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Research article

Computational analysis of phytocompounds in *Centella asiatica* for its antifibrotic and drug-likeness properties - Herb to drug study

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ABSTRACT

Oral submucous fibrosis (OSMF) is a potentially malignant disorder with no permanent cure that affects the quality of life due to trismus. Computational pharmacology has accelerated the discovery of drug candidates for the treatment of incurable diseases. The present study aimed to screen the compounds of the miracle herb *Centella asiatica* with drug-likeness properties based on the absorption, distribution, metabolism, and excretion (ADME) properties. The pharmacological actions of these screened compounds against OSMF were identified by network pharmacology, gene ontology, pathway enrichment analysis, molecular docking, and simulation. Fifteen drug-like ligands were identified after virtual screening viz; asiatic acid, kaempferol, quercetin, luteolin, apigenin, bayogenin, gallic acid, isothankunic acid, madecassic acid, madasiatic acid, arjunolic acid, terminolic acid, catechin, epicatechin, and nobiletin. 850 potential targets were predicted for the ligands, which were analyzed against 354 proteins associated with OSMF. Compound pathway analysis and disease pathway analysis identified 53 common proteins. The GO enrichment analysis identified 472 biological process terms, 76 molecular function terms, and 44 cellular component terms. Pathway enrichment analysis predicted 142 KEGG pathways, 35 Biocarta pathways, and 236 Reactome pathways for the target proteins. The analysis revealed that the herb targets crucial events of fibrosis such as inflammation, oxidative stress, apoptosis, collagen deposition, and epithelial-mesenchymal transition. The common 53 proteins were used for protein-protein interaction (PPI) network analysis, which revealed 4 key proteins interacting with the phytocompounds viz; transforming growth factor-β1 (TGF-β1), mothers against decapentaplegic-3 (SMAD-3), mitogen-activated protein kinase-1 (MAPK-1) and proto-oncogene tyrosine-protein kinase (SRC). Molecular docking revealed that all ligands had a good binding affinity to the target proteins. Bayogenin had the highest binding affinity towards MAPK-1 (−9.7 kcal/mol), followed by isothankunic acid towards SRC protein (−9.3 kcal/mol). Madasiatic acid had the highest binding affinity to SMAD-3 (−7.6 kcal/mol) and TGF-β1 (−7.1 kcal/mol).

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Molecular dynamics simulation demonstrated stable ligand protein interactions of bayogenin and MAPK complex, isothankunic acid and SRC complex. This *in silico* study is the first to identify potential phytochemicals present in *Centella asiatica* and their target molecules, which might be responsible for reversing OSMF.

Abbreviations

ADAM	a disintegrin and metalloproteinase
ADME	absorption distribution metabolism excretion
BC	betweenness centrality
CC	closeness centrality
CDC	Cell division control protein-2
CK	cytokeratin
COX 2	cyclooxygenase 2
DC	degree centrality
ECM –	Extracellular matrix
EGF	epidermal growth factor
GI	gastrointestinal
GO	gene ontology
JNK	Janus kinase
IL-	Interleukin
MAPK	mitogen-activated protein kinase
OSMF	oral submucous fibrosis
PGE 2	prostaglandin E2
PPI	protein-protein interaction
ROS	reactive oxygen species
RMSD	root mean square deviation
RMSF	root mean square fluctuation
SMAD	mothers against decapentaplegic
SRC	proto-oncogene tyrosine-protein kinase
8-IsoP SRC	8 isoprostane SRC
STAT-3	Signal transducer and activator of transcription-3
TGF-β-	transforming growth factor β
TGF-βR	TGFβ receptor
TAK	TGF-β activated kinase-1
TRAF-6	tumor necrosis factor receptor-associated factor-6

1. Introduction

Bioinformatics has revolutionized medical research. The pace of decoding pathophysiology and drug discovery has increased by several folds owing to the application of bioinformatics tools. Oral submucous fibrosis (OSMF) is a chronic, crippling, progressive disorder with a malignancy rate of 7–30 %. Areca nut is reportedly the prime etiological factor [1]. Incessant areca nut chewing can cause tissue abrasion and inflammation. The alkaloids in areca nut imperil numerous cellular pathways, leading to oxidative stress, hypoxia, cell cycle disruption, angiogenesis, apoptosis, autophagy, and DNA damage [2–4]. OSMF is a collagen metabolism disorder wherein collagen synthesis is increased and its degradation is drastically reduced. As a result, an excessive amount of collagen is deposited which leads to fibrotic contracture [5]. It is described as an 'over-healing wound' as the addictive habit of continuous areca nut chewing leads to microtrauma to the oral mucosa and progression to fibrosis [6]. OSMF is a highly prevalent disease among the South Asian population, with high occurrence in India, China, and Taiwan. Current treatment options do not completely cure the disease. Long-term treatment with corticosteroids leads to adverse side effects such as immunodeficiency, which causes secondary infections. Hence, identifying new therapeutic agents to ameliorate fibrosis is of prime importance.

Herbal medicine has a rich history in human practice with excellent pharmacological activity and minimal side effects, unlike synthetic medicines. Secondary metabolites of medicinal herbs have been proven beneficial and are used in traditional alternative medicine for various ailments. *Centella asiatica* (L.) Urban. from the Apiaceae family is a renowned traditional herb employed to treat multiple ailments such as depression, anxiety, gastric ulcer and arthritis, skin disorders, and wound healing [7,8]. *C. asiatica* and its triterpenoid, asiatic acid, were reported earlier to have antifibrotic effects on arecoline-induced fibrosis in human buccal fibroblasts [9].

Fan et al. conducted a network pharmacology study to identify the targets and pathways involved in the therapeutic effect of *Centella asiatica* on hepatic fibrosis. MAPK signaling pathway and relaxin signaling pathway were the crucial pathways identified. The potential targets identified were TGF-β, SRC, epidermal growth factor receptor, tumor necrosis factor, interleukin 6 and 1β, vascular endothelial growth factor A, matrix metalloproteinase 2 and 9, chemokine (C-X-C motif) ligand 8, p53, and JUN among others [10]. Zhao et al. explored the antiangiogenic effect of the herb by network pharmacology. The key targets identified for the antiangiogenic effect include STAT3, SRC, MAPK-1, and AKT-1 [11].

In this study, we have attempted to predict the active phytocompounds of *C. asiatica* interacting with the crucial target molecules of OSMF. PPI network of proteins analogous to the herb and disease was analyzed to identify the closely associated proteins. These proteins were docked against the potential phytocompounds. The docked complex with the highest docking score was analyzed using molecular dynamics simulation studies. The compounds interacting with the disease targets could be used to develop phytomedicines, which could be used to effectively reverse oral submucous fibrosis.

2. Methodology

2.1. Network pharmacology

2.1.1. Preparation of compound library for C. asiatica

The chemical constituents present in *C. asiatica* were identified by exploring literature and different phytochemical databases such as the Duke Phytochemical Database (https://phytochem.nal.usda.gov/) and Chinese Traditional Medicinal Database (https://tcmsp-e.com/tcmsp.php). Virtual screening of compounds was carried out using the Swiss ADME web server (http://www.swissadme.ch/) based on absorption, distribution, metabolism, excretion (ADME) properties such as bioavailability, gastrointestinal (GI) absorption, and blood-brain permeability. A cutoff of 0.55 was set for bioavailability, and high GI absorption was considered [12]. The compounds that did not permeate the blood-brain barrier were selected. Drug-likeness properties such as Lipinski's rule, Veber's rule, and Egan's rule were also included in the screening criteria. As the phytocompounds are usually of higher molecular weight, a violation with respect to molecular weight was allowed.

