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ORIGINAL ARTICLE

Antifibrotic effect of silymarin on arecoline-induced fibrosis in primary human buccal fibroblasts: an in silico and in vitro analysis

Divyambika Catakapatri Venugopal[1] · Paramesh Viswanathan[2] · Soundharya Ravindran[3] · Alan Mathew Punnoose[2] · Madhavan Yasasve[1] · Davis G. Dicky John[4] · Lavanya Prabhakar[4] · Gnanasambandan Ramanathan[5] · Sathasivasubramanian Sankarapandian[1] · Vijayalakshmi Ramshankar[3]

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Abstract

Background This study aimed to assess silymarin's anticancer and antifibrotic potential through in silico analysis and investigate its impact on in vitro arecoline-induced fibrosis in primary human buccal fibroblasts (HBF).

Methods & results The study utilized iGEMDOCK for molecular docking, evaluating nine bioflavonoids, and identified silymarin and baicalein as the top two compounds with the highest target affinity, followed by subsequent validation through a 100ns Molecular Dynamic Simulation demonstrating silymarin's stable behavior with Transforming Growth Factor Beta. HBF cell lines were developed from tissue samples obtained from patients undergoing third molar extraction. Arecoline, a known etiological factor in oral submucous fibrosis (OSMF), was employed to induce fibrogenesis in these HBFs. The inhibitory concentration (IC_{50}) of arecoline was determined using the MTT assay, revealing dose-dependent cytotoxicity of HBFs to arecoline, with notable cytotoxicity observed at concentrations exceeding 50µM. Subsequently, the cytotoxicity of silymarin was assessed at 24 and 72 h, spanning concentrations from 5µM to 200µM, and an IC_{50} value of 143µM was determined. Real-time polymerase chain reaction (qPCR) was used to analyze the significant downregulation of key markers including collagen, epithelial-mesenchymal transition (EMT), stem cell, hypoxia, angiogenesis and stress markers in silymarin-treated arecoline-induced primary buccal fibroblast cells.

Conclusion Silymarin effectively inhibited fibroblast proliferation and downregulated genes associated with cancer progression and EMT pathway, both of which are implicated in malignant transformation. To our knowledge, this study represents the first exploration of silymarin's potential as a novel therapeutic agent in an in vitro model of OSMF.

Keywords OSMF · Silymarin · Arecoline · Fibrosis · Cell culture · Gene expression

Introduction

Oral submucous fibrosis (OSMF) is prevalent most common in Southeast Asia, primarily due to the extensive consumption of areca nut products. While the heightened use of areca nut products is mostly limited to the Asia-Pacific region, it's important to note that 10–20% of the global population utilizes these products, and this can be partially attributed to emigrants from these regions [1–3]. The areca nut constituents, mainly arecoline contribute to fibrosis and

✉ Divyambika Catakapatri Venugopal
cvdivyambika@sriramachandra.edu.in

✉ Vijayalakshmi Ramshankar
r.vijayalakshmi@cancerinstitutewia.org

[1] Department of Oral Medicine and Radiology, Sri Ramachandra Dental College & Hospital, Sri Ramachandra Institute of Higher Education and Research (DU), Porur, Chennai 600 116, India

[2] Stem Cell & Regenerative Biology Laboratory, Faculty of Clinical Research, Sri Ramachandra Institute of Higher Education & Research (DU), Porur, Chennai 600 116, India

[3] Department of Preventive Oncology (Research), Cancer Institute (WIA), Adyar, Chennai 600020, India

[4] Department of Bioinformatics, Sri Ramachandra Institute of Higher Education and Research (DU), Porur, Chennai 600 116, India

[5] Department of Biomedical Science, School of Biosciences and Technology, Vellore Institute of Technology, Vellore 632 014, India

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hyalinisation of sub-epithelial tissues, thereby leading to clinical manifestations of loss of oral mucosal texture and trismus [4]. In vitro studies in human buccal fibroblasts have shown increased proliferation of fibroblasts and deposition of collagen on induction with arecoline, similar to the histopathological appearance of human OSMF tissues [5]. A scoping review has underlined the significance of reactive oxygen species (ROS) and oxidative stress in the etiology of fibrosis [6]. OSMF falls under oral potentially malignant disorders (OPMD) with a 7–30% risk of transforming into OSCC [7]. Current treatment options do not effectively alleviate symptoms or reduce the risk of malignant transformation, posing a global challenge for clinicians. A systematic review of OSMF medical management recommends more high-quality, multicentre trials with larger sample sizes to evaluate medicinal formulations' therapeutic efficacy [8–10]. Hence it is currently a global health priority to explore novel treatment modalities that can combat oxidative stress and reduce the risk of undergoing malignant transformation [8, 9].

