNA1, CCND1 and CCNE1 were significantly downregulated after Tan IIA treatment. The WB observations further verified that in the Tan IIA group, G_0/G_1 phase-related proteins, except for p53 was downregulated, which overall suggested that Tan IIA may decrease cell growth by regulating the p53 pathway in PWs.

This study had certain limitations. Because all research involved in the meta-analysis were RCTs conducted in China, regional bias may exist. All studies showed favorable outcomes, which might indicate publication bias. Second, we conducted experimental validation only on Tan IIA, which was predicted to possess the best bioactivity in the network pharmacology analysis and was also one of the major components of WRXYF. As discussed earlier, Tan IIA resulted in considerable improvement in PWs, suggesting that other major compounds in this formulation, which were predicted to be bioactive, may also be worthy of further investigation. This research is the first to investigate the effectiveness and mechanism of TCM in treating PWs.

Our findings suggest that WRXYF offers a viable option for the painless and non-invasive treatment of palmopalmoplantar warts. In the future, we will conduct in-depth studies around the effects of the remaining monomers in WRXYF on HPV.

5. Conclusion

WRXYF is a topical Chinese medicine prescription that effectively treats palmoplantar warts in clinical practice. Long-term clinical trials have shown positive effectiveness in treating PWs. However, the pharmacological foundation and mechanism of its efficacy in treatment PWs still unexplored. A meta-analysis demonstrated the potential of TCM for PW treatment. Integrated studies have identified the promising bioactive agent Tan IIA from WRXYF and further confirmed that Tan AII in WRXYF could treat PWs by arresting the cell cycle through regulation of the p53 signaling pathway.

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Table 4
Compounds in WRXYF identified by HPLC-ESI/MS.

No.	Compound	Retention time (min)	Mass-to-charge ratio (*m/z*)
1	curcumol	10.8	235.1630 $[M + H]^+$
2	lupenone	13.9	447.1253 $[M + Na]^+$
3	atractylenolide II	16.0	233.1439 $[M + H]^+$
4	atractylenolide I	17.1	231.1356 $[M + H]^+$
5	curdione	26.1	237.1861 $[M + H]^+$
6	cryptotanshinone	28.1	297.1531 $[M + H]^+$
7	tanshinone IIA	31.6	295.1315 $[M + H]^+$
8	gallic acid	2.1	169.0149 $[M - H]^-$
9	protocatechualdehyde	5.8	137.0299 $[M - H]^-$
10	caffeic acid	8.7	179.0377 $[M - H]^-$
11	jaranol	13.6	313.0720 $[M - H]^-$
12	gingerone	14.2	193.0504 $[M - H]^-$
13	astraisoflavan	15.1	463.0908 $[M - H]^-$
14	rosmarinic acid	16.9	359.0824 $[M - H]^-$
15	salvianolic acid B	18.0	717.1534 $[M - H]^-$
16	salvianolic acid N	18.8	493.1188 $[M - H]^-$
17	miltirone	21.8	281.1420 $[M - H]^-$

WRXYF: Weiren Xiaoyou formula; HPLC-ESI/MS: high-performance liquid chromatography with electrospray mass spectrometry.