2.1.2. Identification of potential targets

The targets for screened potential compounds were predicted using Swiss Target Prediction (http://www.swisstargetprediction.ch/) Binding database (https://www.bindingdb.org/rwd/bind/index.jsp), Therapeutic Target database (https://db.idrblab.net/ttd/) and Passonline server (http://www.way2drug.com/passonline/). The targets for the disease were identified using various online servers such as DisGeNET (https://www.disgenet.org/), Therapeutic Target database, and Gene Ontology database (https://www.ncbi.nlm.nih.gov/geo/). Genes respective to protein targets were identified using the Uniprot database and Genbank. Apart from the online servers, literature relevant to the compounds and disease was reviewed from PubMed and handpicked articles. The studies selected from the literature were reviewed individually by two authors. Any discrepancies were resolved by discussion, and conclusions were drawn. Standardized information was extracted from the studies, including the protein name, its respective gene name, and references. Proteins retrieved from different resources were collated, duplicates were removed, and a dataset of target proteins was created for both compounds and targets. The Venn diagram depicted the intersection between the predicted target of compounds and OSMF targets.

2.1.3. Gene ontology (GO) and pathway enrichment analysis

GO and pathway analysis were executed using David software (https://david.ncifcrf.gov/). GO analysis depicts the known molecular functions, biological processes, and cellular components related to the proteins. Signaling pathway enrichment analysis was performed using KEGG, Biocarta, and Reactome pathways. The top 20 GO terms and pathways were depicted in a graph drawn using SrPlot Tools (https://www.bioinformatics.com.cn/en).

2.1.4. Establishment of protein-protein interaction (PPI) network and analysis

The proteins recurrent in both the target datasets of compounds and disease were used to draw a PPI network using the String database (version 11.5). A stringent score of highest confidence (0.900) and a stringent false-discovery rate of 1.00 was used. The network was imported to Cytoscape. MCODE was run to identify hub genes, and network analysis was conducted using parameters such as degree centrality, closeness centrality, and betweenness centrality to recognize closely associated proteins.

2.2. Molecular docking

The crystal structures of potential targets were retrieved from RCSB PDB (https://www.rcsb.org/). The three-dimensional structures of targets were downloaded and saved in.pdb format. The protein structures were prepared for docking by removing water molecules, and heteroatoms, and by adding polar hydrogen atoms and resaving them in.pdb format using Discovery Studio 2021 Client. Structures of screened active ligands were retrieved from PubChem in the 3D SDF format. The 3D SDF format for all ligands was converted to PDBQT file using the PyRx tool to generate atomic coordinates. Molecular docking analysis was performed to explore the binding energy, binding affinity, and bound conformations of all ligands to targets using AutoDock VINA implicated in the PyRx tool. The active site residues of proteins were taken by analyzing the binding site of their co-crystallized ligands retrieved from PDBSum. The grid box was defined using XYZ coordinates to encompass the binding site, allowing the ligand to freely dock within the designated

region. The grid box was assigned around the active sites using the Auto Grid application. The parameters were set to default, and the best binding poses of each protein were identified using the PyRx scoring function. The obtained protein and ligand conformations were thoroughly examined for various types of interactions using Discovery Studio 2021 Client, which was used after the docking steps were completed successfully.

2.3. Molecular simulation

Molecular Dynamics simulations were run for 100ns using Desmond software (Schrodinger LLC) to investigate the stability and flexibility of the ligand-target interactions. The ligands with strong binding affinities to the target proteins were chosen for simulation. To establish the desired framework, a predetermined TIP3P water strategy was developed to establish a specific volume with periodic orthorhombic coordinates separated by 10 mm. 2e necessary ions, for example, 0+ and 0.15 M salts, were randomly added to the solvent solution to neutralize the framework electrically. 2e solvency protein system was constructed using a ligand complex, and the system framework was reduced using the default protocol. This was accomplished inside the Desmond module by applying the force field settings OPLS3e. In NPT assemblies, which were held at 1 atm pressure and 300 K with 50 PS capture sessions totaling 1.2 kcal/mol energy coming before them, the Nosé–Hoover temperature combination and the isotropic approach were used. The stability of the protein-ligand complex structure was evaluated based on the trajectory performance, root mean square fluctuation (RMSF), and root mean square deviation (RMSD). RMSD values indicated fluctuations in the interaction of the ligand with the active site of the target protein, whereas RMSF calculations are employed to assess the extent of atomic movement and flexibility within the simulated structure throughout the simulation time.

3. Results and discussion

The herbal approach in managing various incurable diseases is garnering attention due to high efficacy rates, heterogeneity of herbal composition, and minimal toxicity. The conventional drug discovery approach of targeting a disease with one target and one drug has proven ineffective in curing the disease [13]. Hence, our present study aimed to concatenate *in silico* approaches such as network pharmacology, pathway enrichment, molecular docking, and simulation to discover bioactive ligands from *C. asiatica,* that could exert antifibrotic effect against potentially malignant OSMF.

3.1. Network pharmacology

3.1.1. Preparation of compound library for C. asiatica

The exploration of literature and phytochemical databases revealed 209 compounds after the assimilation of search results and de-duplication. The compounds were screened for ADME and drug-likeness properties. After screening, 15 active compounds comprising terpenoids, flavonoids, and polyphenols were identified (Table 1). The drug-likeness properties and the ADME properties of the compounds are given in Table 2 and Table 3.

Triterpenoids are the most valuable and bioactive group of compounds in *C. asiatica* accounting for 8 % of its total constituents

Table 1
Bio-active phytocompounds of *Centella asiatica*.

Common Name	IUPAC Name	Reference
Asiatic acid	(1S,2R,4aS,6aR,6aS,6bR,8aR,9R,10R,11R,12aR,14bS)-10,11-dihydroxy-9-(hydroxymethyl)-1,2,6a,6b,9,12a-hexamethyl-2,3,4,5,6,6a,7,8,8a, 10,11,12,13,14b-tetradecahydro-1H-picene-4a-carboxylic acid	[14–17,19,20]
Kaempferol	3,5,7-trihydroxy-2-(4-hydroxyphenyl)chromen-4-one	[14–20]
Quercetin	2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one	[14–20]
Luteolin	2-(3,4-dihydroxyphenyl)-5,7-dihydroxychromen-4-one	[14,17,18]
Apigenin	5,7-dihydroxy-2-(4-hydroxyphenyl)chromen-4-one	[14,16,19,20]
Bayogenin	(4aS,6aR,6aS,6bR,8aR,9R,10R,11S,12aR,14bS)-10,11-dihydroxy-9-(hydroxymethyl)-2,2,6a,6b, 9,12a-hexamethyl-1,3,4,5,6,6a,7,8,8a,10,11,12, 13,14b-tetradecahydropicene-4a-carboxylic acid	[19]
Gallic acid	3,4,5-trihydroxybenzoic acid	[18]
Isothankunic acid	8,8a,10-trihydroxy-9-(hydroxymethyl)-1,2,6a, 6b,9,12a-hexamethyl-1,2,3,4,5,6,6a, 7,8,10,11, 12,13,14b-tetradecahydropicene-4a-carboxylic acid	[14–16,19]
Madecassic acid	(1S,2R,4aS,6aR,6aS,6bR,8R,8aR,9R,10R,11R,12aR,14bS)-8,10,11-trihydroxy-9-(hydroxymethyl)-1,2,6a,6b,9,12a-hexamethyl-2,3,4,5,6,6a,7,8,8a,10,11,12,13,14b-tetradecahydro-1H-picene-4a-carboxylic acid	[14–16,19,20]
Madasiatic acid	8,10,11-trihydroxy-1,2,6a,6b,9,9,12a-heptamethyl-2,3,4,5,6,6a,7,8,8a,10,11,12,13, 14b-tetradecahydro-1H-picene-4a-carboxylic acid	[14–16,19]
Arjunolic acid	(4aS,6aR,6aS,6bR,8aR,9R,10R,11R,12aR,14bS)-10,11-dihydroxy-9-(hydroxymethyl)-2,2,6a,6b,9,12a-hexamethyl-1,3,4,5,6,6a,7, 8,8a,10,11,12,13,14b-tetradecahydropicene-4a-carboxylic acid	[16,20]
Terminolic acid	(4aS,6aR,6aS,6bR,8R,8aR,9R,10R,11R,12aR,14bS)-8,10,11-trihydroxy-9-(hydroxymethyl)-2,2,6a,6b,9,12a-hexamethyl-1,3,4,5,6,6a,7, 8,8a,10,11,12,13,14b-tetradecahydropicene-4a-carboxylic acid	[14,16,19,20]
Catechin	(2S,3R)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol	[16,18,20]
Epicatechin	(2R,3R)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol	[16,20]
Nobiletin	2-(3,4-dimethoxyphenyl)-5,6,7,8-tetramethoxychromen-4-one	[16]