The treatment of OSMF starts with cessation of habit; however, cessation alone does not cause reversal of fibrosis [9]. The goals of treatment of OSMF include a reduction in burning sensation, and improvement in mouth opening, thereby producing a better quality of life for OSMF patients [10, 11]. The array of treatment strategies investigated to date in OSMF includes corticosteroids (dexamethasone, betamethasone, hydrocortisone, triamcinolone) [12], enzymes such as hyaluronidase and collagenase [13]; antioxidants such as lycopene, curcumin, spirulina, aloe vera [14–16], cytokines like IFN-γ [17], placental extract [18], vitamins and micronutrients, vasodilators like pentoxifylline, isoxsuprine [19, 20], oral physiotherapy [21] and surgical approaches [22]. While various treatments like physical therapy, steroids, and surgery have been proposed for OSMF, their effectiveness in symptom relief and reducing malignancy transformation is limited [10, 23]. Clinical trials using nutrient antioxidants have shown promise in providing symptomatic relief, including increased mouth opening and reduced burning sensation [6]. Research on herbal antioxidants and antifibrotic agents could prove valuable in assessing their therapeutic potential for OSMF. In silico methods, including docking studies, offer a platform to screen compounds, select those with high activity, and identify novel therapeutic targets for further in vitro and in vivo experiments [24]. An in silico study to identify promising naturally derived compounds with anti-heat Shock Protein 47 (HSP 47) activity revealed silymarin to have the best binding affinity and serve as a potent therapeutic agent in controlling the abundant production of collagen in OSMF [25]. Silymarin, derived from milk thistle (*Silybum marianum*), comprises flavonoids like silydianin, silybin, isosilybin, and silychristin [26, 27] and offers antioxidant, anticancer, and anti-inflammatory benefits, effectively inhibiting hepatic fibrosis and modulating pathways linked to malignant transformation [27]. Enhanced silymarin absorption has led to safety evaluations, showing excellent animal tolerance, with headache and pruritus as the most common side effects in prolonged high-dosage use, without any reported life-threatening adverse events in randomized controlled trials [28]. In vitro studies of silymarin have demonstrated antioxidant, antibacterial, antifungal, and anticancer activity [29–31]. To the best of our knowledge, currently, no in vitro and in vivo studies are available on the assessment of the antifibrotic efficacy of silymarin in OSMF models. Despite two decades of efforts to find effective treatments for OSMF and prevent malignant transformation, no such strategies exist; therefore, we propose exploring the antifibrotic and anticancer activity of silymarin, a potent bioflavanoid, through in silico and in vitro studies.

Materials and methods

Molecular Docking

The chemical structures of compounds namely Silymarin, Curcumin, Quercetin, Baicalein, Luteolin, Lutein, Genistein, Emodin, and Baicalin were retreived from Pubchem (https://pubchem.ncbi.nlm.nih.gov/). The protein structures namely SRY-box transcription factor 2 (SOX-2), E-Cadherin (ECAD), Periostin (PTN), S100A7, Matrix metallopeptidase 9 (MMP-9), Heat Shock Protein 70 (HSP70), Transforming Growth Factor (TGF-β1), Carbonic anhydrase 2 (CA-2), Carbonic anhydrase 1 (CA-1) and 14-3-3 epsilon (14-3-3ε), were obtained from the Protein Data Bank (https://www.rcsb.org/) and the PDB ID is mentioned in Table 1. The protein and ligand structure was edited using BIOVIA Discovery Studio and saved in PDB and SYBL/MOL2 format respectively. Finally, the protein and ligand were docked using iGEMDOCK software [32]. The open chemistry database comprises all the secondary, and tertiary structures and

Table 1 Protein details obtained from PDB

Protein Name	PDB ID
HSP-70	6Z5N
PTN	5YJG
S100A7	1PSR
MMP-9	1I6J
CA-1	1CRM
14-3-3 ε	2BR9
SOX-2	6WX9
CA-2	1A42
TGF β-1	1PY5
ECAD	4ZTE

the molecular information about small molecules as well as large molecules including carbohydrates, nucleotides, lipids, peptides, and chemically modified macromolecules. Each of the compound's three-dimensional structures was downloaded in .sdf format (Table S11).

Protein and ligand preparation

The retrieved protein structure from the PDB database was pre-processed for the removal of water molecules, addition of hydrogen atoms, and elimination of pre-existing ligands, metal ions, and cofactors, resulting in a modified .pdb format for the protein. Ligand molecules were also analyzed in three dimensions and saved in .mol2 format.

iGEM Docking

The compounds were screened using iGEM docking software, which employs 70 generations to optimize binding energy, hydrogen bonding, and Vander Waal's interactions for each compound in the ligand library, and the best binding compound to the protein was selected based on combined pharmacological interactions and iGEMDOCK's energy-based scoring function, with rankings and visualization done by iGEM.

Also, the iGEMDOCK supports a hierarchical clustering method to cluster the screening compounds according to interaction profiles and atomic compositions. The compound similarity was measured by the atomic composition. The amino acid composition of the protein sequence was used for the measuring of the compound similarity. Finally, the software ranks and visualizes the screened compounds by combining the pharmacological interactions and energy-based scoring functions.

Molecular dynamic simulations

Molecular dynamic simulations were performed using GROMACS 2020.2. Topology files were made; topology contained all the information necessary to define the molecule within a simulation. This information included non-bonded parameters (atom types and charges) as well as bonded parameters (bonds, angles, and dihedrals).

Complexes were placed in an orthorhombic box with TIP3P water and salt counterions using gmxgromp and gmxgenion commands, maintaining a > 10 Å distance from box walls to prevent direct interactions with periodic images. Equilibration involved constrained minimizations and MD simulations under NPT (number of atoms N, pressure P, and temperature T) conditions, with the SHAKE algorithm applied for atom and ion restrictions. A 100 ns production MD run was conducted, followed by analysis using the XmGrace tool.

Patient tissue samples

The study was approved by the Institutional Ethical Committee (IEC No. NI/15/AUG/48/46) and was conducted at the Department of Oral Medicine and Radiology, Sri Ramachandra Institute of Higher Education and Research. All the cell culture related and molecular experiments were conducted at Cancer Institute WIA. All individuals provided written informed consent before tissue samples were collected.

