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Identification of the Anti-Atherosclerotic Properties of Benzoinum through Integrative Network Pharmacology and Pharmacological Analyses

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Abstract Benzoinum is a resin derived from Styrax tonkinensis bark that has long been used in clinical settings as a traditional Chinese medicine with cardioprotective properties. The mechanistic basis for these pharmacological properties, however, remains to be demarcated. We used a network pharmacology approach to characterise the mechanism of action that benzoinum used to treat AS. The PharmMapper database was used to find potential benzoin target proteins, and the TCMSP, CTD, TTD, GAD, drug Bank, and pharmGkb databases were used to identify AS-related target genes. Following assessments of pathway enrichment, the functional roles of these genes were discovered, allowing the creation of compound-target and target-pathway networks. HUVEC cells were then used to validate these predicted benzoinum-related mechanisms of action, revealing that an active derivative of this resin was able to modulate signaling activity in TNF-α-exposed HUVECs by regulating the caspase-9 and NF-κB signaling pathways. Overall, our predictive network pharmacology analyses and supporting data highlighted the potential mechanisms whereby benzoinum may exert cardiovascular activity in the context of AS.

Keywords: *network pharmacology, atherosclerosis, cell apoptosis, inflammatory*

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1. Introduction

AS is a major reason for cerebrovascular and cardiovascular mortality and morbidity worldwide. [1,2] While statins have been developed and are widely prescribed to treat AS and hyperlipidemia, they are associated with potentially serious gastrointestinal, muscular, and hepatic adverse effects. [3] There is thus an urgent need for the development of alternative AS treatments. Natural compounds appear to be a promising source for the development of state-of-the-art clinical agents, with over 83% of cancer drugs developed from natural sources. [4] Benzoinum is a resin derived from the bark ofthe Styrax tonkinensis (Pier.) Craib tree found throughout Southeast Asia. Owing to its purported anti-inflammatory and anticancer characteristics, benzoinum is often implemented for the therapy of AS and other cardiovascular diseases. [5,6,28,29] The mechanistic basis for the activity of this resin, however, remains to be established.

The relevant components of TCM can have either antagonistic or synergistic effects. TCM is a complicated system with numerous targets. [7] As a result, TCM research may not be able to use traditional pharmacological techniques for identifying its distinct mode of action through tests. A powerful tool for defining the strategy of complex pharmaceutical systems in great detail, from the molecular level to the pathway level, is the emerging area of network pharmacology, which is based on enormous datasets and driven by advancements in bioinformatics. [8] Network pharmacology is congruent with the core concepts of Chinese medicine's holistic philosophy. [7] As a result of the new study approach, the research concept is updated from a "single target, single drug" to a "network target, multi-component" model. The network of compounds, targets, and target pathways aids in determining the rationale and compatibility of TCM.

To develop an understanding of the potential active constituents and pharmacological targets associated with the benzoinum, we here in sought to conduct a series of network pharmacology and systems biology analyses as these approaches have previously been leveraged to successfully clarify the activity. Herein, we employed a series of bioinformatics approaches to attempt to clarify the pharmacological mechanisms whereby benzoinum influences specific genes and pathways in the framework of AS. Additionally, using a TNF-treated HUVEC model, we were able to screen for active benzoinum-derived compounds, which led to the chromatographic separation of a brand-new natural product that we have given the working name of Stybenpropol B (The compound is No.9). Stybenpropol B treatment of HUVECs was sufficient to significantly inhibit TNF-induced cellular damage, possibly through mechanisms linked to the NF-B/caspase-9 signalling pathways that were discovered through our network pharmacology investigations.

The arrangements of the paper are as follows: Section 2 defines the materials and methods. Section 3 examines the experimental result validation. Section 4 evaluates the results. Sections 5 analyze the experimental Validation. Section 6 discusses the discussion. Section 7 concludes an article.

2 Materials and Methods

2.1. Reagents

Benzoinum was obtained from Bo Zhou, Anhui Province. Compounds were separated from benzoinum that had been extracted with EtOH (95%), and their structures were determined using mass spectrometry and NMR. HPLC was used to determine that the chemicals' purity was greater than 98 percent. The ECM (which included basal medium for HUVECs, bovine endothelial cell growth supplement (ECGS) growth factor, streptomycin, and penicillin) was provided by ScienCell Biotechnology (Carlsbad, CA, USA); trypsin (0.25 percent), PBS, and foetal bovine serum (FBS), were purchased from Thermo Fisher Scientific; and Peprotech (Princeton, NJ, USA) provided the recombin (Waltham, MA, USA). The Japan Tongren Company provided the CCK-8 reagents (Dojindo, Japan). The kits of NO were supplied by Nanjing Institute of Bioengineering. The ICAM-1, VCAM-1, IL-8, and IL-Iβ ELISA kits were provided through Wuhan Cloud-Clone CORP (Wuhan, China). ICAM-1 antibody of rabbit, VCAM-1 antibody of mouse, IκB-α antibody of rabbit, IKK-β antibody of rabbit, Bcl-2 antibody of mouse, caspase-9 antibody of rabbit, and Bax antibody of mouse, were obtained from Proteintech (Sanying, Wuhan, China). Beyotime (Shanghai, China) provided the annexin-FITC kit for detecting apoptosis.