[21]. The potential triterpenoids identified in this study include asiatic acid, arjunolic acid, madecassic acid, madasiatic acid, isothankunic acid, bayogenin, and terminolic acid. Flavonoids are another group of biologically active compounds with phenol in their structure [22]. The bioactive flavonoids identified in this study included apigenin, catechin, epicatechin, kaempferol, luteolin, quercetin, and nobiletin. Gallic acid, a potent natural antioxidant, and an excellent radical scavenger is also identified as an active ligand in our study [23,24].

Previous studies have reported the interaction of phytochemicals present in *C. asiatica* with identified targets. Asiatic acid, apigenin, kaempferol, luteolin, quercetin, catechin, epicatechin, nobiletin, and gallic acid reportedly exerted antifibrotic activity by reducing collagen deposition, epithelial-mesenchymal transition and targeting TGF β/Smad signaling [9,25–33]. Asiatic acid, arjunolic acid, madecassic acid, kaempferol, luteolin, quercetin, catechin, epicatechin, and nobiletin regulated MAPK pathway thereby reducing inflammation, collagen deposition, modulating apoptosis and oxidative stress [34–40].

SRC pathway is reportedly targeted by asiatic acid, apigenin, nobiletin, catechin, epicatechin, and gallic acid to ameliorate angiogenesis and cell proliferation [28,32,41,42,46]. Kaempferol and luteolin were found to compete with ATP molecules and attach to the ATP binding site of Src, thereby suppressing its activity [43,44]. Gallic acid restored renal function in diabetic rats by inhibiting MAPK and mitigating colon cancer by suppressing SRC phosphorylation [45,46].

Very few studies have reported the cellular factors that play a role in mitigating the anti-inflammatory and anti-proliferative effects of bayogenin, madasiatic acid, and isothankunic acid. The anticancer activity of asiatic acid and bayogenin via interaction with hexokinase II was demonstrated in an *in silico* study [47]. Another *in silico* study demonstrated the interaction of madasiatic acid, asiaticoside, termolinic acid, madecassic acid, asiatic acid, with proinflammatory cytokines viz; IL-1α, IL-1β, IL-6, IL-4 and exhibited anti-inflammatory properties [48]. Antiviral properties and anti-hyperuricemic activity of isothankunic acid have been studied [49,50]. However, the role of isothankunic acid in antifibrotic activity is yet to be studied.

The bioactive phytocompounds protect against various ailments when used in moderation. Rarely, allergic reactions and burning sensations may occur on skin during topical administration. The intake of herbal components might induce headache, dizziness, upset stomach, or nausea at higher doses. Prolonged administration of higher doses might decelerate the metabolism of compounds causing toxicity [7]. All the bioactive compounds except quercetin had an LD 50 value of ≥2000 mg/kg (Table 3). Hence, the compounds are safe for therapeutic regimens when used in moderation.

Dunnick and Hailey administered 40–1900 mg/kg/day of quercetin to the rats in their feed for two years. They reported no treatment related effect on the survival of rats. At the interim stages of 6 and 15 months, no toxic lesions were observed in the rats. However, neoplastic lesions were observed in the kidneys after 2 years of administration. Thus, they concluded that toxic adverse effects appeared among experimental rats only when an extremely high dose of quercetin was administered for a considerably longer duration [51].

3.1.2. Identification of potential targets

Targets for the screened active ligands and disease predicted using online servers and literature were integrated, and duplicates were removed. 850 targets were predicted for ligands and 354 targets for disease (Source: Supplementary Tables 1 and 2). The intersection of compound and disease targets yielded 53 proteins (Fig. 1 and Table 4). UniProt identifiers and gene names were retrieved for these proteins, and a dataset was created for further analysis.

3.1.3. Geo and KeGG enrichment analysis

GO enrichment analysis using David software predicted 472 biological process terms, 76 molecular function terms, and 44 cellular component terms. Pathway enrichment analysis identified 142 KEGG pathways, 35 Biocarta pathways, and 236 Reactome pathways

Table 2
Drug likeness properties of bioactive phytocompounds of *Centella asiatica*.

Compound name	Molecular Weight (≤500 Da)	Hydrogen Bond Donor (≤5)	Hydrogen Bond Acceptor (≤10)	Rotatable bonds ≤10	Polar surface area ≤131.6 Å2	No. of Violations
Asiatic acid	488.7	4	5	2	97.99	0
Kaempferol	286.24	4	6	1	111.13	0
Quercetin	302.24	5	7	1	131.36	0
Luteolin	286.24	4	6	1	111.13	0
Apigenin	270.24	3	5	1	90.9	0
Bayogenin	488.7	4	5	2	97.99	0
Gallic acid	170.12	4	5	1	97.99	0
Isothankunic acid	504.7	5	6	2	118.22	1
Madecassic acid	504.7	5	6	2	118.22	1
Madasiatic acid	488.7	4	5	1	97.99	0
Arjunolic acid	488.7	4	5	2	97.99	0
Terminolic acid	504.7	5	6	2	118.22	1
Catechin	290.27	5	6	1	110.38	0
Epicatechin	290.27	5	6	1	110.38	0
Nobiletin	402.39	0	8	7	85.59	0

The values were predicted using SwissADME.

Table 3
ADMET properties of bioactive phytocompounds of *Centella asiatica*.