Establishment of human oral buccal fibroblast cell line (HBF)

Healthy tissue samples were collected from buccal mucosa, collected during third molar extraction, were processed in Phosphate Buffer Saline (PBS) and 2x concentration of penicillin, amphotericin, streptomycin, ciprofloxacin, and gentamycin followed by buccal stromal cells isolation through collagenase digestion. Cells from passages 3 to 7 were cultured in 10% Fetal Bovine Serum (FBS), Dulbecco's Modified Eagle Medium (DMEM) at 37 °C with 5% CO_2 as described previously[33, 34].

Cytotoxicity Assessment

Cell viability and proliferation were assessed using an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) colorimetric test with 5×10^4 cells per well in 96-well plates exposed to various arecoline and silymarin concentrations for 24 and 72 h, followed by colorimetric measurement at 590 nm after MTT and DMSO treatments [35] with triplicates conducted for each condition, while cytotoxicity evaluation followed the same protocol.

Recovery using silymarin of arecoline treated cells

Buccal fibroblast cells (approximately 1,08,000 cells/well) were cultured in 10% FBS-DMEM with 1% penicillin, streptomycin, and amphotericin, then incubated at 37 °C with 5% CO_2 for initial attachment. Arecoline induction at 25 µM for 72 h was followed by treatment with 143 µM silymarin for 72 h to recover from fibrosis, with triplicates at all concentrations; the control medium contained no samples [36, 37].

RNA isolation and cDNA Conversion

RNA isolation using RNA iso Plus was performed by adding 750 µL onto the adherent cells in a 6-well plate, ensuring coverage, followed by RNA dissolution in nuclease-free water and storage at − 80 °C [38]. The Quantitect Reverse Transcription Kit (Qiagen Inc.) was used to convert 2 µg of RNA into cDNA, involving genomic DNA elimination with 2 µL of gDNA wipe-out buffer at 42 °C for 2 min, followed

by reverse transcription at 42 °C and inactivation at 95 °C; the resulting cDNA was stored at − 40 °C [39].

Real time PCR (RT-PCR)

The QuantiNova SYBR Green RT-PCR kit (Qiagen Inc.) was employed for SYBR Green-based real-time amplifications, using a 20 µL reaction with 10 µL of 2x QuantiNova SYBR Green RT-PCR Master Mix, 1 µL of each forward and reverse primer, 6 µL of nuclease-free water, and 2 µL of cDNA, following a thermal profile consisted of 30 min at 50 °C, 15 min at 95 °C, 45 cycles of 15 s at 94 °C, 30 s at Tm, and 30 s at 72 °C, followed by a melting curve ranging from 60 to 90 °C and run on a Rotor-Gene Q Real-Time PCR system, with triplicate reactions for gene expression studies namely TGF β, Col3A1, OCT3/4, SOX 2, COL1A1, COL1A2, PTN, VIM, LAMC2, MMP9, GLUT 1, CA1 and HSP 70 (Table S12), and relative expression levels were calculated using the (2-ddct) technique [40].

Statistical analysis

Clinicopathological parameters and gene expression-based statistical correlations were done using SPSS (IBM Corporation version 16) [41]. Student's t-test was used to calculate statistically significant differences between the groups in gene expression during real-time PCR. The statistical significance was determined using a p value less than 0.05.

Results

Docking studies

In silico studies employing iGEMDOCK software identified silymarin and baicalein as top compounds with maximum affinity for proven malignancy biomarkers. Specifically, silymarin exhibited the highest affinity for TGF-β1, a key player in OSMF pathogenesis and malignant transformation, further validated through Molecular Dynamic Simulation (MDS) in GROMACS over 100ns, confirming strong binding energy, amino acid interactions, and hydrogen bonds. The antifibrotic potential of silymarin was assessed using arecoline-induced human primary buccal fibroblast cell lines, serving as a relevant OSMF model for drug efficacy studies (Table S1 to S10; Figure S1 to S10).

Molecular dynamic simulation

Molecular dynamics simulations of TGF-β (PDB ID: 1PY5) and silymarin over 100 ns revealed stable behavior with a root-mean-square deviation (RMSD) value of 0.35 nm. The RMSD values remained low for both the native protein and the 1PY5-silymarin complex, indicating stable docking (Fig. 1). In Gyration plots, both the native protein and 1PY5 – silymarin complex showed minimal fluctuations in compactness, with the complex maintaining tight uniformity (Fig. 2).

RMS fluctuations highlighted increased compactness in the 1PY5-silymarin complex, particularly at residue 325 (Fig. 3). The results depicted that the binding of silymarin had made the protein more compact. Hydrogen bonds remained strong with minor fluctuations, ranging from 2 to 5 bonds (Fig. 4). Binding energy calculations identified key residues like Glycine, Tryptophan, Leucine, and Isoleucine contributing to complex stability as depicted in Fig. 5, while residues such as Glutamine, Tyrosine, Asparagine, and Lysine had destabilizing effects (Table 2).

Cytotoxicity Assessment

Arecoline concentrations ranging from 25 to 200 µM (Fig. 6) induced fibrosis in human buccal fibroblasts, with an IC_{50} of 25 µM for cell death seen in Fig. 7. Silymarin exhibited higher cytotoxicity to HBFs above 140 µM, with an IC_{50} of 143 µM and inhibition of cell proliferation at 50, 100, and 200 µM concentrations and inhibited up to 34.2%, 45.2%, and 66.31% (Fig. 8).

Assessment of gene markers on silymarin-treated arecoline-induced fibrosis in HBF

Arecoline (25 µM) induced fibroblasts showed upregulation of HSP 70, laminin, GLUT 1, OCT3/4, SOX 2, vimentin, PTN, MMP 9, CA1, COL1A1, COL1 A2, COL3A1, TGF-β, which was downregulated after treatment with silymarin (143 µM) at 72 h (Fig. 9). We found a significant downregulation of the genes previously upregulated by arecoline post-silymarin treatment with a p value. Interestingly, the genes involved in collagen metabolism namely COL1A1, COL1A2, and COL3A1 were significantly downregulated implying the antifibrotic potential of silymarin. The differential expression profiles are depicted in the heatmap as well (Fig. 10).