1. Triterpenes [8,27,29]:

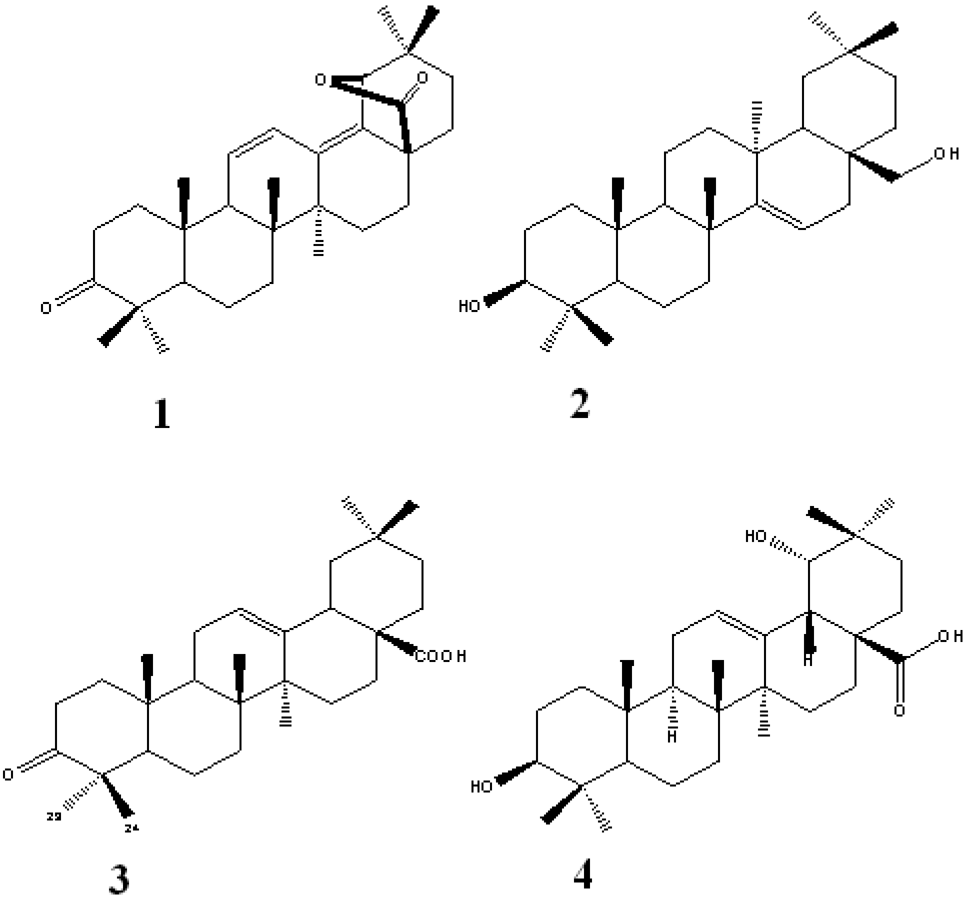


Figure 1-1. Structures of compounds 1-4

2. Phenylbutane derivatives [10]:

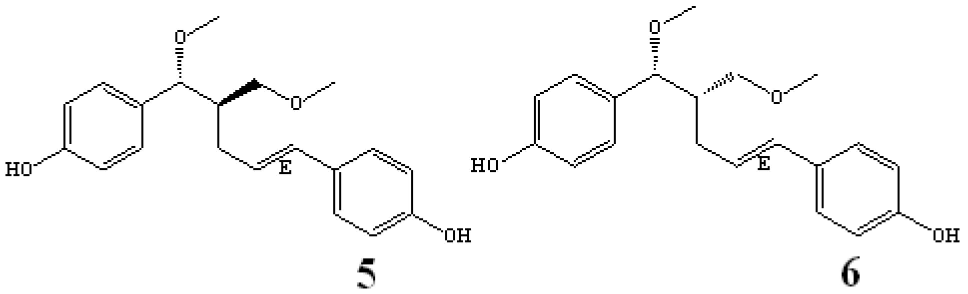


Figure 1-2. Structures of compounds 5-6

3. Phenylpropane derivatives [8,9,28]:

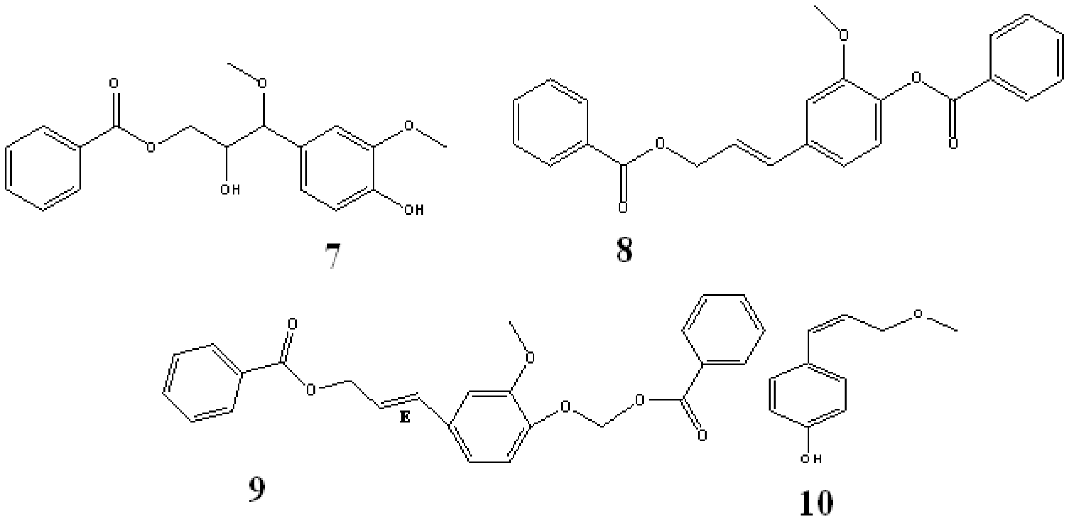


Figure 1-3. Structures of compounds 7-10

4. lignanoids [10]:

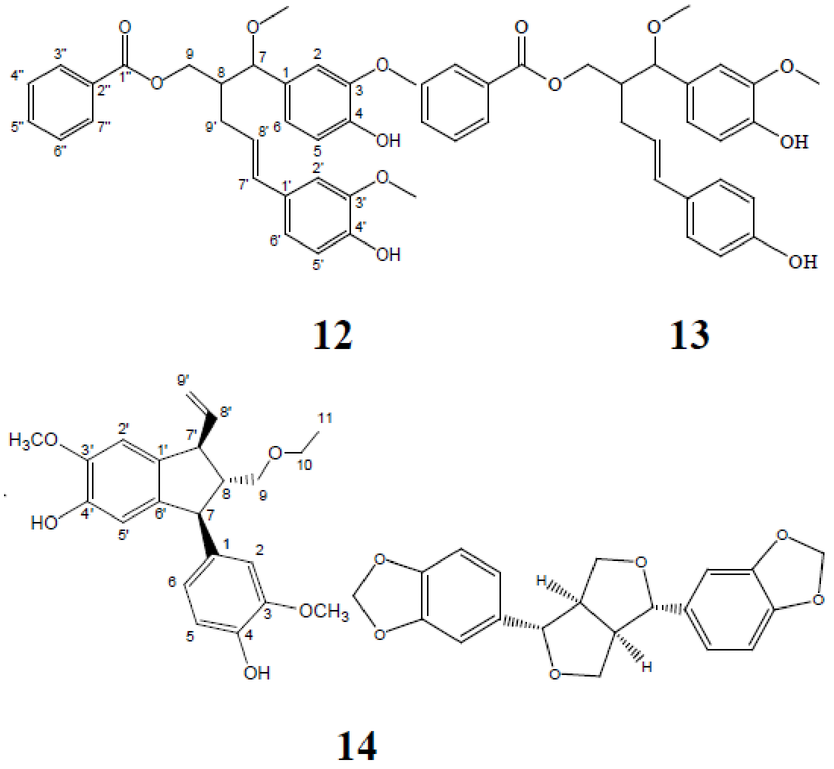
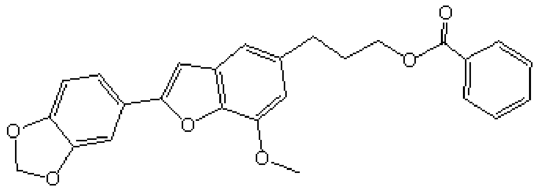


Figure 1-4. Structures of compounds 11-14

5. Stilbenes [8]:



15

Figure 1-5. Structures of compounds 15

6. Another [8]:

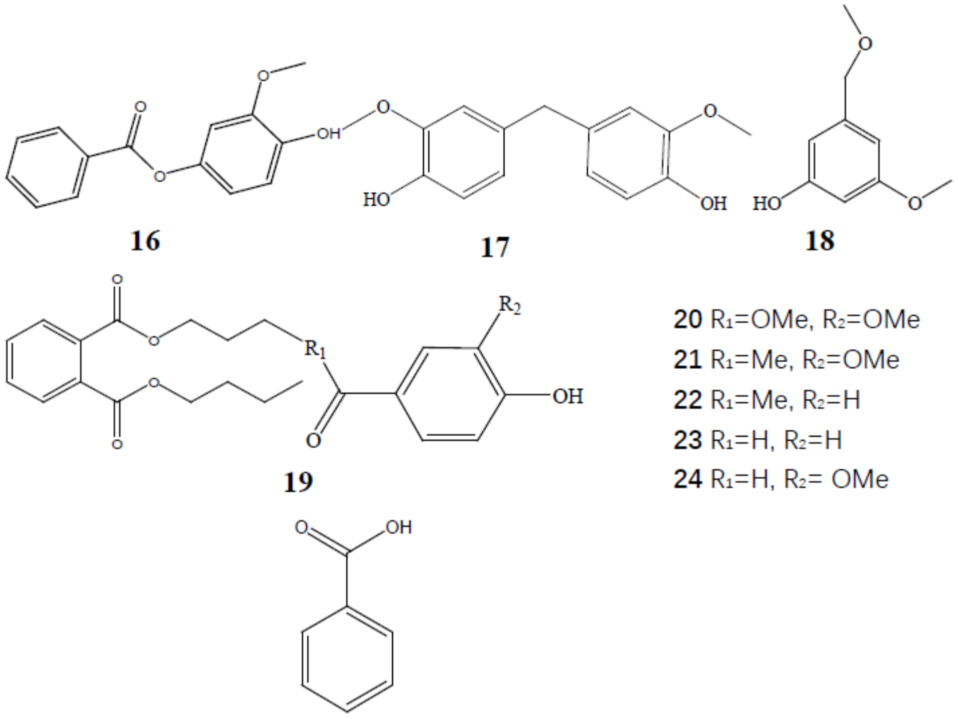


Figure 1-6. Structures of compounds16-25

The names of 25 components

1	3-oxo-olean-11，13(18) -dien-28，19β-olide
2	Myricadiol
3	3-keto-oleanonic acid
4	Tara resin acid
5	(4E) -1，5-bis (4-hydroxyphenyl) -1-methoxy-2-(methoxy-methyl) -4-pentene(1S,2R)
6	(4E) -1，5-bis (4-hydroxyphenyl) -1-methoxy-2-(methoxy-methyl) -4-pentene (1S,2S)
7	Stytonkinol A
8	Stybenpropol A
9	Stybenpropol B
10	(E) -p-coumaryl alcohol γ-O-methyl ether
11	2-[(4-hydroxy-3-methoxyphenyl)-methoxymethyl]-5-(4-hydroxy-3-methoxyphenyl)-pent-4-enyl benzoate
12	2-[(4-hydroxy-3-methoxy-phenyl)-methoxymethyl]-5-(4-hydroxyphenyl)-pent-4-enyl benzoate
13	1-(3-hydroxy-4-methoxyphenyl)-2-ethoxymethyl-3-vinyl-6-methoxyindan-5-ol
14	Sesamin
15	5-(3″-ben-zoyloxypropyl) -7-methoxy-2-(3'，4'-methylenedioxyphenyl) –benzofuran
16	1,4-benzenzenedol,2-methoxy-,4-benzoate
17	Bis(4-hydroxy-3-methoxyphenyl)methane
18	3-methoxy-5-(methoxy methyl)phenol
19	Dibutyl phthalate
20	Vanillic acid methyl ester
21	p-hydroxy benzaldehyde
22	Para hydroxyacetophenone
23	Vanilla ethyl ketone
24	Vanillic aldehyde
25	Benzoic acid

2.2. Network Pharmacology-Based Analysis:

Initially, compounds in benzoinum preparations were extracted and separated, leading to the identification of 25 total compounds through a combination of column chromatography and spectral data analysis.

