

In Silico Molecular Docking of Chelidonic Acid across Multiple Targets Associated with Carcinogenesis

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ABSTRACT:

Background: Cancers can result from dysfunction of interrelated molecular pathways, including oxidative stress, inflammation, proliferation, apoptosis, and angiogenesis. Chelidonic acid (CA) is a naturally occurring organic heterocyclic acid with reported antioxidant and anti-inflammatory effects. However, its molecular interactions with cancer-related targets remain unexplored. **Objective:** This study aims to investigate CA's molecular interaction profile against various proteins associated with carcinogenesis. **Methods:** We conducted molecular docking studies using AutoDock Vina with 23 cancer-associated protein targets, including proteins involved in oxidative stress and antioxidant defense, inflammatory responses, apoptosis, cell proliferation and cell-cycle regulation, PI3K/AKT/mTOR signaling, invasion, and angiogenesis. The interactions between proteins and ligands were visualized using BIOVIA Discovery Studio Visualizer. Ligand efficiency (LE) was calculated as docking score/number of heavy atoms in CA. The docking protocol was further validated by redocking suitable co-crystallized/native ligands using the same docking parameters, and RMSD values were calculated using PyMOL. **Results:** CA showed predicted docking scores ranging from -7.5 to -5.4 kcal/mol across the 23 target proteins. The most negative predicted docking score was observed for CAT (-7.5), followed by GR (-6.9), caspase-9 (-6.7), COX-2 (-6.6), MMP-9 (-6.6), and PI3K α (-6.5). Other predicted interactions were observed with p53 (-6.4), caspase-3 (-6.3), AKT1 (-6.3), Keap1 (-6.3), MMP-2 (-6.2), HO-1 (-6.1), VEGFR2 (-6.1), GST (-6.0), c-Myc/Max (-5.9), IL-6 (-5.9), mTOR (-5.9), TNF- α (-5.8), NF- κ B p52 (-5.6), iNOS (-5.6), GPX4 (-5.5), PCNA (-5.5) and SOD (-5.4). Various binding interactions were observed with the protein targets, including hydrogen, carbon-hydrogen, π -mediated interactions, van der Waals forces and hydrophobic contacts. Redocking validation was performed for 10 targets, showing RMSD values below 2.0 Å. Ligand efficiency values ranged from -0.577 to -0.415 kcal/mol/HA, with the more negative LE observed for CAT. **Conclusion:** These findings indicate that CA can interact with several interconnected molecular pathways involved in carcinogenesis.

KEYWORDS: Angiogenesis, Apoptosis, Cancer, Cell proliferation, Chelidonic acid, Molecular docking, Oxidative stress.

RECEIVED ON:
04-09-2026

REVISED ON:
18-09-2026; 07-10-2026

ACCEPTED ON:
11-10-2026

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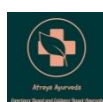
<https://doi.org/10.70066/jahm.v14i9.3041>

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CITE THIS ARTICLE AS

Harish Krishnan, Sivasankaran Shanmugam Manoharan, Vidhyambigai Balasubramaniyan, Manoharan Shanmugam. In Silico Molecular Docking of Chelidonic Acid across Multiple Targets Associated with Carcinogenesis. Journal of Ayurveda and Holistic Medicine (JAHM) 2026; 14(9):98-110.



1. INTRODUCTION

Identifying novel, safer molecules that can modulate multiple molecular events involved in carcinogenesis remains an important research priority. Carcinogenesis is associated with the dysregulation of oxidative stress, inflammation, cellular proliferation, cell cycle progression, apoptosis, and angiogenesis. At the molecular level, dysregulated signaling pathways such as phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR), along with alterations in tumor suppressor genes and cell cycle regulators (p53, cyclin-dependent kinases (CDKs), and c-Myc), contribute to cancer development and progression. Moreover, activation of inflammatory mediators such as nuclear factor kappa B (NF- κ B), cyclooxygenase-2 (COX-2), interleukins, and inducible nitric oxide synthase (iNOS) contributes to a pro-inflammatory microenvironment. In addition, dysregulation of matrix metalloproteinases and vascular endothelial growth factor (VEGF) can promote extracellular matrix (ECM) degradation, angiogenesis, and tumor invasion, thereby contributing to uncontrolled cellular proliferation and survival. [1,2] Thus, the close mechanistic links between the molecular events warrant study of the compounds to modulate various pathways together rather than focusing on a single molecular target for anticancer treatments.