Discussion

In silico methods enable the screening of potential therapeutic compounds against molecular targets, facilitating the selection of candidates for further experiments [24]. Bioflavonoids known for their antifibrotic and antioxidant properties, including quercetin, baicalein, genistein, lutein, and luteolin, were identified in the literature search for its potential role in in renal and lung fibrosis [42–45]. These compounds, along with silymarin, curcumin, and others

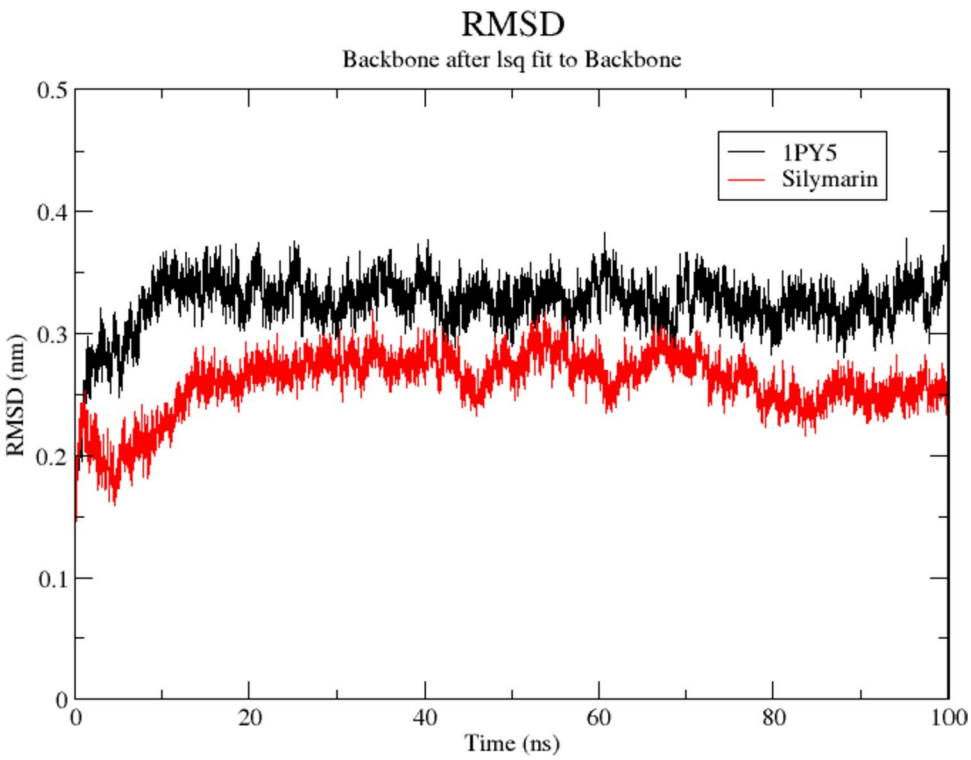

Fig. 1 RMSD plot for 100ns time period to check conformational stability of 1PY5 – silymarin complex (Red) and native protein (Black). (Color figure online)

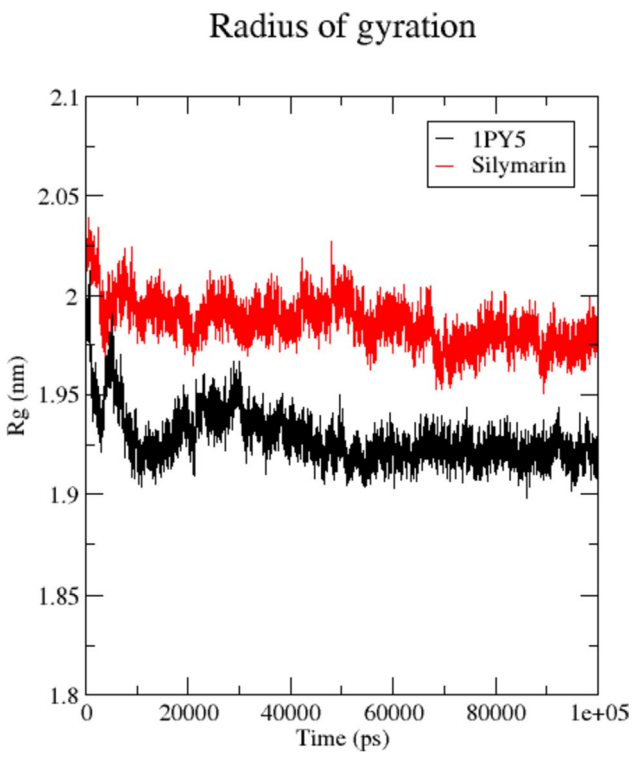

Fig. 2 The radius of Gyration of the protein Ca backbone atoms of 1PY5 – silymarin complex (red) and native protein (black) for 100 ns time scale. (Color figure online)

recognized for their anticancer and antifibrotic effects, were docked with various targets, including the HSP 70, CA 1, CA 2, 14-3-3ε, SOX 2, ECAD, PTN, S 100 A7, MMP-9, and TGF β1. TGF β1 in OSMF pathogenesis and carcinomatous transformation was validated through molecular dynamics simulations.