2.3. Benzoinum Drug Target and AS-related Gene Target Predictive Analyses

The 25 benzoinum-derived compounds were discovered, and the structures corresponding to these compounds were generated using the ChemBiodraw tool. These structures were then rendered in 3D using the ChemBio3D programme. Then, based on the 3D structural characteristics of these chemicals, the PharmMapper database was used to discover probable protein targets of these substances. Genes related to human AS were identified through analyses of the TCMSP, CTD (http://ctdbase.org/), TTD http://bidd.nus.edu.sg/group/cjttd/TTD_HOME.asp), GAD, drugBank, and PharmGKB (https://www.pharmgkb.org/) databases. [11] The targets of metabolites and prototypes with a probability more than 0.5 in the Swiss Target Prediction, a Z score greater than 1 in the PharmMapper, a score greater than 20 in the BATMAN, and all targets in TCMSP were chosen. All targets were summarized and UniProt database was employed for standardizing the names of the gene for all targets via the limitation of the species to "Homo sapiens".

2.4. Pathway Enrichment Analyses of AS-Related Benzoinum Targets

The DAVID Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov/) On the basis of functional annotations tool, Gene Ontology (GO) was carried out and pathway enrichment analyses of AS-related benzoinum target genes.[12]

2.5. Network Construction and Analysis

To gain more detailed insights regarding the mechanisms whereby benzoinum may influence AS, Cytoscape 3.3.0 was used for compound-target and target-pathway network construction, with compounds, proteins, and pathways being represented by nodes within the resultant networks, while interactions between these nodes were represented by edges. [13]

3. Experimental Result Validation

3.1. Cell Culture

HUVECs (P_0) were obtained from AllCells (VA, USA) and cultured in ECM media containing FBS (10%), ECGS of 10% (Sigma-Aldrich, MO, USA), and streptomycin/penicillin in an incubator set to 5% of CO_2 and 37°C with cells being used following 3-8 in vitro passage. [14]

3.2. Cell Viability Assay

The influence of **Stybenpropol B** therapy on HUVEC viability was determined by plating these cells in 96-well plates (7×10^3/well) overnight, and then treating them with a range of phenyl propane derivatives absorptions for 24 h, Following that, 10 µL CCK-8 reagent was poured into each well. Following an additional incubation for at 37°C 2 h, a 450 nm absorbance value was detected using a microplate reader (Bio-Rad, USA), and survival was quantified as a percentage of untreated cell viability. [14]

3.3. NO Level Analysis

A Nitrate/Nitrite Fluorometric Assay kit (Cayman Chemical Company, MI, USA) was utilized for the assessment of NO production via a nitrate reductase method based on provided directions. [15]

3.4. ELISAs

IL-8, IL-1β, VCAM-1 and ICAM-1 protein levels were evaluated in cell lysates employing directions provided with the ELISA kits detailed above. [16]

3.5. Apoptosis Analysis

Apoptotic HUVEC death was evaluated using Annexin V-FITC (Sigma-Aldrich; Merck KGaA)/PI (Sigma-Aldrich; Merck KGaA) dual-staining. Briefly, cells were further to 6-well plates ($2x10^6$/well) and treated as above, after which they were stained for 10 min with Annexin V-FITC (5 µl) followed by a 15 min stain with PI (5 µL) at ambient temperature in the dark. Following that, samples were processed employing a FACS Calibur flow cytometer (BD Biosciences) and the resultant outcomes were evaluated employing Cell Quest computer program (v5.1). [17]

3.6. Western Blotting

Following treatment for 24 h with a range of concentrations of **Stybenpropol B** (0-100 µM), HUVECs were processed for 12 h with TNF-α (10 ng/ml), and a Cytoplasmic Protein Extraction kit (Beyotime Institute of Biotechnology, Shanghai, China) was then utilized for protein extraction. Protein samples equivalent (20 µg) were subsequently separated via SDS-PAGE (8-10%) and added to membranes of PVDF that had been blocked for 1 h with VSA and next, the primary antibodies described above were used to probe the membrane. Chemi luminescent protein band detection was then performed, with the Quantity One computer program (Bio-Rad, CA, USA) being used to quantify band density. [7]

3.7. Statistical Analysis

The outcomes are the means ± SEM of 3 or more independent studies, and were evaluated by one-way ANOVAs or Student's t-tests using SPSS. $P < 0.01$ was regarded meaningful in this work.

4. Results

4.1. Identification of AS-related Benzoinum Targets

The Pharmmapper database was first used to formulate a list of candidate targets for the 25 compounds tentatively isolated from benzoin extracts. The TCMSP, CTD, drugbank, TTD, GAD, and PharmGKb databases were then queried to identify 227 AS-related target genes (Table 1). The overlap between these two target lists was then mapped using the TTD, CTD, and PharmGKB database tools, leading to the identification of 35 shared AS-related targets of the 25 compounds identified in benzoin extracts (Table 2).

Table 1. CTD/Drugbank/TTD/GAD/PharmGKbonline database to arrange to get the AS targets (227)

TNF	JμN	ATHS	FGFR1	MMP9	CCL4	LDLR	CYBA	CASP9	SOSTDC1
IL6	FOS	TSC1	SQLE	SCARB1	SIRT1	PON1	ESR1	CYP1A1	TYS
PTGS2	NFE2L2	ALS2CR12	F10	PPARG	IGF2	PPARG	AHR	GPT	SSCA1
CCL2	CDKN1A	OTSC1	HTR1A	MTTP	AGER	ADIPOQ	GSTM1	NCF1	EXOSC9
NOS2	CXCL8	OTSC3	HTR2B	ALOX15B	SOCS3	PLAT	MMP1	LEP	EXOSC10
VEGFA	PPARA	OTSC6	ADORA2A	PLA2G4A	ALOX5	NR1H4	BMP8A	TRPV1	CES2
ICAM1	NFKBIA	OTSC9	LTA4H	CELA2A	PLA2G2A	APOA4	LPA	MMP2	Rxrb
VCAM1	IL10	OTSC4	PCSK9	PPARD	ALOX5AP	NPC1	SELP	EDN1	CCN3
AGT	CPT1A	OTSC7	ADRB1	CASP3	HSPA1B	ALOX15	NOS3	AKT1	BCL2
APOE	INS1	OTSC5	ITGA2B/ITGB3	MPO	ABCG5	CETP	SOD2	HMOX1	MAPK3
IFNG	NFKB1	MPV17	LCAT	F3	HP	CNR2	CCL3	CCN2	CARF
SERPINE1	TP53	MS	MAPK14	MMP12	BCL3	IL1B	FASLG	APP	C2CD6
ABCA1	ACTA2	ALS5	PCSK9	NR1H2	CYP27A1	RELA	STAT3	FAS	SOST
PARP1	HMGCR	ALS3	APOC3	PLA2G2A	HRH1	CAT	TSC2	MYC	CES
SOD1	FASN	ALS2	LPA	PAFAH1B2	TTPA	MAPK1	RAPH1	FGF1	NR1H3
TLR4	CCND1	WRN	SREBF1	CFB	GRIA	p38	PK	APOC2	NECTIN2
LMNA	COG2	LDLRAP1	ELN	FBN1	APOA2	LOX	APOA5	ABCG1	EPHX2
APOA1	PLTP	TGFBR2	INS	ENPP1	CYP7A1	SREBF2	F2	TGFB2	SERPINC1
APOB	TGFBR1	BAX	ACE	CDKN2B-AS1	CRP	PRKG1	VWF	ITGB3	LIPC
ABCG8	PECAM1	APOC3	SPP1	PLA2G7	SELE	CD14	AGPAT2	OLR1	BGN
BMP2	TGFB1	ALB	THBD	SMAD3	LIPA	MFAP5	BSCL2	MAPT	ANGPTL3
LPL	ZMPSTE24	MYH11	SCARB1	NOTCH3	MYLK	RETN	ENG	CAV1	COL3A1
MTHFR	CXCL2	TNFAIP3	TNFRSF1A	BCL2A1	BCL2L1	PIDD1			

Table 2. Target genes of the intersection of benzoin and AS diseases

NO	Gene	Gene description	ID
1	BMP2	Bone morphogenetic protein 2	P12643
2	ALB	Serum albumin	P02768
3	MAPK1	Mitogen-activated protein kinase 1	P28482
4	F2	Prothrombin	P00734
5	PPARG	Peroxisome proliferator-activated receptor gamma	P37231
6	CASP3	Caspase-3	P42574
7	MAPK14	Mitogen-activated protein kinase 14	Q16539
8	ESR1	Estrogen receptor;	P03372
9	NOS3	Nitric oxide synthase	P29474
10	F10	Coagulation factor X	P00742
11	NR1H4	Bile acid receptor;	Q96RI1
12	CXCL2	C-X-C motif chemokine 2	PStybenpropol B875
13	CXCL8	Interleμkin-8	P10145
14	IL1B	Interleμkin-1 beta	P01584
15	CASP9	Caspase-9	P55211
16	TNFAIP3	Tumor necrosis factor alpha-induced protein 3	P21580
17	CCL4	C-C motif chemokine 4;	Q8NHW4
18	ICAM1	Intercellular adhesion molecule 1	P05362
19	PARP1	Poly [ADP-ribose] polymerase 1	P09874
20	PTGS2	Prostaglandin G/H synthase 2;	P35354
21	TLR4	Toll-like receptor 4;	O00206
22	TNF	Tumor necrosis factor;	P01375
23	VCAM1	Vascular cell adhesion protein 1	PStybenpropol B320
24	FASLG	Tumor necrosis factor ligand superfamily member 6	P48023
25	BCL2	Apoptosis regulator Bcl-2	Q16548
26	SOD2	Superoxide dismutase [Mn],	P04179
27	FGFR1	Fibroblast growth factor receptor 1	P11362
28	GSTM1	Glutathione S-transferase Mμ 1	P09488
29	ACE	Angiotensin-converting enzyme;	Q9BYF1
30	TNFRSF1A	Tumor necrosis factor receptor superfamily member 1A;	P19438
31	BCL2A1	Bcl-2-related protein A1	Q16548
32	BCL2L1	Bcl-2-like protein 1	Q07817
33	NFKBIA	NF-kappa-B inhibitor alpha	P25963
34	PIDD1	P53-indμced death domain-containing protein 1	Q9HB75
35	RELA	Transcription factor p65	Q04206