Oxidative stress is a significant parameter in chemically induced cancer development. Overproduction of reactive oxygen species (ROS) leads to redox imbalance in cells and oxidative injury to lipids, proteins, and nucleic acids. Disruption of the antioxidant system, including superoxide dismutase (SOD), Catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), and GST, promotes oxidative stress and cellular injury. Prolonged oxidative stress activates inflammatory and survival-signaling pathways, which may contribute to apoptotic resistance and abnormal cell proliferation. [3,4] Thus, investigating natural products that target oxidative stress and cancer-

associated signaling pathways may provide promising candidates for further anticancer research.

Small molecule drugs are low molecular weight compounds that can support a cellular response upon binding to a target. Their low molecular weight and distinct physicochemical properties may improve their cellular permeability, oral bioavailability, tissue distribution and selective recognition by target molecules. Consequently, small molecules have remained a primary class of drug discovery and development in modern drug development, including anticancer drug discovery. [5] However, conventional small-molecule-based anticancer drugs have certain disadvantages such as poor therapeutic efficacy, side effects and drug resistance. Therefore, the search for new anticancer agents that are more effective and safer is required. Natural products are an excellent source of small molecules and exhibit great structural diversity. Plant-derived agents represent a promising starting point for the design of novel anticancer drugs because of their ability to interact with multiple biological targets. Several studies reported that phytochemicals regulate a variety of mechanisms implicated in cancer. [6,7]

Chelidonium majus L. (greater celandine) is a traditionally used medicinal plant in the Papaveraceae family and has a diverse phytochemical composition. Several bioactive constituents of *C. majus*, including chelidonine, sanguinarine, berberine, and chelidonic acid (CA), have attracted scientific interest because of their reported pharmacological properties. [8] Chelidonic acid, chemically known as 4-oxo-4H-pyran-2,6-dicarboxylic acid ([Figure 1](#)), has a molecular weight of 184.10 g/mol and is a naturally occurring pyran-derived organic acid mainly found in *C. majus* and also reported in *Sorghum vulgare*, *Cassia spectabilis*, and *Gloriosa superba*. Earlier studies have identified potent antioxidant, anti-inflammatory, and immunomodulatory properties of CA, suggesting that it could be a biologically active compound. [9] However, its molecular interactions with proteins involved in cancer have not been comprehensively investigated. In particular,

data remain limited on its interactions with protein targets involved in oxidative stress, inflammation, apoptosis, cell proliferation, cell-cycle regulation, intracellular signaling, ECM remodeling, and angiogenesis. Therefore, a systematic investigation of CA's interaction profile against a broad spectrum of carcinogenesis-associated proteins may provide valuable insights into its potential molecular mechanisms.

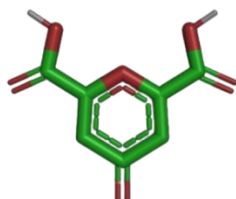


Figure 1: 3D molecular structure of CA used for molecular docking analysis.

In silico approaches are valuable tools in the early stage of drug discovery. Molecular docking is a widely used computational approach for predicting ligand binding interactions within a target protein's binding pocket and identifying interacting amino acid (AA) residues and noncovalent interactions. [10] Such structural insights may provide a preliminary molecular basis for understanding the potential biological activity of natural compounds and help in identifying promising molecular targets for experimental models. As evaluated by Khairnar et al. [11], the ADMET of CA was predicted to be favorable, water-soluble, GI-absorbable, with a bioavailability score of 0.56 and no major alerts associated with toxicity. Thus, the current study aimed to analyze the *in silico* molecular interactions of CA against 23 cancer-associated protein targets using molecular docking with AutoDock Vina.

2. MATERIALS AND METHODS

Ligand selection and preparation: We downloaded the 3D structure of chelidonic acid (CID: 7431) from PubChem in Legacy PDB format. The PubChem3D conformer represents a computationally generated, energetically accessible structure. Therefore, we retained the downloaded conformer as the standardized initial structure without applying additional independent geometry optimization or

energy minimization. This approach avoided additional conformational modification before docking while allowing ligand positional, orientational, and torsional conformational sampling during the docking calculation. The ligand was prepared using AutoDockTools (MGL Tools v1.5.7) by adding hydrogen atoms and assigning Gasteiger partial charges, followed by conversion to PDBQT format. Chelidonic acid was docked in its neutral protonation state with the carboxylic acid groups represented as COOH.