In this study, iGEMDOCK was chosen for its integrated virtual screening capabilities [32], cluster analysis, and graphical interface. We have shown molecular dynamic simulations of TGF β and silymarin interactions showing stable behaviour and stable docking. Silymarin and baicalein emerged as top compounds in molecular docking, with silymarin showing a high affinity for TGF-β1, a key pathway in OSMF. Molecular Dynamics Simulation (MDS) was employed to validate silymarin's stability, demonstrating its significance in understanding ligand-protein interactions for drug design [46]. GROMACS software was used for Molecular Dynamics Simulation (MDS) due to its user-friendly nature. The study demonstrated silymarin's conformational stability with TGF β1, highlighting hydrogen bonds and favorable binding and electrostatic energies. Comparative analysis with previous research underscored silymarin's superior binding affinity to relevant targets, reinforcing its potential as a therapeutic candidate for OSMF.

Silymarin has been found to exhibit anti-inflammatory properties through the ability to suppress inflammatory cytokines, COX-2, 5-lipooxygenase (LOX), and NF-κB activation [27]. Besides anti-inflammatory and anti-oxidant

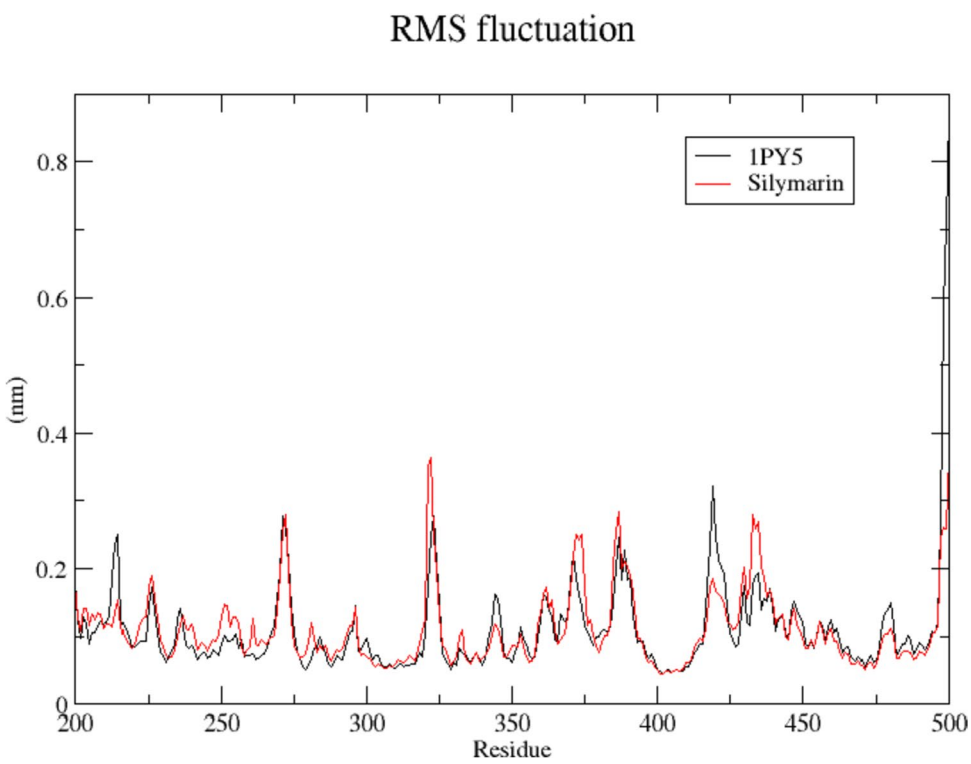

Fig. 3 RMSF plot of 1PY5 – silymarin complex (red) and native protein (black) for 100 ns time scale. (Color figure online)

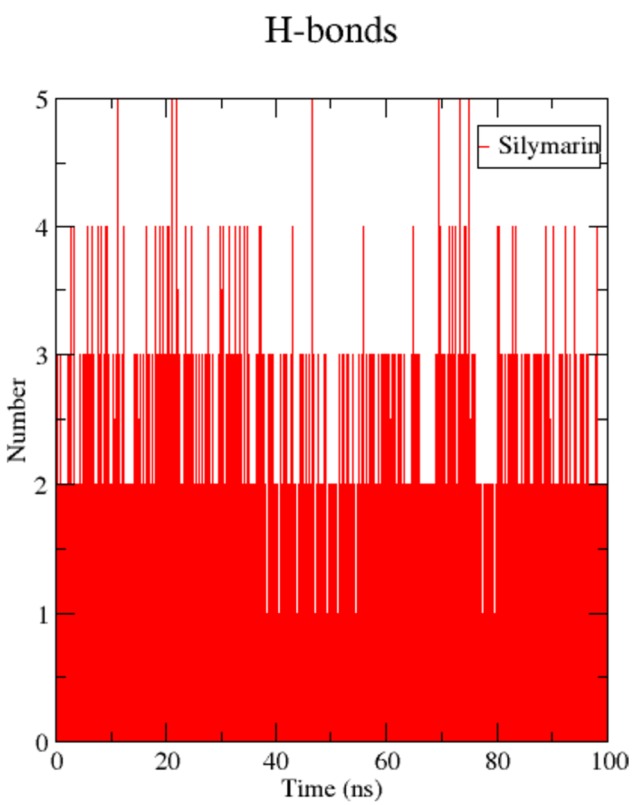

Fig. 4 H– bond formed between 1PY5- Silymarin complex during 100 ns time scale

properties, silymarin has been shown to demonstrate anticancer activity. Silymarin has been shown to express anticancer activity by induction of DR5/caspase-8 apoptotic signalling in cell lines and also suppressing the tumor activity in vivo without causing hepatic or renal toxicity [31]. Primary human buccal fibroblasts were established from healthy volunteers undergoing third molar extraction, and arecoline was used to induce fibrosis in vitro to establish an OSMF model. The IC_{50} value for arecoline was determined to be 25 µM at 24 and 48 h, confirming fibrosis induction. Silymarin was administered at an IC_{50} of 143 µM for 72 h to assess its mRNA expression using qPCR, suggesting its potential therapeutic role in OSMF.