Compound name	Absorption and Distribution			Metabolism					Toxicity LD 50 (mg/Kg)
	BA	GIA	BBB penetration	CYP1A2 inhibitor	CYP2C19 inhibitor	CYP2C9 inhibitor	CYP2D6 inhibitor	CYP3A4 inhibitor	
Asiatic acid	0.56	High	No	No	No	No	No	No	2000
Kaempferol	0.55	High	No	Yes	No	No	Yes	Yes	3919
Quercetin	0.55	High	No	Yes	No	No	Yes	Yes	159
Luteolin	0.55	High	No	Yes	No	No	Yes	Yes	3919
Apigenin	0.55	High	No	Yes	No	No	Yes	Yes	2500
Bayogenin	0.56	High	No	No	No	No	No	No	2000
Gallic acid	0.56	High	No	No	No	No	No	Yes	2000
Isothankunic acid	0.56	High	No	No	No	No	No	No	2000
Madecassic acid	0.56	High	No	No	No	No	No	No	2000
Madasiatic acid	0.56	High	No	No	No	No	No	No	2000
Arjunolic acid	0.56	High	No	No	No	No	No	No	2000
Terminolic acid	0.56	High	No	No	No	No	No	No	2000
Catechin	0.55	High	No	No	No	No	No	No	10000
Epicatechin	0.55	High	No	No	No	No	No	No	10000
Nobiletin	0.55	High	No	No	No	Yes	No	Yes	5000

BA: Bioavailability; GIA: Gastrointestinal absorption; BBB: Blood-brain barrier; CYP: Cytochrome P450. The values were predicted using SwissADME and ProTox II.

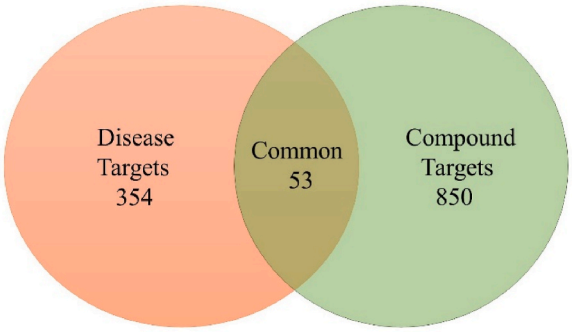

Fig. 1. Venn diagram of common potential targets.

for the target proteins. The top 20 GO terms and pathways are shown in Supplementary Figs. S1(a-c) and S2(a-c). The top GO biological processes identified in this study include 'positive regulation of pri-miRNA transcription from RNA polymerase II promoter', 'positive regulation of smooth muscle cell proliferation', 'protein kinase B signaling', 'transforming growth factor beta receptor signaling pathway', and 'wound healing'.

The top GO cellular components identified include 'platelet alpha granule lumen', extrinsic component of cytoplasmic side of plasma membrane', 'caveola', 'collagen trimer', 'ruffle membrane', and 'extracellular matrix'. The top GO molecular functions identified were 'transcription coactivator binding', 'collagen binding', 'endopeptidase activity', 'protease binding', and 'protein phosphatase binding'. The major pathways include 'AGE-RAGE signaling pathway', 'pancreatic cancer', 'EGFR tyrosine kinase inhibitor resistance', 'STAT-3 signaling pathway', 'IL-10 anti-inflammatory signaling pathway', 'Telomeres, Telomerase, Cellular Aging, and Immortality', 'Interleukin-4 and Interleukin-13 signaling', 'collagen degradation', and 'degradation of the extracellular matrix'. The findings correlate with the pathogenesis of OSMF reported earlier [58,113].

3.1.4. Establishment of PPI network for OSMF targets and network analysis

String analysis of the proteins revealed a network comprising 53 nodes and 164 edges with an enrichment p-value of $<$1.0e-16. The average node degree was 6.19, and the average local clustering coefficient was 0.482. The PPI network was imported to Cytoscape. Important clusters of 24 proteins were identified using MCODE. Network analysis was done using degree centrality (DC $\geq$ 20), betweenness centrality (BC $\geq$ 1), and closeness centrality (CC $\geq$ 0.5). The potential targets identified post-network analysis were MAPK-1, SRC, SMAD-3, and TGF-β1 (Fig. 2). These compounds have been reported earlier to contribute to the pathogenesis of OSMF. Areca nut chewing causes assault of oral mucosa, and it disrupts the tissue architecture and cellular pathways.

TGF-β interacts with receptor TGF-β receptor-2 (TGF-βR-2), which in turn recruits and phosphorylates TGF-β receptor-1 (TGF-βR-1). Further signaling may be via canonical or non-canonical pathways. In the canonical pathway, SMAD proteins are phosphorylated sequentially, activating genes that promote inflammation, extracellular matrix (ECM) remodeling, and α-smooth muscle actin (α-SMA)

Table 4
Common potential targets.

S.No.	Protein Name	Abbreviation	Source for disease target
1	Actin, aortic smooth muscle	ACTA2	[52–63]
2	Serine/threonine protein kinase	Akt	[58,64]
3	Apolipoprotein-A	APOA	[65]
4	Androgen receptor	AR	[66]
5	Bone morphogenetic protein-7	BMP7	[66]
6	Carbonic anhydrase-9	CA9	[66]
7	Capsase-3	CASP3	[67]
8	Caspase-8	CASP8	[66]
9	E-cadherin	CDH1	[58,68–70]
10	Collagen 1A2	COL1A2	[54,58,63,66,70–72]
11	Collagen 3A1	COL3A1	[58,70]
12	Epidermal growth factor receptor	EGFR	[3,58]
13	Prothrombin	F2	[6]
14	Fatty acid-binding protein	FABP	[73]
15	Fibronectin	FN	[52,54,58,74,75]
16	Hypoxia-inducible factor-1 alpha	HIF1A	[58,66,76–79]
17	Heme oxygenase-1	HMOX1	[66]
18	Insulin-like growth factor-1	IGF1	[74,80]
19	Insulin-like growth factor-1 receptor	IGF1R	[72,74]
20	Interleukin-1 beta	IL1B	[58,81,82]
21	Interleukin-6	IL6	[58,66,81,83–86]
22	Lysyl oxidase	LOX	[6,66,87]
23	Mitogen-activated protein kinase-1	MAPK-1	[88,89]
24	met - Hepatocyte growth factor receptor	MET	[58]
25	Matrix metalloproteinase-1	MMP1	[6,66]
26	Matrix metalloproteinase-2	MMP2	[6,58,66,72,90]
27	Matrix metalloproteinase-3	MMP3	[66,72,91]
28	Matrix metalloproteinase-9	MMP9	[6,58,66,72]
29	Matrix metalloproteinase-12	MMP12	[92]
30	Matrix metalloproteinase-13	MMP13	[93]
31	Mammalian target of rapamycin	MTOR	[64]
32	Nuclear factor kappa B	NF-κB	[58]
33	Nitric oxide synthase	NOS	[94]
34	NADPH oxidase-4	NOX4	[83]
35	Plasminogen	PLG	[66]
36	Peroxisome proliferator-activated receptor gamma	PPARG	[95,96]
37	Prostaglandin F2-alpha receptor	PTGFR	[96]
38	Prostaglandin G/H synthase 2	PTGS2	[66]
39	Ras-related C3 botulinum toxin substrate-1	RAC1	[74]
40	RAR-related orphan receptor C	RORC	[85]
41	Plasminogen activator inhibitor 1	SERPINE1	[79]
42	Mothers against decapentaplegic homolog-2	SMAD2	[57,58,63,66,70,71,97,98]
43	Mothers against decapentaplegic homolog-3	SMAD3	[57,70,71,97]
44	Mothers against decapentaplegic homolog-7	SMAD7	[99]
45	Superoxide dismutase	SOD	[100]
46	Proto-oncogene tyrosine-protein kinase	SRC	[3]
47	Signal transducer and activator of transcription	STAT3	[6]
48	Telomerase reverse transcriptase	TERT	[101]
49	Transforming growth factor beta-1	TGFB1	[6,9,57,58,61,66,72,102–107]
50	Tumor necrosis factor-alpha	TNFa	[6,58,66,82]
51	Cellular tumor antigen p53	TP53	[58,66,76,88,94,108–110]
52	UDP-glucuronosyltransferase 1-6	UGT1A6	[111]
53	Vascular endothelial growth factor receptor-2	VEGFR2	[112]

upregulation ultimately leading to fibrosis. On the other hand, non-canonical pathway results in tumor necrosis factor receptor-associated factor-6 (TRAF-6) mediated phosphorylation of MAPK, which leads to inflammation, apoptosis, cell proliferation, and differentiation (Fig. 3) [3,114–116]. Reactive oxygen species (ROS) are generated due to abrasion of oral mucosa by the areca nut components, which trigger epidermal growth factor and stimulate SRC. This leads to the upregulation of various downstream proteins, including MAPK. The chain of reactions leads to various cellular changes, ultimately resulting in fibrosis (Fig. 3) [117,118].