Table 3. Potential AS target genes of benzoin chemical constituents

Compound	Gene										number
1			F2	CXCL8	BMP2	MAPK1	ALB				5
2			BMP2	ALB	BCL2A1	BCL2L1	MAPK1				5
3			BMP2	ALB	F2	BCL2L1	PPARG				5
4					BMP2	ALB					2
5				F2	CXCL8	NFKBIA	ALB				4
6			F2	ESR1	MAPK1	VCAM1	BMP2				5
7	NFKBIA	BMP2	PPARG	ESR1	F2	NOS3	MAPK14	FGFR1	ALB	ICAM1	10
8	MAPK1	ALB	PIDD1	CXCL8	VCAM1	F2	CASP3	ESR1	MAPK14	CCL4	15
			ICAM1	PARP1	PTGS2	TNF	CXCL2				
9	CASP3	PIDD1	VCAM1	F2	MAPK1	F10	MAPK14	CCL4	TNF	TLR4	13
				BCL2	CASP9	NFKBIA					
10		PPARG	SOD2	MAPK14	ALB	F10	ESR1	F2	FGFR1	TNFAIP3	9
11				CASP3	F2	ESR1	CXCL8				4
12			CFB	ESR1	F2	MAPK14	IL1B				5
13				F2	FASLG	ESR1					3
14					CASP3	MAPK1					2
15					CFB	ESR1					2
16			ESR1	CXCL8	F2	CCL4	ICAM1	PARP1	PTGS2		7
17					BMP2						1
18					SOD2						1
19				F2	FASLG	MAPK1	CFB				4
20					TNFRSF1A	F2					2
21					TNFRSF1A						1
22				SOD2	RELA	MAPK14	ICAM1				4
23				PIDD1	MAPK14	GSTM1	ICAM1				4
24					SOD2	CASP9					2
25				ACE	MAPK14	CASP9					3

4.2. Compounds-Target Network Analysis

Given that we recognized multiple pharmacological properties associated with multiple components of benzoinum, we next sought to better comprehend the underlying mechanisms governing these complex regulatory interactions. To that end, we construct a compound-target network corresponding to the benzoinum-mediated treatment of AS (Table 3). The resultant network incorporated 60 nodes, including 35 protein targets and 25 candidate bioactive compounds. Four of these bioactive benzoinum-derived compounds, which were identified as phenylpropanoids, exhibited the highest degree values, suggesting that they may be essential to the therapeutic efficacy of benzoinum as a treatment for AS.

4.3. Functional Enrichment Analysis

Next, GO and pathway enrichment analyses were carried out to more thoroughly understand the biological functions of identified AS-related targets of benzoinum. These analyses revealed that these targets are enriched in the GO categories molecular function, cellular component, and biological system, Overall, these GO terms suggested that benzoinum may target signal receptors, cytokine receptors, enzymes, the lipopolysaccharide response, and the extracellular portion of membrane rafts to exert therapeutic efficacy in the context of AS. Subsequent KEGG pathway analyses further revealed these targets to be enriched in specific pathways

4.4. Target-Pathway Network Analyses

Individual genes and proteins do not influence cellular physiology or pharmacological activity in isolation, and instead function in dynamic, interactive molecular pathways (Figure 2). To better understand how benzoinum and its derivatives can influence AS, therefore, we developed a network of target-pathway comprising all identified proteins and their relevant signaling pathways (Figure 3). The resultant network was composed of 80 nodes (35 protein nodes; 20 pathway nodes), of which the NF-κB pathway exhibited the highest degree value. F2, CASP9, CASP3, ESR1, NFKBIA, ICAM1, BCL2, MAPK14, TNF, TLR4, CXCL8, and IL1B were identified as potential high-degree protein targets associated with this pathway. Since these targets were connected to cell proliferation, they were chosen as potential biomarkers of benzoinum-mediated AS treatment. The cardioprotective effects of benzoinum in the setting of AS may be related to its capacity to affect important targets like F2, CASP9, CASP3, ESR1, NFKBIA, ICAM1, BCL2, MAPK14, TNF, TLR4, CXCL8, and IL1B, according to these integrated network, target prediction, and network analyses, thereby influencing cellular survival and proliferation (Figure 4 and Table 4).