Target selection strategy: The target proteins were selected using a literature-guided and pathway-based approach based on their established roles in carcinogenesis associated processes such as oxidative stress and antioxidant responses (CAT, GR, HO-1, GST, GPX4 and SOD), apoptosis and cell cycle control (p53, caspase-9, caspase-3, c-Myc/Max and PCNA), intracellular survival pathways (PI3K α , AKT1, mTOR and Keap1), inflammatory mediators (COX-2, IL-6, NF- κ B, TNF- α and iNOS) and tumor invasion and angiogenesis (MMP-2, MMP-9 and VEGFR2). A systematic literature search was conducted using PubMed, Google Scholar, ScienceDirect, Scopus, and Web of Science, using combinations of keywords such as "cancer", "carcinogenesis", cancer with "oxidative stress", "inflammation", "apoptosis", "cell cycle", "PI3K/AKT/mTOR signaling pathway", "tumor invasion," and "angiogenesis" along with the names of individual candidate targets. We conducted the search from Jan 2020 to May 2026. Targets were included when (i) published evidence supported a relevant role in carcinogenesis, (ii) the target was a human protein (*Homo sapiens*), and (iii) a suitable experimentally determined three-dimensional structure was available in the RCSB Protein Data Bank. We excluded targets when evidence for carcinogenesis-related relevance was insufficient, when no suitable human protein structure was available, or when available structures lacked an appropriate, interpretable binding/catalytic site for molecular docking. For the final structural screening, we evaluated PDB entries based on structural resolution, absence of mutations, well-defined binding/catalytic sites,

structural completeness, appropriate chain/domain selection, and retention of biologically relevant cofactors, prosthetic groups, or catalytic metals where required. Following this screening, we selected 23 proteins representing interconnected carcinogenesis-associated pathways for the present study.

Protein selection and preparation: The 3D structures of 23 protein targets related to carcinogenesis were retrieved from RCSB PDB: CAT (1DGF), GR (3GRS), HO-1 (3CZY), GST (5J41), GPX4 (6HN3), SOD (5VF9), p53 (2OCJ), caspase-9 (1JXQ), caspase-3 (5I9B), c-Myc/Max (6G6K), PCNA (1AXC), PI3K α (4JPS), mTOR (4JSV), AKT1 (3O96), Keap1 (4IQK), COX-2 (5KIR), IL-6 (1ALU), NF- κ B p52 (1A3Q), TNF- α (2AZ5), iNOS (3E7G), MMP-9 (1GKC), MMP-2 (7XJO) and VEGFR2 (3VO3).

We selected PDB structures based on the availability of experimentally determined human (*Homo sapiens*) protein structures, suitable/high structural resolution, absence of relevant mutations, well-defined biologically relevant binding or catalytic sites, and acceptable ligand structure quality. We also assessed structural completeness, appropriate cofactor availability, and suitability for molecular docking (Supplementary Table 1).

We prepared the experimentally determined protein structures using BIOVIA Discovery Studio (v25.1.0.24284). We removed water molecules and other unnecessary crystallographic constituents. We included biologically significant cofactors, prosthetic groups, and catalytic metals where required, based on their roles in the corresponding target proteins. We further processed the prepared protein structures in AutoDockTools (MGL Tools v1.5.7), then converted them to the PDBQT format by adding polar hydrogen atoms and assigning Kollman partial charges.

Binding-site prediction and grid-box definition: We predicted the binding cavities for each protein using BIOVIA Discovery Studio (v25.1.0.24284). We selected the most appropriate pocket for each target protein and used its geometric center as the docking box center. We used a 40 $\times$ 40 $\times$ 40 Å grid box for all targets, set exhaustiveness to 8,

and set the energy range to 4 kcal/mol. We determined target-specific grid-center coordinates based on the selected cavity. Supplementary Table 2 provides the corresponding grid-center coordinate for all 23 targets.