3.2. Molecular docking

The crystal structures of identified potential proteins were retrieved for MAPK-1 (PDB ID: 1TVO), SRC (PDB ID: 2H8H), SMAD-3 (PDB ID: 1MK2), and TGF-β1 (PDB ID: 5VQP). Also, the SDF files of ligands were retrieved from PubChem. As the SDF format of 2 ligands, namely madasiatic acid and isothankunic acid, were unavailable in PubChem, their SMILES formats were used to draw and download the structure in the SDF format using the Marvin Js tool. The active site residues of MAPK-1, SRC, SMAD-3, and TGF-β1 were

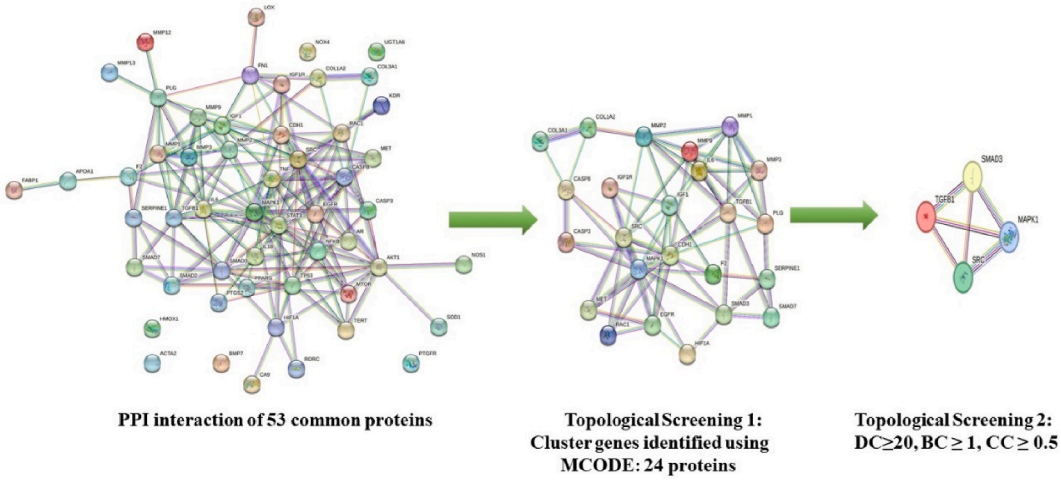

Fig. 2. Network analysis of PPI network.

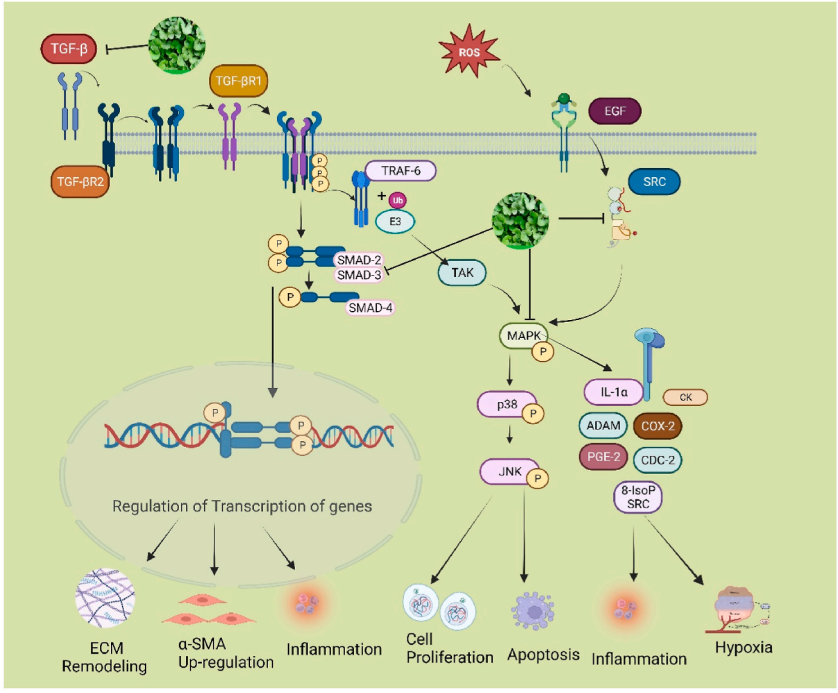

Fig. 3. Molecular Pathways involving the antifibrotic activity of *C. asiatica*.
Phytoconstituents of *C. asiatica* exert their antifibrotic activity by targeting MAPK, SRC, TGF-β and SMAD-3 signaling. CDC2, cell division control protein 2; CK, Cytokeratin; COX 2, cyclooxygenase 2; EGF, Epidermal growth factor; IL-1α, Interleukin-1α; JNK, Janus kinase; MAPK-1, Mitogen-Activated Protein Kinase 1; OSMF, Oral submucous fibrosis; PGE 2, Prostaglandin E2; ROS, Reactive oxygen species; SRC, Proto-oncogene tyrosine protein kinase; TGF-β, Transforming growth factor β; TGF βR, TGFβ receptor; SMAD, mothers against decapentaplegic; TRAF-6, tumor necrosis factor receptor-associated factor 6; TAK, TGF β activated kinase 1; ADAM, a disintegrin and metalloproteinase; 8-IsoP SRC; 8 isoprostane SRC. Image created with BioRender.com.

taken by analyzing the binding site of their co-crystallized ligands retrieved from PDBSum.

Molecular docking studies demonstrated good binding affinity of the drug-like phytocompounds with target proteins (binding energy - 5.1 to −9.7 kcal/mol). Docking of the target and ligands generated 9 different conformations for each ligand based on the binding affinity (Kcal/mol). Among 15 ligands docked with the targets, the best binding compounds for each target were identified by the least binding energy (Kcal/mol) and root mean square deviation (RMSD), and that conformation was considered the most suitable docking pose of compounds (Table 5).

MAPK-1 protein had a good docking score of less than −7 kcal/mol for 14 ligands. Bayogenin presented the highest binding affinity with a dock score of −9.7 kcal/mol. The center x, y, and z of the active cavity box model were set to 5.92, −4.23, and 18.19. The conventional hydrogen bond occurs between the oxygen atom in the ligand and the hydrogen atom of the aspartic acid (ASP 149) residue in the protein by approximately 2.97 Å, which is indicative of a favorable interaction (Fig. 4 a-c). Isothankunic acid had exceptional binding potency against SRC protein, achieving a docking score of −9.3 kcal/mol. The carbon-hydrogen bond interaction occurred between the carbon atom of the ligand and the hydrogen atom of the glutamate residue (GLU:339) in the protein 2H8H at 3.4 Å. The strong carbon-hydrogen bond interaction between target and ligand is expected to contribute to the stability and specificity of the interaction significantly. The center x,y, and z of the active cavity box model were set to 18.52, 18.40, and 24.57, respectively (Fig. 5 a-c).