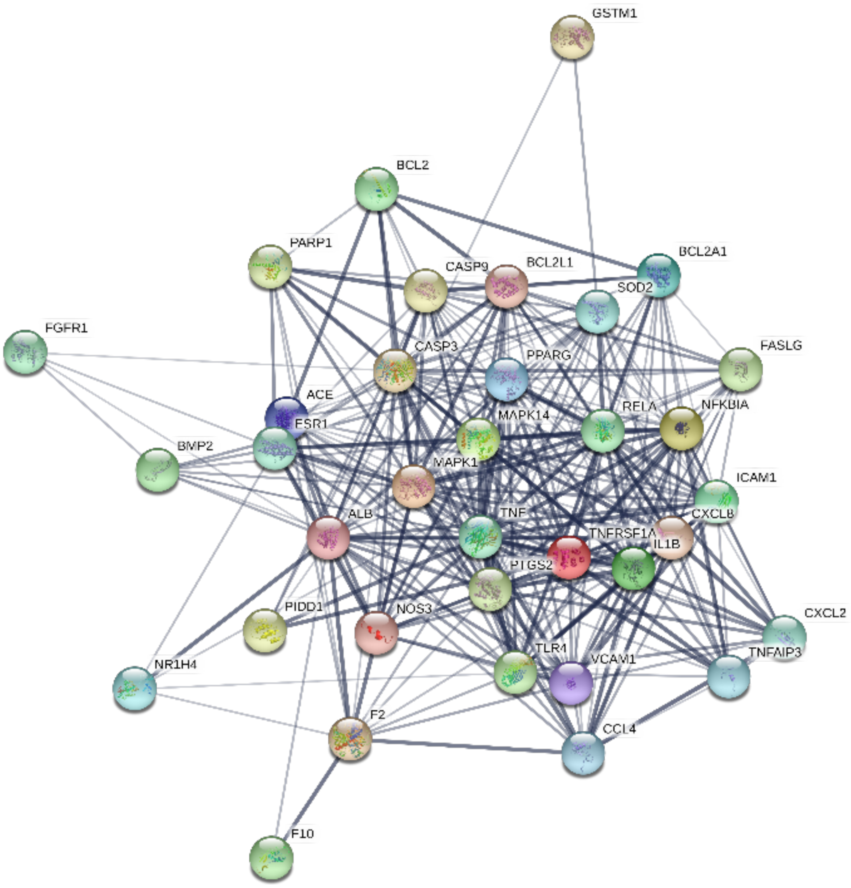


Figure 2. The PPI interaction network of 35 identical genes through STRING

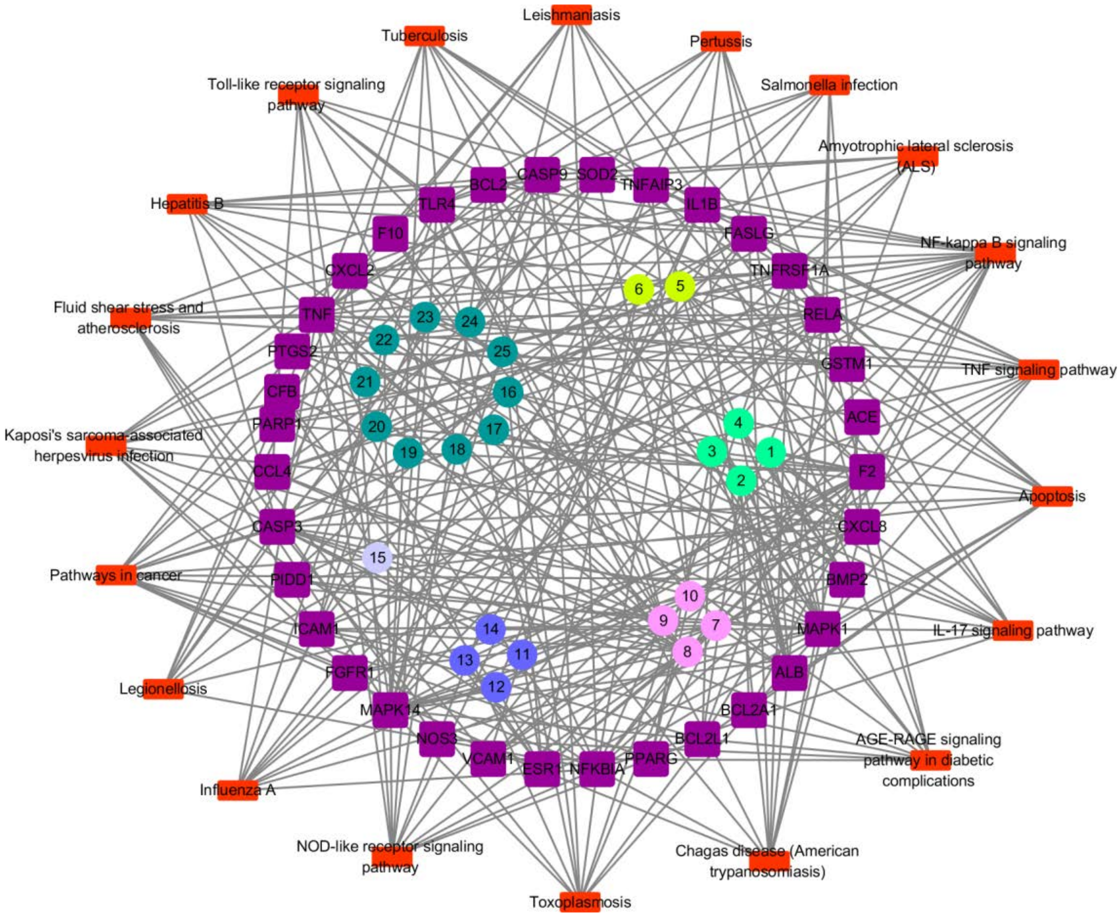


Figure 3. The “Compound-Target-Pathway” interaction network

Table 4. Pathway information of benzoin acting on AS

NO		**Biological pathway**	**Count**	**P-Valμe**
1	hsa04064	NF-kappa B signaling pathway	18	6.37E-30
2	hsa04668	TNF signaling pathway	13	1.15E-18
3	hsa04210	Apoptosis	13	1.16E-17
4	hsa04657	IL-17 signaling pathway	11	8.24E-16
5	hsa04933	AGE-RAGE signaling pathway in diabetic complications	11	1.26E-15
6	hsa05142	Chagas disease (American trypanosomiasis)	11	1.43E-15
7	hsa05145	Toxoplasmosis	11	2.69E-15
8	hsa04621	NOD-like receptor signaling pathway	12	3.39E-15
9	hsa05164	Influenza A	12	3.45E-15
10	hsa05134	Legionellosis	9	2.40E-14
11	hsa05200	Pathways in cancer	15	8.02E-14
12	hsa05167	Kaposi's sarcoma-associated herpesvirus infection	11	3.42E-13
13	hsa05418	Fluid shear stress and atherosclerosis	10	6.76E-13
14	hsa05161	Hepatitis B	10	1.17E-12
15	hsa04620	Toll-like receptor signaling pathway	9	3.23E-12

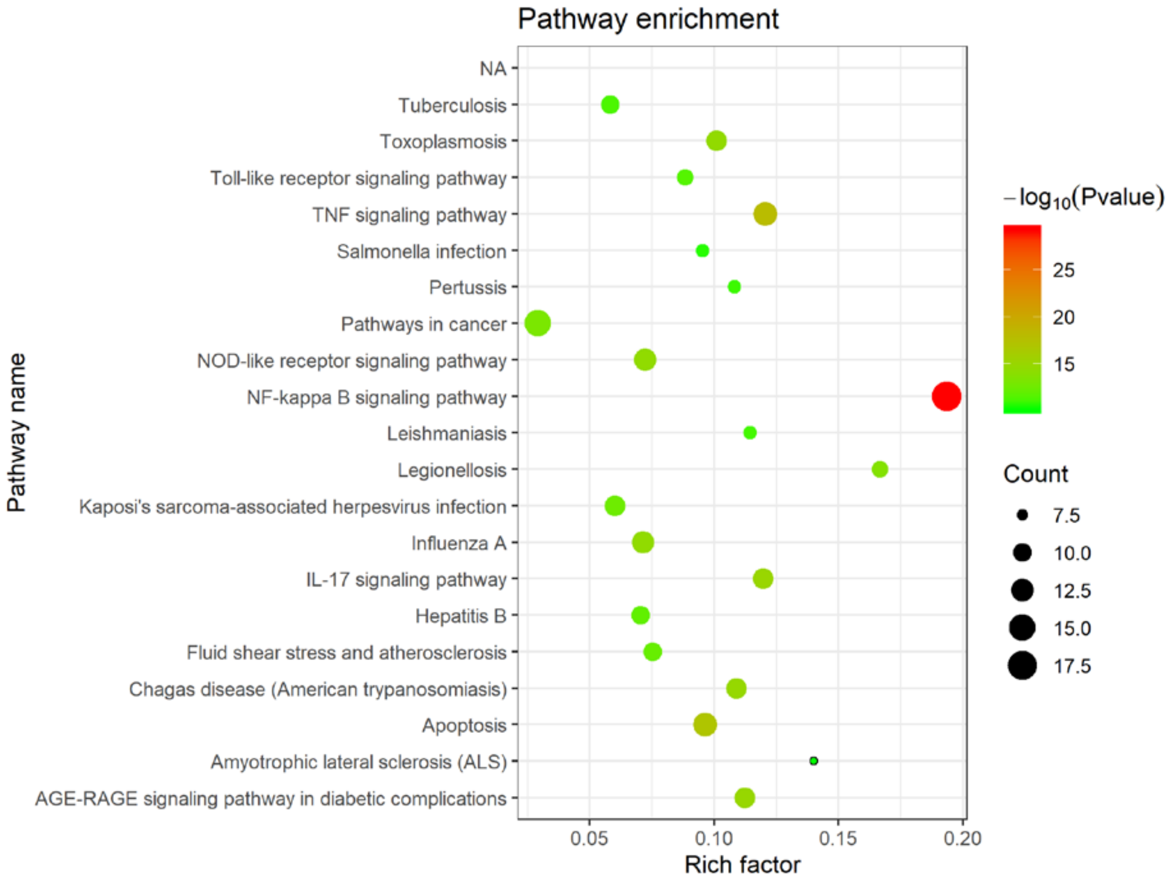


Figure 4. Bubble diagram of Benzoin anti-AS enriched KEGG pathway

5. Experimental Validation

5.1. HUVEC Treatment with Stybenpropol B Does not Induce Significant Cytotoxicity

We began by utilizing a CCK-8 assay approach to assess HUVEC viability following a 24 h treatment with phenylpropane derivatives (0-200 μM), revealing that even at the highest tested dose the viability of these cells was > 90%. As such, phenylpropane derivativeswere used at a dose of Maximum safe in follow-up experiments.

5.2. HUVEC Treatment with Stybenpropol B Protects against TNF-α Injury

We next explored the ability of phenylpropane derivatives to preserve HUVEC viability in the context of TNF-αtreatment, which reduced cell survival, we can see that compound 9 (Stybenpropol B) has the best protective effect on HUVECs. Pretreatment of these cells with Stybenpropol B improved their cell survival in a dose-dependent fashion, as measured via CCK-8 assay (Figure 5), and such pretreatment also reversed TNF-α-related changes in the production of nitric oxide (NO) (Figure 6).

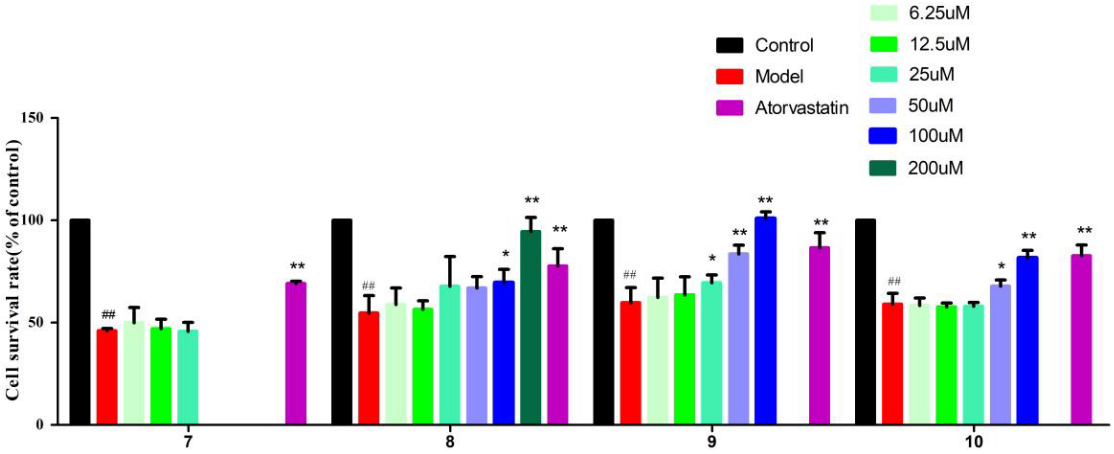


Figure 5. The abscissas 7, 8, 9, 10 mean serial number of phenylpropane derivatives from benzoin. HUVECs were pretreated for 24 h with 0-200 μmol/L phenylpropane derivatives or 8 μmol/L atorvastatin, then TNF-α was added for 12 h and viability was measured. $^{\#\#}P < 0.01$ vs. control; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. model group.