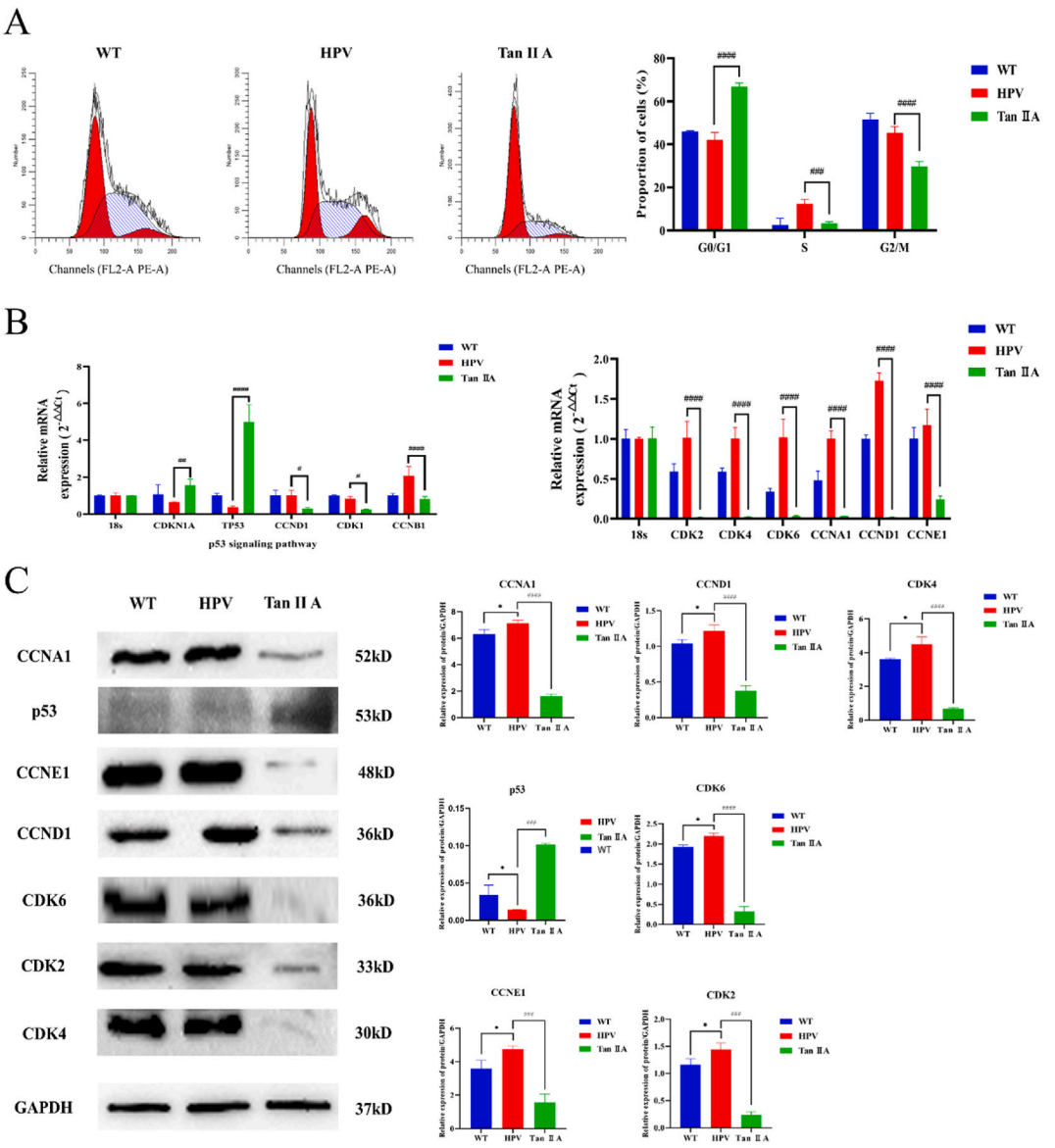

Fig. 5. Tan IIA affected the cell cycle in HPV-infected HaCaT cells. (A). Tan IIA increased the proportion of the G0/G1 phase. ###P < 0.001, ####P < 0.0001. **(B).** The RT-qPCR results showed that Tan IIA regulated the expression of p53 signaling pathway and cell cycle-related proteins (mean ± SD, n = 3). #P < 0.05, ##P < 0.01, ####P < 0.0001. **(C).** WB validated the regulation of the expression of cell cycle-related proteins by Tan IIA. WT vs. HPV groups: *P < 0.05, HPV vs. Tan IIA groups: ###P < 0.001, ####P < 0.0001. WT: wild-type; Tan IIA: tanshinone IIA; HPV: human papilloma virus; RT-qPCR: real-time quantitive polymerase chain reaction; WB: Western blotting; CCNA1: cyclin A1; CDKN1A: cyclin-dependent kinase inhibitor 1A; P53: Tumor Protein P53; CCND1: cyclin D1; CCNE1: cyclin E1; CDK2: cyclin dependent kinase 2; CDK4: cyclin dependent kinase 4; CDK6: cyclin dependent kinase 6.

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Data availability statement

Data from our research has been posted in a public repository.

The components of WRXYF were sourced from TCMSP, a system pharmacology platform developed for the purpose of conducting thorough studies on TCM (http://tcmspw.com/tcmsp.php).In order to get the verruca vulgaris targets, we obtained the datasets (GSE72140) from the GEO database.

CRediT authorship contribution statement

Xin Liu: Writing – review & editing, Writing – original draft, Data curation, Conceptualization. **Ruofan Xi:** Data curation, Conceptualization. **Linyan Cheng:** Formal analysis. **Yi Wang:** Writing – original draft. **Yiwen Nie:** Writing – review & editing. **Ge Yan:** Writing – review & editing. **Dongjie Guo:** Formal analysis. **Wanjun Guo:** Software. **Ting Du:** Writing – review & editing. **Hanzhi Lu:** Writing – review & editing. **Peiyao Wang:** Formal analysis. **Jianyong Zhu:** Writing – review & editing. **Fulun Li:** Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e31376.

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