Madasiatic acid demonstrated the highest binding affinity towards two target proteins viz; SMAD-3 and TGF-β1 with dock scores of −7.6 kcal/mol and −7.1 kcal/mol, respectively. SMAD-3 interacted with madasiatic acid with the aid of a conventional hydrogen bond formed between the asparagine residue (ASN: 320) in the protein to the oxygen atom of the ligand producing weaker interactions. Strong interactions were formed with the aid of glutamine residue (GLN:364). The center x, y, and z of the active cavity box model was set to 9.13, 22.85, and 24.59, respectively (Fig. 6 a-c).

For TGF-β1, center x, y, and z of the active cavity box model was set to 23.90, 25.5, 25.5 and docked with the ligands. The protein interacted with madasiatic acid through a strong and stable hydrogen bond between the hydrogen atom of the valine residue (VAL:79) and the oxygen atom of the ligand at a bond distance of 2.68 Å (Fig. 7 a-c).

3.3. Molecular simulation

The simulation complex of MAPK-1 protein and bayogenin comprised 53,682 atoms and 15,964 water molecules. RMSD plot showed initial disturbance and convergence at ~15 ns. The ligand RMSD values were within the range of 0.83–2.95 Å with an average RMSD of 1.82 Å. The complex was stable after 15 ns throughout the simulation time of 100 ns, and the ligand remained firmly bound to the receptor (Fig. 8a). The RMSF plot showed minimum fluctuations of the amino acids present in MAPK-1 protein between ~ 0.39 Å and ~ 5.99 Å. The trajectory remained stable throughout the simulation. (Fig. 9a).

SRC protein and isothankunic acid simulation complex comprised of 66,316 atoms with 19,688 water molecules. RMSD plot displayed convergence at ~40 ns. The ligand RMSD values were within the range of 0.15 Å to 0.80 Å, with an average RMSD of 0.54 Å. The trajectory had disturbance again between 50 and 60 Å. Then it maintained stability throughout the simulation (Fig. 8b). The RMSF plot showed fluctuations of the amino acids present in SRC protein between ~ 0.49 Å to 6.06 Å. (Fig. 9b).

The simulation system of SMAD-3 protein and madasiatic acid simulation complex comprised 35,670 atoms and 10,814 water molecules. RMSD plot displayed convergence at 5 ns. The ligand RMSD values were within the range of 0.13 Å and 0.43 Å with an average RMSD of 0.22 Å. The RMSD plot fluctuated throughout simulation time (Fig. 8c). The RMSF plot showed fluctuations of the amino acids present in SMAD-3 protein between 0.43 Å to 6.73 Å. (Fig. 9c).

The simulation complex of TGF-β1 and madasiatic acid had 76,193 atoms with 23,615 water molecules. RMSD plot displayed convergence at 10 ns. The ligand RMSD values were within the range of 0.13 Å to 0.66 Å with an average RMSD of 0.50 Å. The plot showed fluctuations throughout the simulation time (Fig. 8d). The RMSF plot showed fluctuations of the amino acids present in TGF-β1 protein between 0.85 Å to 10.94 Å (Fig. 9d).

Molecular docking revealed that all ligands had a good binding affinity to the target proteins. Molecular simulation studies for 100ns demonstrated stable interaction of MAPK-1 protein and bayogenin complex and SRC protein and isothankunic acid complex. Fluctuations were observed when complexes of madasiatic acid with TGF-β1 and SMAD-3 were simulated. Further experimental

Table 5
Binding energy (kcal/mol) of protein-ligand interaction.

Ligands	Targets			
	MAPK-1 (1TVO)	SRC (2H8H)	SMAD-3 (1MK2)	TGF-β1 (5VQP)
Bayogenin	**−9.7**[a]	−9.1	−7.1	−6.0
Terminolic acid	−9.6	−8.2	−6.8	−6.2
Arjunolic acid	−9.5	−9.1	−7.4	−5.5
Asiatic acid	−9.1	−9	−6.9	−5.6
Madasiatic acid	−9.1	−7.9	**−7.6**[a]	**−7.1**[a]
Isothankunic acid	−9	**−9.3**[a]	−7.6	−6.9
Madecassic acid	−8.9	−9.1	−6.7	**−5.1**[b]
Luteolin	−8.7	−9	−6.8	−6.8
Apigenin	−8.6	−8.9	−6.8	−6.6
Epicatechin	−8.4	−8.9	−6.4	−6.0
Quercetin	−8.4	−9.1	−6.9	−6.9
Catechin	−8.3	−8.6	−7.1	−6.0
Kaempferol	−8.1	−8.8	−6.8	−6.9
Nobiletin	−7.5	−8.7	−5.8	−5.9
Gallic acid	**−5.5**[b]	**−5.8**[b]	**−5.2**[b]	−5.5

[a] -highest binding energy.
[b] -lowest binding energy.

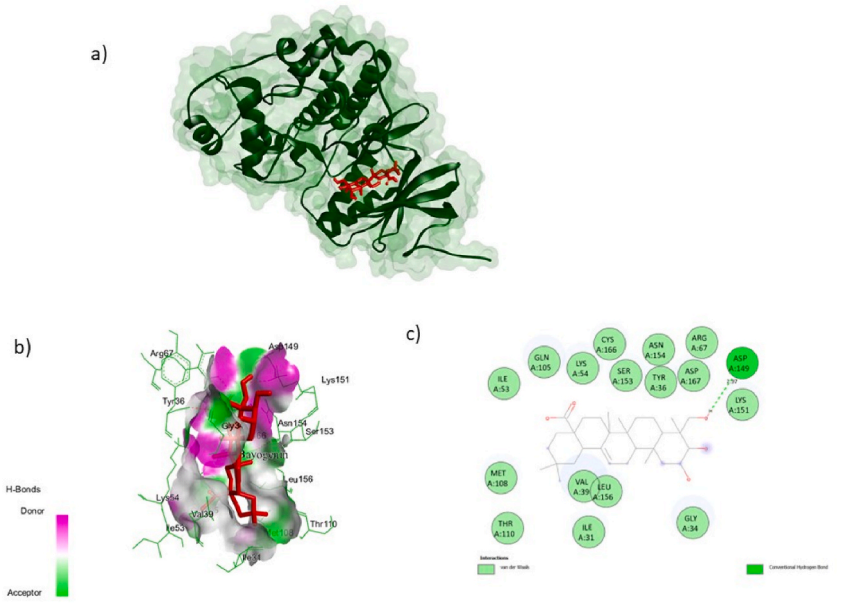

Fig. 4. Molecular docking of MAPK-1 protein with Bayogenin a) Bayogenin bound to the catalytic site of MAPK-1 protein. b) Residues at the binding site of the ligand and Hydrogen-bond donors and acceptors regions of target-ligand interaction c) 2D interaction of the ligand with the target protein.