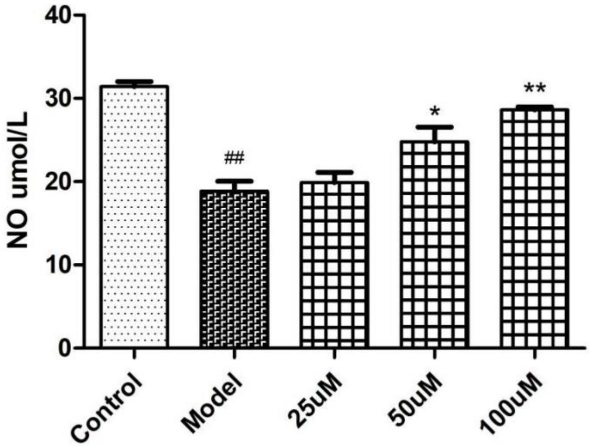


Figure 6. The influences of Stybenpropol B on NO secretion in TNF-α damaged HUVECs model. $^{\#\#}P < 0.01$ vs. control; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. model group

5.3. Treatment with Stybenpropol B Reduces Expression of HUVEC ICAM-1 and VCAM-1

VCAM-1 and ICAM-1 are the primary molecules that mediate monocyte adhesion to HUVECs, and we therefore explored the impact of Stybenpropol B treatment on ICAM-1 and VCAM-1 expression [18]. To that end, we treated HUVECs for 4 h with a range of Stybenpropol B concentrations (0-100 μM), after which they were treated for 24 h with or without TNF-α. While TNF-α therapy enhanced ICAM-1 and VCAM-1 expressions significantly (Figure 7A), Stybenpropol B pretreatment reduced the upregulation of both of these adhesion molecules (Figure 7B).

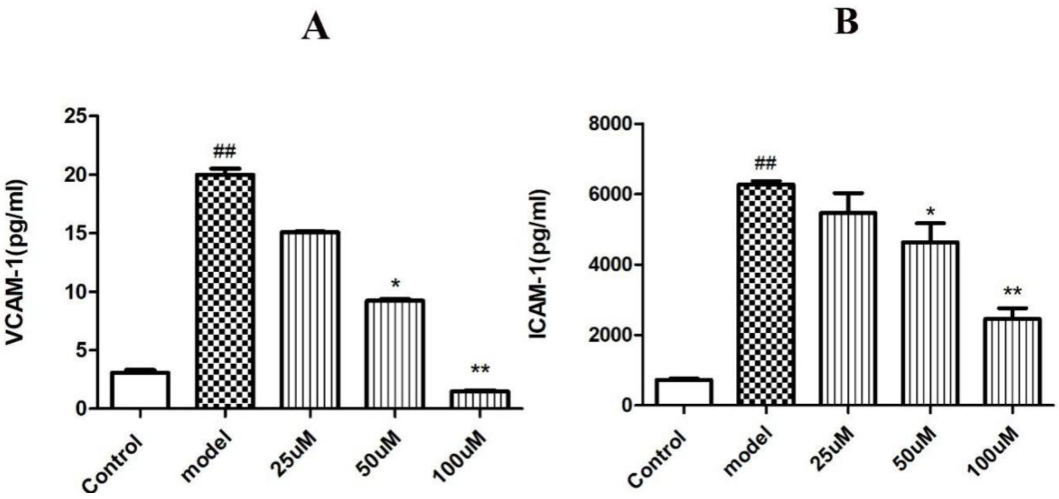


Figure 7. After a pretreatment of 24 h with Stybenpropol B, TNF-α was added to HUVECs and the levels of VCAM-1 and ICAM-1 was determined through ELISA. The outcomes are the means ± SD of three independent studies. $^{\#\#}P < 0.01$ vs. control; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. model group

5.4. Treatment with Stybenpropol B Suppresses IL-8 and IL-1β Production by HUVECs

As IL-8 and IL-1β are primary mediators of inflammatory HUVEC responses [19], we next evaluated the impact of Stybenpropol B pretreatment for 4 h (0-100 μM) on the production of these cytokines following a 24 h incubation without or with TNF-α for 24 h. While TNF-α treatment resulted in marked IL-1β and IL-8 upregulation (Figure 8A-B), Stybenpropol B pretreatment was sufficient to largely reverse this inflammatory cytokine response.

5.5. Treatment with Stybenpropol B Suppresses TNF-α-induced HUVEC Apoptosis

Apoptosis is a primary mechanism of AS-related cell death, and we therefore employed a flow cytometry approach to measure HUVEC apoptosis, revealing that Stybenpropol B pretreatment was sufficient to markedly attenuate TNF-α-induced apoptotic cell death (Figure 9 A-B).

5.6. Treatment with Stybenpropol B reduces the Expression of NF-κB and Modulates Apoptosis-related TNF-α-Treated HUVECs

We next evaluated the expression of principal proteins correlated with apoptotic cell death including Bax and Bcl-2. [20] Following treatment of TNF-α, at the protein level, HUVECs demonstrated a downregulation of Bcl-2 and an upregulation of Bax whereas Stybenpropol B pretreatment reversed these changes and increased the Bcl-2/Bax ratio (Figure 10). Treatment with Stybenpropol B was thus sufficient to decrease pro-apoptotic protein expression in these endothelial cells while enhancing anti-apoptotic protein expression, thus protecting these cells against inflammation-mediated apoptotic death.

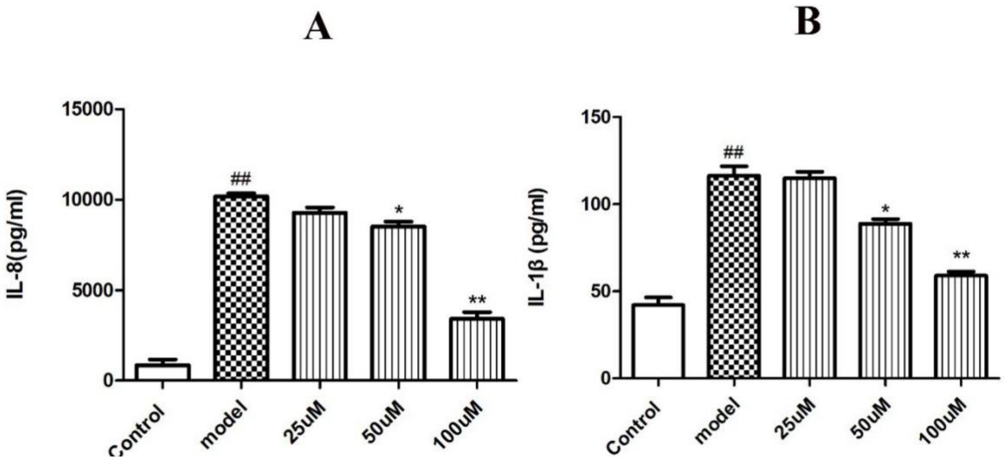


Figure 8. After a pretreatment of 24 h with Stybenpropol B, TNF-α was added to HUVECs and the levels of IL-8 and IL-1β were evaluated employing an ELISA. The outcomes are the means $\overline{X}$ ± SD of three independent studies. $^{\#\#}P$< 0.01 vs. control; $^{*}P$ < 0.05, $^{**}P$< 0.01 vs. model group.