For targets with appropriate co-crystallized ligands, we compared the selected docking region with the corresponding co-crystallized ligand-binding region. For targets lacking a suitable experimentally defined ligand-binding site, we identified the docking pocket from computationally predicted cavities using BIOVIA Discovery Studio. Supplementary Table 3 lists the basis for binding-site selection for each target, whether co-crystallized ligand sites or computationally predicted cavities.

Molecular docking: We performed docking using AutoDock Vina (v1.2.0), docking the prepared CA ligand into each of the 23 receptor proteins in flexible-ligand and rigid-receptor configurations, respectively. We used protein-specific configuration files, including receptor and ligand PDBQT files, grid-box parameters, exhaustiveness, and energy range, for docking. We generated nine conformations for each protein-ligand complex and ranked them by predicted docking score. We then selected the conformation with the lowest binding energy to analyze CA binding interactions.

Visualizing and analyzing protein-ligand interactions: The selected docking poses were evaluated in BIOVIA Discovery Studio Visualizer/Client 2025 to visualize ligand binding (2D and 3D diagrams) and identify key AA residues and their interactions. [Figure 2](#) shows the molecular docking workflow.

Docking validation-RMSD: We validated docking by re-docking the co-crystallized/native ligands into their corresponding protein pockets using the same AutoDock Vina procedure used for chelidonic acid. We used the same target-specific grid center coordinates (X, Y, and Z), grid dimensions, exhaustiveness, energy range, and docking parameters for re-docking. We assessed the protocol's reproducibility by comparing the redocked ligand pose with the experimentally determined crystallographic pose. PyMOL (v 3.1.6.1) calculated the RMSD by aligning

corresponding atoms of the crystallographic and redocked ligand poses. An RMSD of <2.0 Å indicated acceptable reproduction of the experimentally observed binding mode. Redocking of the native ligands was carried out for 10 targets where appropriate native/co-crystallized ligands were available, including: HO-1 (3CZY), AKT1 (3O96), COX-2 (5KIR), Keap1 (4IQK), MMP-9 (1GKC), MMP-2 (7XJO), PI3Kα (4JPS), VEGFR2 (3VO3), iNOS (3E7G), and TNF-α (2A25). Supplementary Table 4 provides target-specific justification for proteins not selected for RMSD validation.

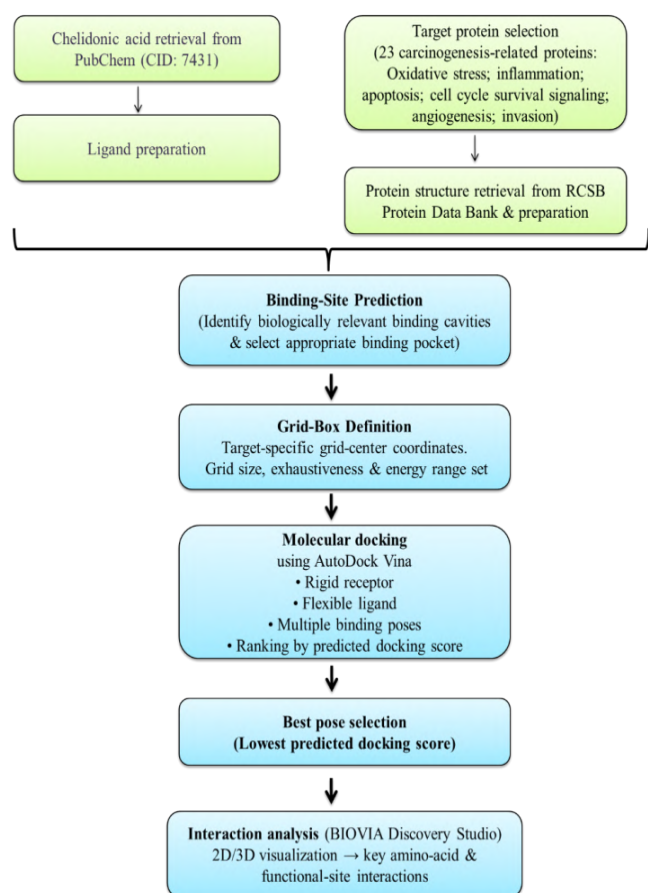


Figure 2: Computational workflow for molecular docking

Ligand efficiency analysis: We calculated the number of heavy atoms in CA from its molecular structure and excluded hydrogen atoms. For CA (C₇H₄O₆; 13 heavy atoms), ligand efficiency (LE) was calculated as:

$$LE = \frac{\text{Binding affinity (kcal/mol)}}{\text{Number of heavy atoms}}$$

The calculated LE values were expressed as kcal/mol/heavy atom (kcal/mol/HA) and represent predicted docking scores normalized by the number of HA in the ligand. [12]