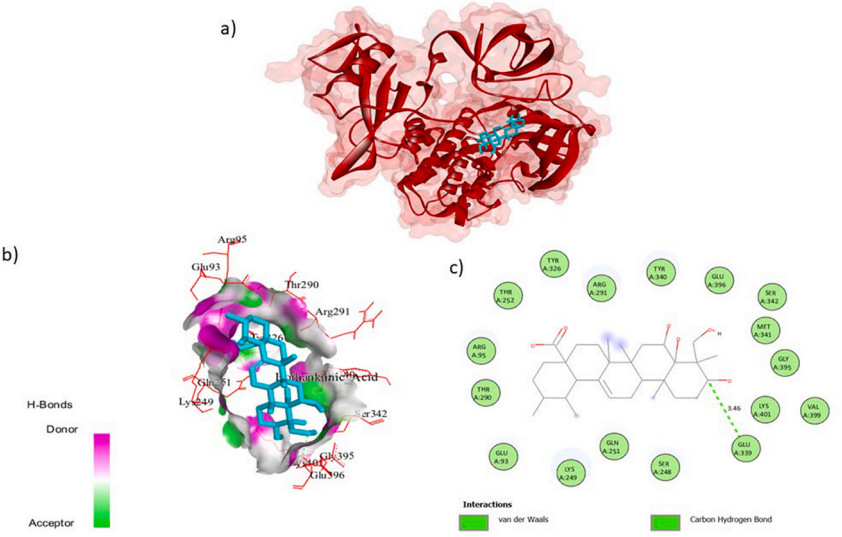

Fig. 5. Molecular docking of Src protein with Isothankunic acid (a) Isothankunic acid bound to the catalytic site of Src protein. b) Residues at the binding site of the ligand and Hydrogen-bond donors and acceptors regions of target-ligand interaction c) 2D interaction of the ligand with the target protein.

studies are required to validate the results.

Previous studies had reported the interaction of phytochemicals such as asiatic acid, arjunolic acid, apigenin, madecassic acid, kaempferol, luteolin, quercetin, catechin, epicatechin, nobiletin, and gallic acid present in *C. asiatica* with identified targets [9,25–47]. Very few studies have reported the cellular factors that come into play in the anti-inflammatory and anti-proliferative effects of bayogenin, terminolic acid, madasiatic acid, and isothankunic acid [47–50]. However, their role in antifibrotic activity is yet to be studied. Interestingly, in our study, the highest docked complexes were MAPK-1 with bayogenin, SRC with isothankunic acid, SMAD-3 with madasiatic acid, and TGF-β1 with madasiatic acid. These findings suggest that bayogenin, isothankunic acid, and madasiatic acid might interact with the key target proteins of OSMF, viz. MAPK-1, SRC, TGF-β1 and SMAD-3. The phytocompounds may modulate the cellular response to hypoxia, smooth muscle cell proliferation, apoptosis, and expression of interleukins and cytokines to inhibit

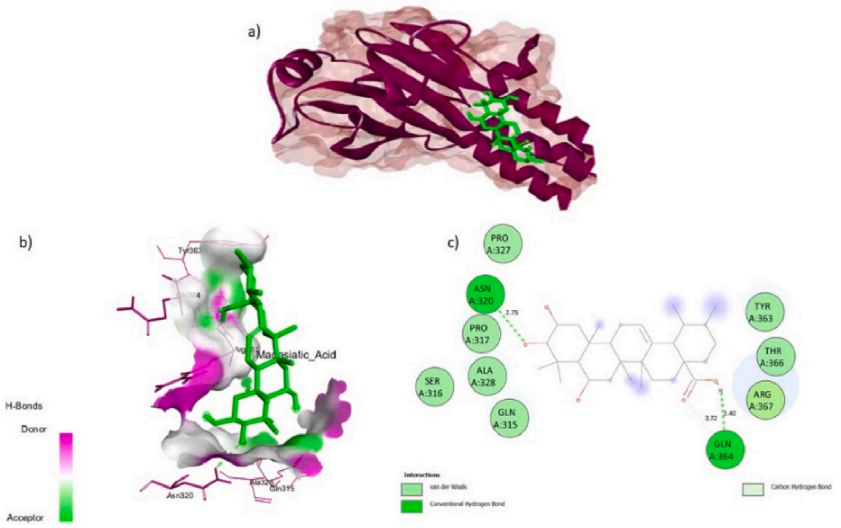

Fig. 6. Molecular docking of SMAD-3 protein with Madasiatic acid (a) Madasiatic acid bound to the catalytic site of SMAD-3 protein. b) Residues at the binding site of the ligand and Hydrogen-bond donors and acceptors regions of target-ligand interaction c) 2D interaction of the ligand with the target protein.

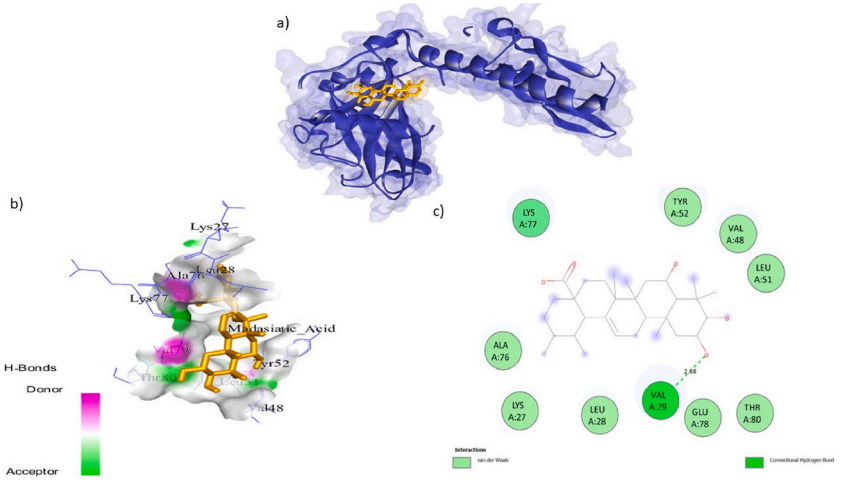

Fig. 7. Molecular docking of TGF-β1 protein with Madasiatic acid. (a) Madasiatic acid bound to the catalytic site of TGF-β1 protein. b) Residues at the binding site of the ligand and Hydrogen-bond donors and acceptors regions of target-ligand interaction c) 2D interaction of the ligand with the target protein.

collagen accumulation, thereby ameliorating fibrosis.

4. Conclusion

OSMF is a collagen metabolism disorder that is classified as potentially malignant. Despite extensive research and the availability of treatment options, OSMF still impacts the quality of life of several patients. Hence, it is of prime importance to develop a therapeutic strategy that cures the disease and prevents malignant transformation. Conventional treatment with corticosteroids and surgery has several ramifications. The herbal approach will negate these side effects and help in the provision of cost-effective treatment to the patients. *C. asiatica* is a miracle herb enriched with numerous phytocompounds which helps to exude a protective effect against OSMF by modulating hallmarks of fibrosis such as oxidative stress, inflammation, collagen deposition, epithelial-mesenchymal transition, and apoptosis by interaction with TGF-β1, MAPK-1, SRC, and SMAD-3. A total of 15 compounds were identified with drug-likeness properties, among which bayogenin, isothankunic acid, and madasiatic acid had the highest binding affinity with the target proteins. Further deeper exploration of the interaction of phytochemicals with various molecular players of pathogenesis is required. The compounds with drug-likeness properties could be used to develop herbal drugs for treating OSMF in the future. The identified targets