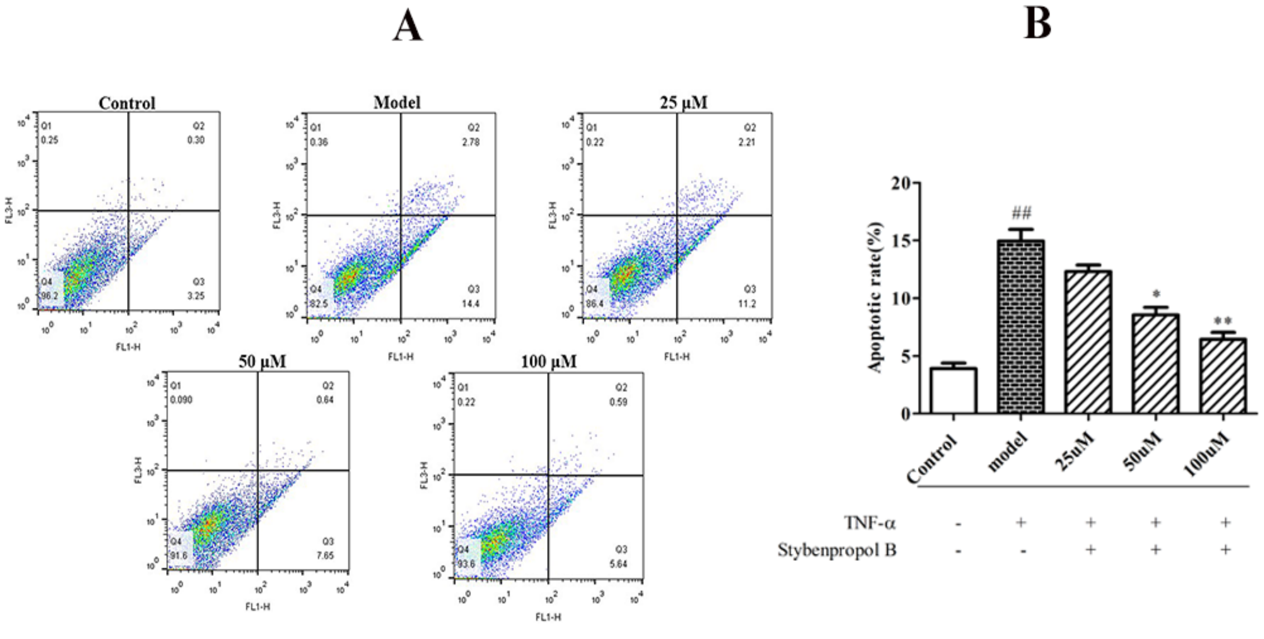


Figure 9. Stybenpropol B inhibited TNF-α induced HUVEC apoptosis. After a pretreatment of 24 h with 9 (12.5, 50, 100 μM), TNF-α was added to HUVECs and apoptosis was ascertained using Annexin V/PI staining. (A). Quantification of the rates of apoptosis (B). The outcomes are the means $\overline{X}$ ± SD of three independent studies. $^{\#\#}P$< 0.01 vs. control; $^{*}P$< 0.05, $^{**}P$< 0.01 vs. model group

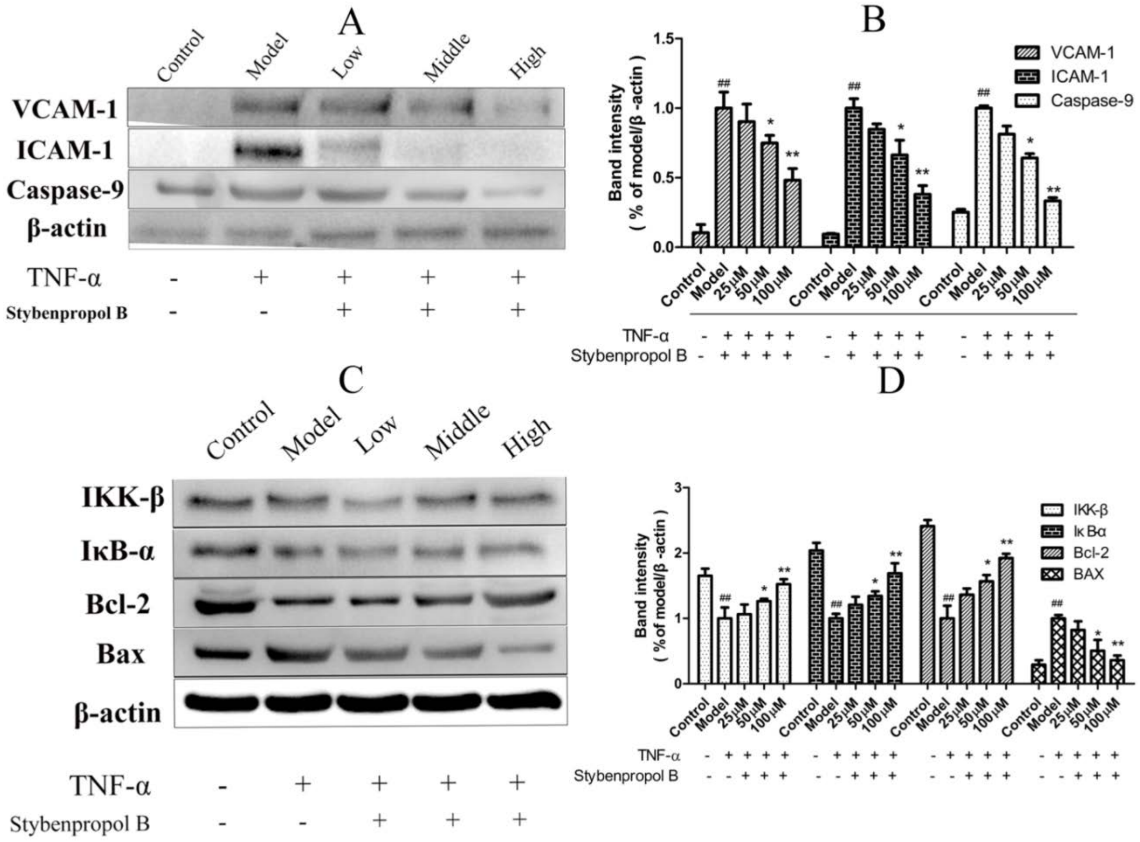


Figure 10. Stybenpropol B regulated the expression of apoptosis-associated proteins and downregulated the NF-κB nuclear transcription. After a pre-treatment of 24 h with 9 (12.5, 50, 100μM), TNF-α was added to HUVECs and levels of protein were accessed using western blotting (A) (B). Quantification of protein levels (C) (D). The outcomes are the means $\bar{X}$± SD of three independent studies. $^{\#\#}P$< 0.01 vs. control; $^{*}P$< 0.05, $^{**}P$ < 0.01 vs. model group

NF-κB is an essential regulator of inflammatory signaling and can drive apoptotic cell death. [21] Herein, we monitored the activation of this signaling pathway by assessing IκBα levels in treated HUVECs. Under steady-state situations, NF-κB is inactive within the cytoplasm where it is sequestered by IκB. Following TNF-α treatment, IκB undergoes phosphorylation and subsequent degradation, after which NF-κB is released is capable of translocating into the nucleus and to thereby alter gene expression. Reductions in IκB protein levels are thus indicative of an increase in NF-κB activity. We found that TNF-α treatment was associated with marked IκBα degradation in HUVECs, whereas Stybenpropol B pretreatment disrupted this IκBα degradation, suggesting that this compound was able to suppress TNF-α-induced NF-κB translocation into the nucleus.

6. Discussion

AS is a complex and debilitating disease that is regulated by a wide range of proteins and pathways. [16,22] Traditional Chinese medicines such as benzoinum are composed of many different compounds with the potential to exert pharmacological activity on multiple different pathways in order to suppress AS development. However, it is still difficult to explore the mechanism of action for a specific TCM preparation due to their complex makeup and multi-target activity. A useful method to comprehending these mechanisms is provided by network pharmacology approaches, which integrate a number of systems biology techniques and other in silico tools. [13,24] In order to investigate the probable pharmacological mechanisms via which benzoin can reduce AS, we used a network pharmacology technique in this article.

Our predictive target and pathway network analyses suggested that phenylpropane derivatives of benzoinum may be the primary bioactive compounds therein with anti-AS activity suppressing inflammatory processes and endothelial cell dysfunction in vitro. We found that benzoinum was able to target the NF-κB and apoptotic pathways in HUVECs, potentially thereby enhancing their ability to survive in the context of inflammatory stimulation. When TNF-α-stimulated HUVECs were treated with Stybenpropol B, we found that it was capable of regulating caspase-9 and NF-κB signaling activity and to thereby downregulate adhesion molecule and proinflammatory cytokine expression while enhancing NO production. NF-κB is a central inflammatory mediator of pathological procedures related with AS. [11] TNF-stimulation causes the release of NF-κB from IκB and IKK-β after their phosphorylation and subsequent degradation, allowing it to translocate to the nucleus to affect gene expression. NF-κB is ordinarily sequestered by IB in an inactive state in the cytoplasm. While IKK-β and IκB-levels were significantly greater in Stybenpropol B-treated cells, the TNF-induced production of ICAM-1 and VCAM-1 was reduced as a result of treatment with Stybenpropol B. This thus suggests that this benzoinum derivative can suppress the NF-κB pathway to disrupt pathological monocyte adhesion and associated inflammation. Endothelial cell apoptotic death is thought to be a key driver of AS pathogenesis [14], and we determined that Stybenpropol B was able to preserve TNF-α-treated HUVEC cells by suppressing apoptotic signaling activity. The caspase-9 protease serves as a central regulator of apoptosis induction such that blocking its activation can disrupt the entire apoptotic process. While TNF-α-treated HUVECs exhibited marked caspase-9 upregulation, Stybenpropol B pretreatment significantly reduced these levels, suggesting that Stybenpropol B can modulate caspase-9 activation or expression in the context of inflammation to augment endothelial cell survival. Bax and Bcl-2 are important regulators of apoptotic signaling. While Bcl-2 disrupts caspase activation and thereby inhibits apoptosis, Bax is a mitochondrial membrane ion channel component that facilitates cytoplasmic cytochrome c accumulation and consequent caspase-9 activation. [23,24] When HUVECs were treated with TNF-α, they exhibited a shift in the ratio of Bax/Bcl-2 in agreement with the induction of mitochondrial apoptosis, whereas Stybenpropol B pretreatment reversed the aforesaid influence by decreasing the levels of pro-apoptotic Bax in these cells. Together, our results thus illuminated that Stybenpropol B can protect HUVECs versus TNF-α-induced inflammation and apoptosis through suppressing NF-κB and caspase-9 signaling.