3. RESULTS

Molecular docking analysis of CA against cancer-associated target proteins:

The docking analysis showed predicted interactions between CA and all 23 cancer-associated proteins, with docking scores ranging from -7.5 to -5.4 kcal/mol. The most negative predicted docking score was observed for CAT (-7.5), followed by GR (-6.9), caspase-9 (-6.7), COX-2 (-6.6), MMP-9 (-6.6), and PI3Kα (-6.5). The docking poses revealed interactions involving multiple amino acid residues through hydrogen, carbon-hydrogen (C-H), π-alkyl, π-cation, π-anion, π-sigma, π-donor H, π-sulfur, π-π, T-shaped, and π-π interactions.

Binding interaction with oxidative stress-associated antioxidant proteins:

CA exhibited predicted interactions with oxidative stress and antioxidant defense proteins ranging from -7.5 to -5.4 kcal/mol. CAT showed the most negative predicted docking score (-7.5) interacting through Arg72, Arg112, Ser114, Ala133, Val146, Phe334 and His362 followed by GR (-6.9) stabilized by Gly29, Gly32, Thr57, Gly157, Asp331, Leu338, Thr339 and Ala342. Heme oxygenase-1 (HO-1) (-6.1) interacted with Lys148, Glu162, Gln145, Phe169, Pro99, Thr168 and Ala94, while GST showed contact with Arg74 (2), Gly80, Tyr79 and Ala86 with predicted docking score of -6.0 kcal/mol. GPX4 showed a score of -5.5 with Lys90, Ala94, Lys99, Phe100, and Met102. SOD showed the least negative predicted docking score (-5.4), with contacts to Thr55, Val54, Ile72, Gln47, and Ser75. The protein-ligand complexes were stabilized through various interactions, including hydrogen, carbon-hydrogen, π-anion, π-sigma, π-π stacking, π-cation, and π-alkyl interactions (Figure 3 and Table 1).

Binding interaction with apoptosis-associated proteins:

CA exhibited relatively more negative predicted interactions with apoptosis-related proteins and predicted docking scores between -6.7 and -6.3 kcal/mol. caspase-9 protein demonstrated the most negative predicted docking score (-6.7), followed by p53 (-6.4) and caspase-3 (-6.3). Stabilization of caspase-9 occurred through interactions with Ala376, Val379, Trp360, Arg372 and Ile356. The p53

protein had interactions with Tyr107, Asn239, Leu137 and His179. Caspase 3 interacted with Arg64, Gln161, Ser120, Arg207, and Cys163. These protein-ligand complexes are

stabilized through hydrogen, π -cation, π -sigma, and π -alkyl interactions ([Figure 4](#) and [Table 2](#)).

Table 1: The binding interactions profile and ligand efficiency of CA with oxidative stress-associated antioxidant protein targets.

Protein target	PDB ID	Predicted docking score (kcal/mol)	Ligand efficiency (kcal/mol/HA)	AA residues	Contacts
CAT	1DGF	-7.5	-0.577	Arg72, Arg112, Ser114, Ala133, Val146, Phe334, His362	H and π -alkyl
GR	3GRS	-6.9	-0.531	Gly29, Gly32, Thr57, Gly157, Asp331, Leu338, Thr339, Ala342	H; C-H; π -anion; π -alkyl
HO-1	3CZY	-6.1	-0.469	Lys148, Glu162, Gln145, Phe169, Pro99, Thr168, Ala94	H; C-H; π -sigma; π -alkyl
GST	5J41	-6.0	-0.462	Arg74 (2), Gly80, Tyr79, Ala86	H; C-H; π - π stacked; π -alkyl
GPX4	6HN3	-5.5	-0.423	Lys90, Ala94, Lys99, Phe100, Met102	H; π -cation; π -alkyl bond
SOD	5VF9	-5.4	-0.415	Thr55, Val54, Ile72, Gln47 and Ser75	H; C-H; π -sigma

Table 2: The binding interactions profile and ligand efficiency of CA with apoptosis-associated proteins.