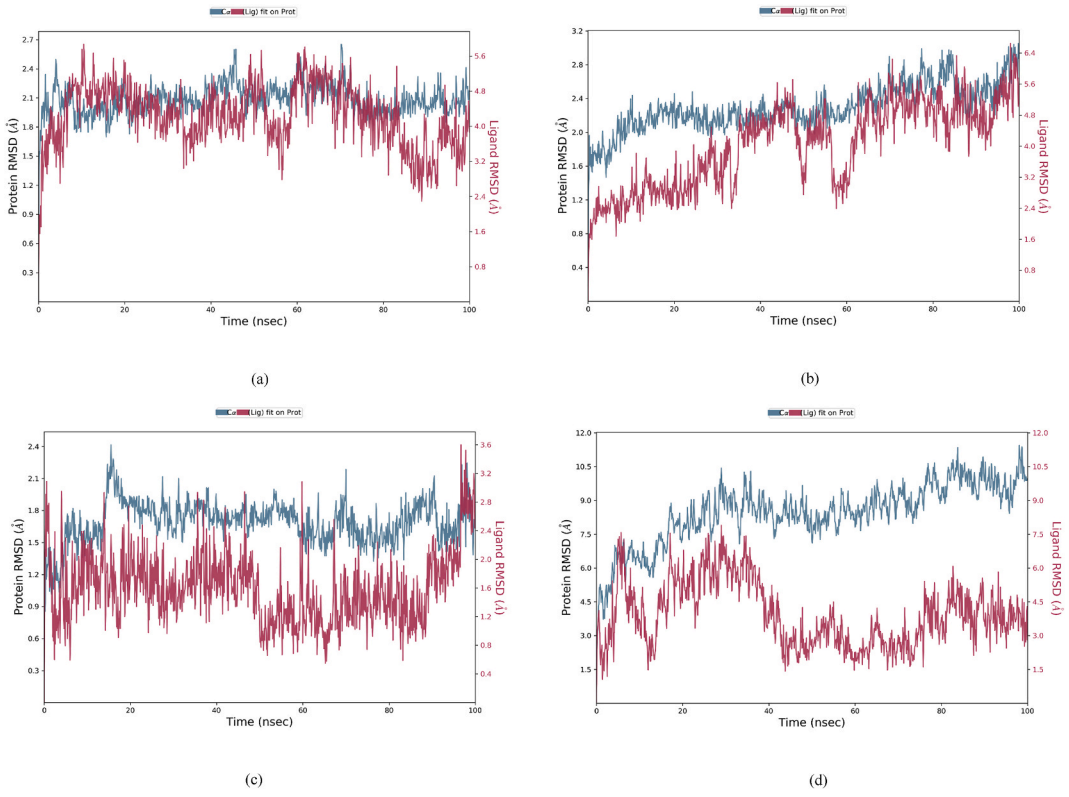

Fig. 8. Protein Ligand Root Mean Square Deviation (RMSD) Plot. (a) MAPK-1 and bayogenin complex (b) SRC and isothankunic acid complex (c) SMAD-3 and madasiatic acid complex (d) TGF-β1 and Madasiatic acid complex.

and pathways will aid in developing target-based therapeutic strategies for OSMF. Our study is the first of its kind and has aimed to provide novel insights into the active compounds of *C. asiatica* and their mechanistic antifibrotic effect on the potential targets of OSMF.

Ethical statement

NA.

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Data availability statement

For more information: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE64216; https://www.disgenet.org/browser/0/1/1/C0029172/source_ALL/_b./

CRediT authorship contribution statement

K. Gayathri: Writing – original draft, Visualization, Software, Methodology, Formal analysis, Data curation, Conceptualization. **P. A. Abhinand:** Writing – review & editing, Validation, Supervision, Software, Investigation, Formal analysis, Conceptualization. **V. Gayathri:** Writing – review & editing, Visualization, Validation, Methodology, Investigation, Conceptualization. **V. Prasanna Lakshmi:** Writing – original draft, Software, Methodology, Formal analysis, Data curation. **D. Chamundeeswari:** Writing – review & editing, Visualization, Supervision, Methodology, Investigation. **Li Jiang:** Writing – review & editing, Validation, Supervision, Methodology, Conceptualization. **Zhen Tian:** Writing – review & editing, Visualization, Validation, Supervision, Methodology. **N. Malathi:** Writing – review & editing, Visualization, Validation, Supervision, Investigation, Conceptualization.

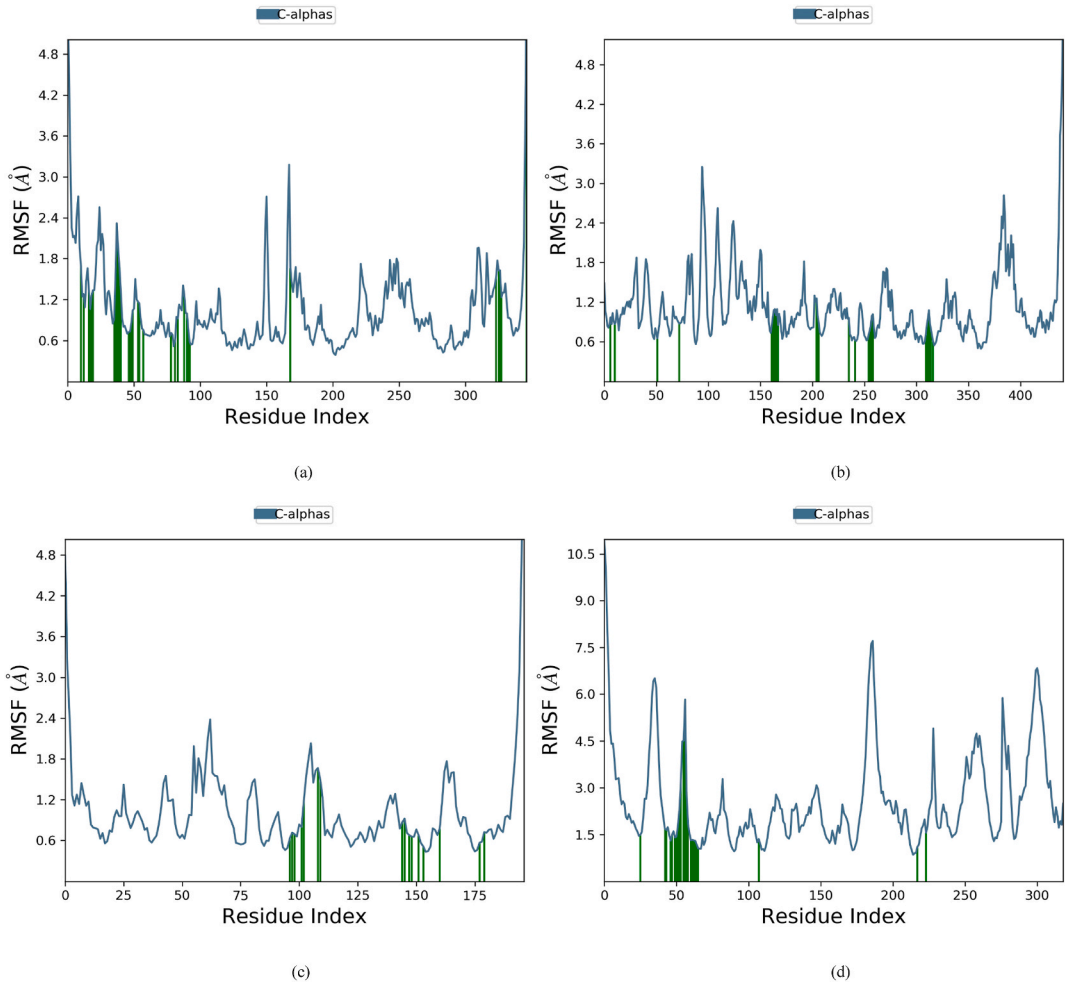

Fig. 9. Root mean square fluctuation (RMSF) plot for proteins. (a) MAPK-1 (b) Src (c) SMAD-3 (d) TGF-β1.

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.

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I hereby acknowledge “Bio render” software which was helpful in drawing the graphical content in this article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e33762.

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