The study demonstrated significant downregulation of key markers including collagen markers (COL1A1, COL1A2, COL1A3), cancer stem cell markers (OCT3/4, SOX2), EMT markers (LAMC2, vimentin, MMP9), hypoxia marker (GLUT1, CA1), angiogenesis and invasion marker (PTN), and stress marker (HSP70) in silymarin-treated arecoline-induced primary buccal fibroblast cells. This aligns with the findings from a study using ethanolic leaf extract of *Ocimum basilicum* and linalool, which also showed significant downregulation of collagen markers (COL1A2, COL3A1) and TGF-beta [47].

Furthermore, the increased expression of cancer stem cell (CSC) markers like SOX2 and Bmi1 in OSMF suggests their role in abnormal proliferation associated with OSCC transformation. Extensive research on SOX2 and OCT3/4 in OSCC indicates their significance as prognostic markers,

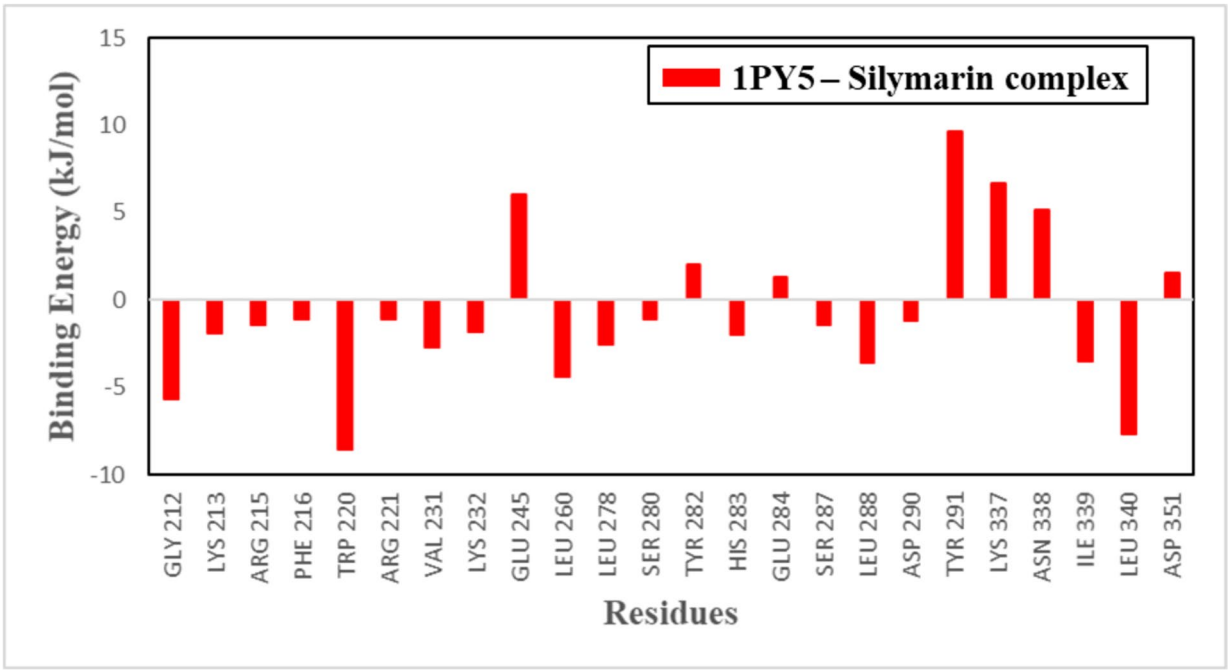

Fig. 5 Contribution of important binding residues of the 1PY5-Silymarin complex to the total binding free energy through MM/PBSA binding energy calculation method during 100 ns MD-simulation. The (− ve) values indicate stable complex formation of the complexes while the (+ ve) values indicate a destabilizing effect

Table 2 The energy contribution of 1PY5- Silymarin complex was calculated using the MM/PBSA method

Energy parameter (kJ/mol)	1PY5-Silymarin
Binding energy	−149.35 +/− 46.60
Electrostatic energy	−18.57 +/− 6.90
Polar solvation energy	121.28 +/− 39.43
SASA energy	−22.54 +/− 6.52
Van der Waal energy	−229.51 +/− 66.04

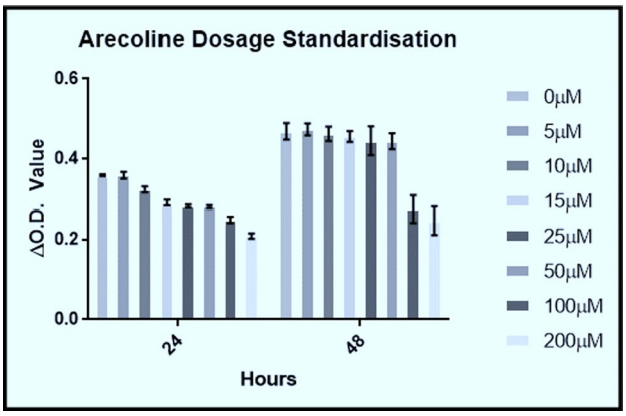

Fig. 6 Arecoline dosage standardisation in various concentrations

reflecting disease severity and chemoresistance. The study highlights silymarin's potential in preventing malignant transformation through significant downregulation of these markers and its role in reducing abnormal collagen deposition associated with OSMF. Additionally, the downregulation of stress marker HSP70 indicates silymarin's ability to mitigate oxidative stress, thereby reducing ROS-related DNA damage and malignant transformation, as observed in OSMF. Overexpression of CA1 in OSMF and OSMF with malignant changes underscores its role as a potential marker in the disease [48].