Protein target	PDB ID	Predicted docking score (kcal/mol)	Ligand efficiency (kcal/mol/HA)	AA residues	Contacts
Caspase-9	1JXQ	-6.7	-0.515	Ala376, Val379, Trp360, Arg372, Ile356	H; π -sigma; π -alkyl
p53	2OCJ	-6.4	-0.492	Tyr107, Asn239, Leu137, His179	H; π -alkyl
Caspase-3	5I9B	-6.3	-0.485	Arg64, Gln161, Ser120, Arg207, Cys163	H; π -cation; π -alkyl

Binding interaction with cell proliferation and cell-cycle regulatory proteins: CA showed predicted interactions with proteins involved in cell proliferation and cell-cycle regulation, with predicted docking scores ranging from -5.9 to -5.5 kcal/mol. c-Myc/Max showed a predicted docking score of -5.9; interactions occurred with Arg214, Leu210, Lys944, Thr947, and Ser224. PCNA showed a predicted docking score of -5.5, involving Lys13, Lys80, Arg146, Arg149, and Ala82. The protein-ligand complexes were stabilized through various contacts, including hydrogen, carbon-hydrogen, π -sigma, and π -alkyl bonds ([Table 3](#) and [Figure 5](#)).

Binding interaction with PI3K/AKT/mTOR and Keap1-associated signaling proteins: CA shows predicted

interactions with key intracellular signaling proteins, with docking scores ranging from -6.5 to -5.9 kcal/mol. The most negative predicted docking scores were observed for PI3K α (-6.5). The PI3K α showed contacts with His670, Phe666, Arg818, Met811 and Cys838. The predicted docking scores of AKT1 and Keap1 were found to be -6.3 and occurred with Trp80, Ser205, Leu210, Thr211, Ala212 and Leu213 in AKT1 and Val606, Gly367, Gly558, Ala366 and Val465 in Keap1. The predicted docking score of mTOR (-5.9) showed contacts with Ile2237, Leu2185, Ile2356, Gly2238, Val2240, Tyr2225. These protein-ligand complexes were stabilized through various contacts, including hydrogen, carbon-hydrogen, π -cation, π - π T-shaped, π -sigma, π - π stacking, and π -alkyl ([Figure 6](#) and [Table 4](#)).