Endothelial NO synthase (eNOS) is an important mediator of anti-atherosclerotic activity, with reductions in endothelial cell NO levels being linked to endothelial damage and dysfunction.[25,26] Stybenpropol B treatment was adequate to increase TNF-α-treated HUVECs' capacity to generate NO, perhaps promoting increased vasodilation and maintaining endothelial integrity. In the setting of ongoing inflammation, sustained production of proinflammatory cytokines and adhesion molecules can help monocytes cling to and infiltrate the endothelium, causing the start or progression of AS. [2,20] As such, the expression of proinflammatory cytokines and adhesion molecules can predict AS lesion development and cardiovascular risk. It was demonstrated that Stybenpropol B pretreatment decreased the upregulation of VCAM-1, ICAM-1, IL-1β, and IL-8 through HUVECs exposed to TNF-α, indicating that this benzoinum derivative can suppress inflammatory cell damage and thereby prevent apoptotic death.

7. Conclusion

In conclusion, we herein identified a series of benzoinum-derived phenylpropane compounds predicted to intersect with AS disease-related target genes in network pharmacology analyses, indicating that these compounds may be valuable active compounds useful for the treatment of this debilitating disease. Benzoinumtarget genes identified in this study included F2, CASP9, CASP3, ESR1, NFKBIA, ICAM1, BCL2, MAPK14, TNF, TLR4, CXCL8, IL1B (degree ≥ 13), suggesting that these compounds may function by targeting the apoptosis, TNF signaling, and NF-κB signaling pathways. Our in vitro analyses also revealed that the novel benzoinumextract derivative Stybenpropol B was able to inhibit inflammatory damage, reductions in NO production, vascular adhesion, pro-inflammatory cytokine production, and apoptotic death associated with the TNF-α treatment of HUVECs, indicating that this pharmacologically active substance can improve the survival of vascular endothelial cells. Notably, the protective action was associated with increased Bcl-2 protein expression and NF-κB suppression. Stybenpropol B might therefore be a promising candidate chemical for the prevention or treatment of AS, while more preclinical and clinical research is required to establish this possibility.

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Data Availability

Data will be provided upon request to the authors

Conflict of Interest

The authors have no competing interests.

Abbreviations

AS	Atherosclerosis
TNF-*α*	Tumor necrosis factor
CCK-8	Cell counting kit - 8
NO	Nitric Oxide
ELISA	Enzyme-linked immunosorbent assay
VCAM-1	Vascular cell adhesion molecule
ICAM-1	Intercellular adhesion molecule
IL-8	Interleukin-8
IL-1*β*	Interleukin-1*β*
HUVECs	Human umbilical vein endothelial cells
LPS	Lipopolysaccharide
NF-*κ*B	Nuclear factor kappa B
ET-1	Endothelin-1

References

[1] Khan, R., Spagnoli, V., Tardif, J. C. & L'Allier, P. L. Novel anti-inflammatory therapies for the treatment of atherosclerosis. *Atherosclerosis* 240, 497-509 (2015).

[2] Zhou, P. *et al.* Attenuation of TNF-α-induced inflammatory injury in endothelial cells by ginsenoside Rb1 via inhibiting NF-κB, JNK and p38 signaling pathways. *Front. Pharmacol.* 8, 1-13 (2017).

[3] Weber, C., Badimon, L., Mach, F. & van Der Vorst, E. P. C. Therapeutic strategies for atherosclerosis and atherothrombosis: Past, present and future. *Thromb. Haemost.* 117, 1258-1264 (2017).

[4] Zhang, F. *et al.* Biemamides A-E, Inhibitors of the TGF-β Pathway That Block the Epithelial to Mesenchymal Transition. *Org. Lett.* 20, 5529-5532 (2018).

[5] Ren, H. *et al.* Selenium inhibits homocysteine-induced endothelial dysfunction and apoptosis via activation of AKT. *Cell. Physiol. Biochem.* 38, 871-882 (2016).

[6] Bruikman, C. S., Stoekenbroek, R. M., Hovingh, G. K. & Kastelein, J. P. New Drugs for Atherosclerosis. *Can. J. Cardiol.* 33, 350-357 (2017).

[7] Choi, E. S. *et al.* Ligustilide attenuates vascular inflammation and activates Nrf2/HO-1 induction and, NO synthesis in HUVECs. *Phytomedicine* 38, 12-23 (2018).

[8] Fei-, W. Chemical Constituents of Benzoinum and Its Anti- tumor Activities in Vitro. 1-7 (2020).

[9] Zhang, L. *et al.* Anti-inflammatory and anti-apoptotic effects of stybenpropol a on human umbilical vein endothelial cells. *Int. J. Mol. Sci.* 20, (2019).

[10] Paralleloneurum, S. Trzy lignany / neolignany z żywicy benzoinowej. 2, 1000-1004 (2020).

[11] Guo, W. *et al.* Integrating network pharmacology and pharmacological evaluation for deciphering the action mechanism of herbal formula Zuojin Pill in suppressing hepatocellular carcinoma. *Front. Pharmacol.* 10, 1-21 (2019).

[12] Huang, D. W., Sherman, B. T. & Lempicki, R. A. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.* 4, 44-57 (2009).

[13] Smoot, M. E., Ono, K., Ruscheinski, J., Wang, P. & Ideker, T. Cytoscape 2. 8 : new features for data integration and network visualization. 27, 431-432 (2011).

[14] Zhou, Y. qiang *et al.* Hyperoside Suppresses Lipopolysaccharide-induced Inflammation and Apoptosis in Human Umbilical Vein Endothelial Cells. *Curr. Med. Sci.* 38, 222-228 (2018).

[15] Mason, R. P., Dawoud, H., Jacob, R. F., Sherratt, S. C. R. & Malinski, T. Eicosapentaenoic acid improves endothelial function and nitric oxide bioavailability in a manner that is enhanced in combination with a statin. *Biomed. Pharmacother.* 103, 1231-1237 (2018).

[16] Wu, S. *et al.* Potent anti-inflammatory effect of dioscin mediated by suppression of TNF-α-induced VCAM-1, ICAM-1 and EL expression via the NF-κB pathway. *Biochimie* 110, 62-72 (2015).

[17] Guo, S. *et al.* Role of A20 in cIAP-2 protection against tumor necrosis factor α (TNF-α)-mediated apoptosis in endothelial cells. *Int. J. Mol. Sci.* 15, 3816-3833 (2014).

[18] Galkina, E. & Ley, K. Vascular adhesion molecules in atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* 27, 2292-2301 (2007).

[19] Xu, T. *et al.* Systematic Understanding of the Mechanism of Baicalin against Ischemic Stroke through a Network Pharmacology Approach. 2018, (2018).

[20] Sun, L., Chen, S., Gao, H., Ren, L. & Song, G. Visfatin induces the apoptosis of endothelial progenitor cells via the induction of pro-inflammatory mediators through the NF-κB pathway. *Int. J. Mol. Med.* 40, 637-646 (2017).

[21] Gwon, W. G. *et al.* Sargaquinoic Acid Inhibits TNF-α-Induced NF-κB Signaling, Thereby Contributing to Decreased Monocyte Adhesion to Human Umbilical Vein Endothelial Cells (HUVECs). *J. Agric. Food Chem.* 63, 9053-9061 (2015).

[22] Wang, M., Wang, Q., Du, Y. & Zhang, X. Network Pharmacology-Based Strategy to Investigate Pharmacological Mechanisms of Qiaoshao Formula for Treatment of Premature Ejaculation. *Evidence-based Complement. Altern. Med.* 2020, 1-12 (2020).

[23] González, D. *et al.* Caspase-3 and -9 are activated in human myeloid HL-60 cells by calcium signal. *Mol. Cell. Biochem.* 333, 151-157 (2010).

[24] Chen, T. L. *et al.* Protective effects of isorhamnetin on apoptosis and inflammation in TNF-α-induced HUVECs injury. *Int. J. Clin. Exp. Pathol.* 8, 2311-2320 (2015).

[25] Förstermann, U., Xia, N. & Li, H. Roles of vascular oxidative stress and nitric oxide in the pathogenesis of atherosclerosis. *Circ. Res.* 120, 713-735 (2017).

[26] Förstermann, U. & Sessa, W. C. Nitric oxide synthases: Regulation and function. *Eur. Heart J.* 33, 829-837 (2012).

[27] Wang F. et al. Neolignan and phenylpropanoid compounds fromthe resin of Styrax tonkinensis.J. Asian. Nat. Prod. Res.23, 1-8 (2021).

[28] Xie Q. et al. Advances in studies on chemical constituents and pharmacological effects of benzoin. Chin. Med. Mat. 43, 243-248 (2020).

[29] Wang Y. et al. Research progress of pharmacological action of benzoin.Asia-Pacific Traditional Medicine. 11, 3 (2015).