The current study revealed that silymarin effectively downregulated collagen, cancer stem cells, EMT, hypoxia, angiogenesis, and stress markers in arecoline-induced human buccal fibroblast cells, suggesting its potential as a novel therapeutic antioxidant for OSMF, thus making it worthwhile to further explore silymarin as a novel therapeutic antioxidant compound for the treatment of OSMF.

Conclusion

Silymarin has shown promising anti-fibrotic potential in the in silico studies, by displaying better binding affinity with the targets, when compared to other antifibrotic compounds and demonstrating good conformational stability

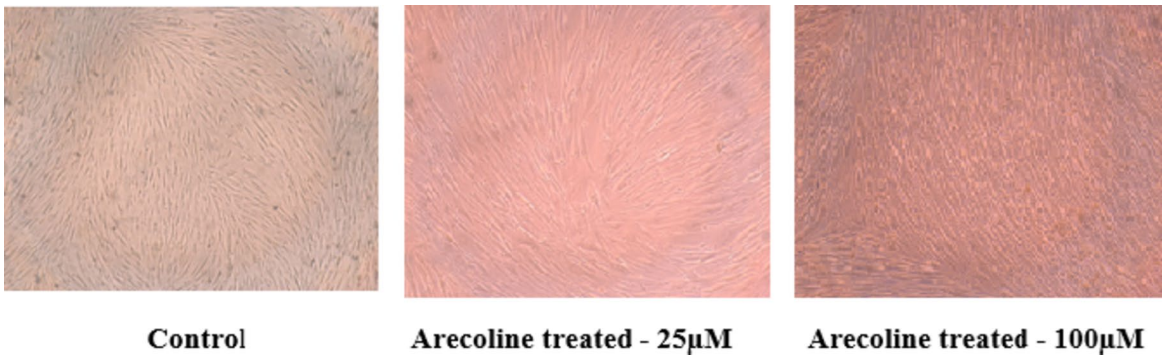

Fig. 7 Induction of fibrosis using arecoline in human buccal fibroblast cells at various concentrations

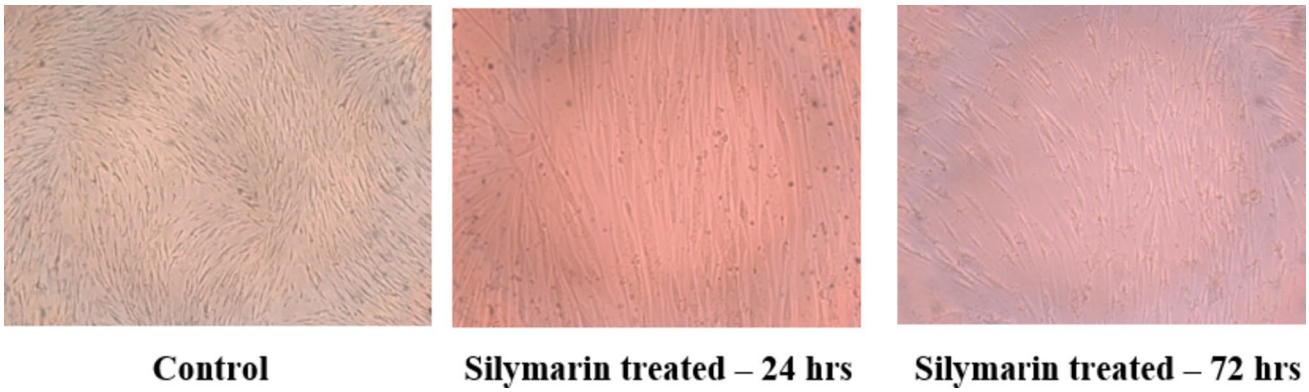

Fig. 8 Silymarin treated human buccal fibroblast cells in different time intervals

in molecular dynamic simulation studies. Silymarin was found to be a promising drug with in silico studies and hence, the current study further evaluated its antifibrotic effect using in vitro studies employing arecoline-induced human primary buccal fibroblast cell lines. The downregulation of EMT markers by silymarin in arecoline-induced HBF proves the potent role of silymarin in arresting the EMT pathway in OSMF. Moreover, downregulation of genes involved in cancer progression and cancer stem cell markers proves silymarin to be a viable bioflavonoid with potent anticancer activity. Thus, with the literature support favoring antioxidant, antifibrotic, and anticancer activity and the promising results of downregulation of genes involved in fibrogenesis and tumorigenesis, it is worthwhile to conduct future research including clinical trials on the therapeutic efficacy of novel bioflavonoid, silymarin in OSMF.

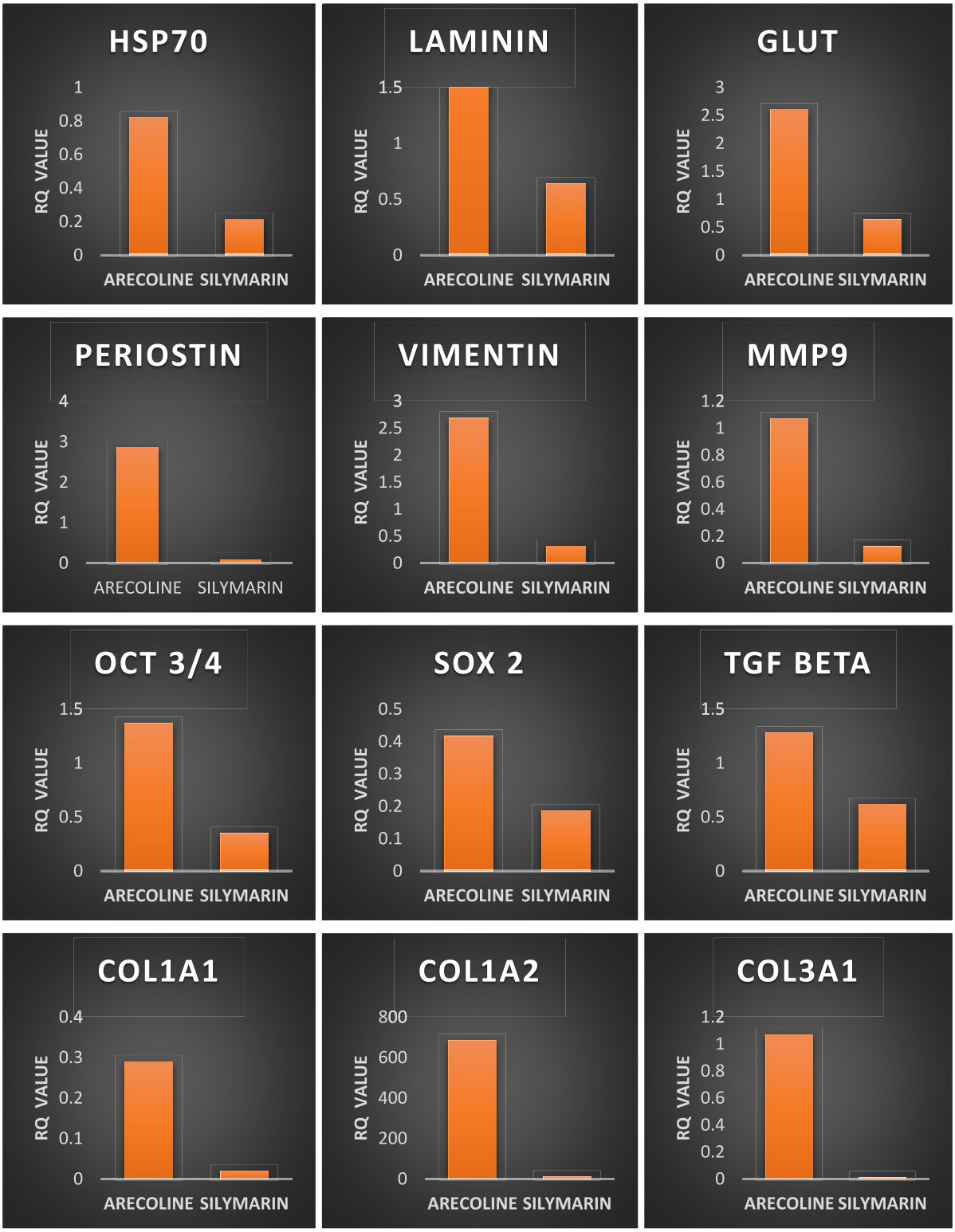

Fig. 9 Gene expression of various markers in arecoline-induced and silymarin-treated HBF

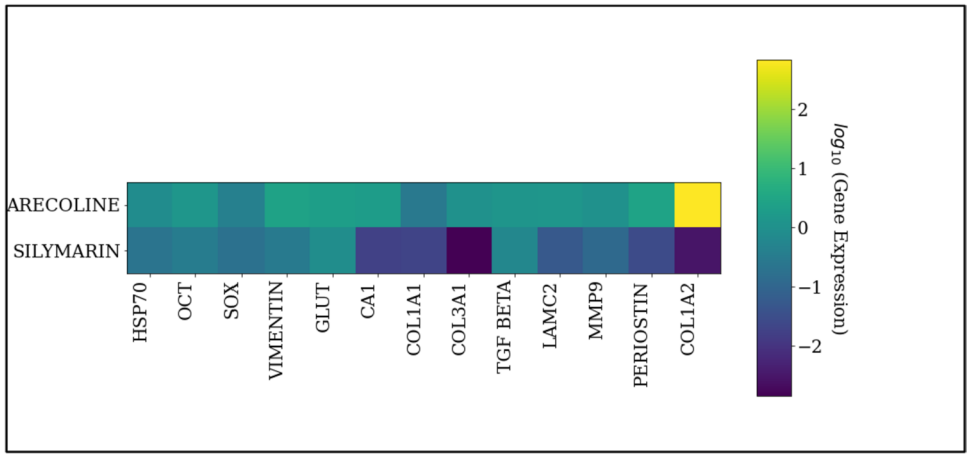

Fig. 10 Heatmap representation of gene expression of various markers in arecoline-induced and silymarin-treated HBF

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11033-023-09177-8.

Author contributions Conceptualization: DCV, VR and SS; Methodology: DCV, SR, PV, AMP, MY and VR; Validation: DGDJ and LP; Resources: GR and SS; Writing—original draft preparation: DCV and MY; Writing—review and editing: SR and VR; Supervision: SS and VR; Project administration: VR. All authors have read and agreed to the published version of the manuscript.

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Data availability All data supporting the findings of this study are available within the paper and its Supplementary Information.

Declaration

Competing interests The authors declare no competing interests.

Ethical approval The study was approved by the Institutional Ethical Committee (IEC No. NI/15/AUG/48/46), SRIHER.

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