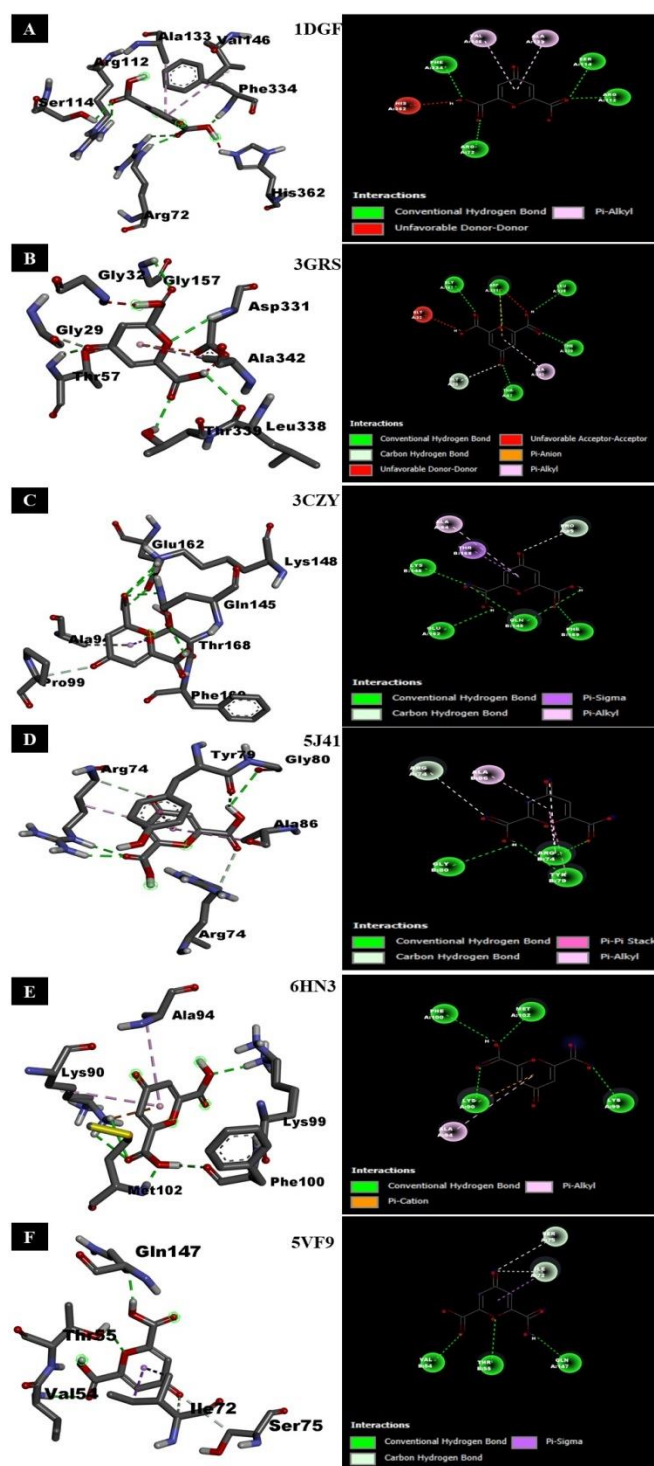


Figure 3: Molecular interactions of CA with oxidative stress-associated antioxidant proteins. 3D (left) and 2D (right) interaction profiles of CA with (A) CAT (1DGF), (B) GR (3GRS), (C) HO-1 (3CZY), (D) GST (5J41), (E) GPX4 (6HN3) and (F) SOD (5VF9).

Table 3: Binding interaction profile and ligand efficiency of CA with proliferation and cell cycle regulatory proteins.

Protein target	PDB ID	Predicted docking score (kcal/mol)	Ligand efficiency (kcal/mol/HA)	AA residues	Major contacts
c-Myc/Max	6G6K	-5.9	-0.454	Arg214, Leu210, Lys944, Thr947, Ser224	H; C-H; π -sigma; π -alkyl
PCNA	1AXC	-5.5	-0.423	Lys13, Lys80, Arg146, Arg149, Ala82	H; C-H; π -alkyl

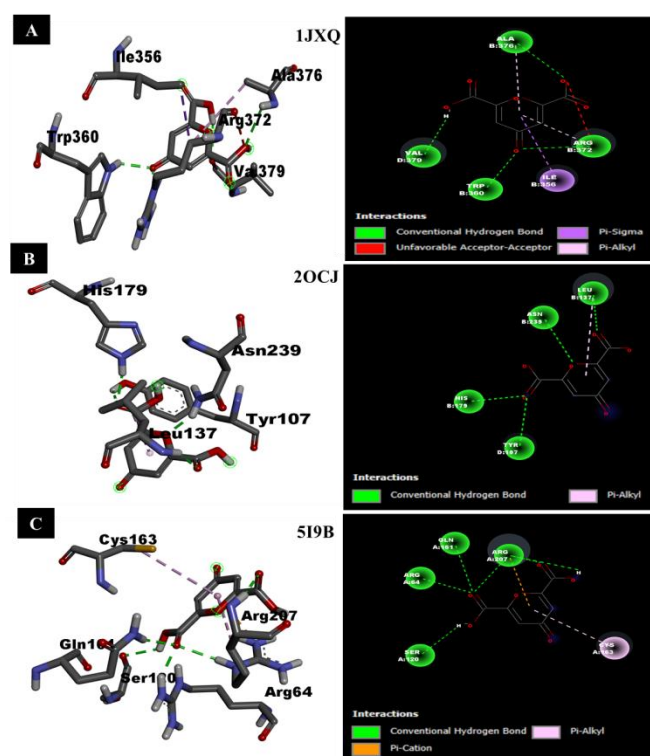


Figure 4: Binding interaction of CA with apoptosis-related proteins. 3D (left) and 2D (right) binding interaction profiles of CA with (A) caspase-9 (1JXQ), (B) p53 (2OCJ), and (C) caspase-3 (5I9B).

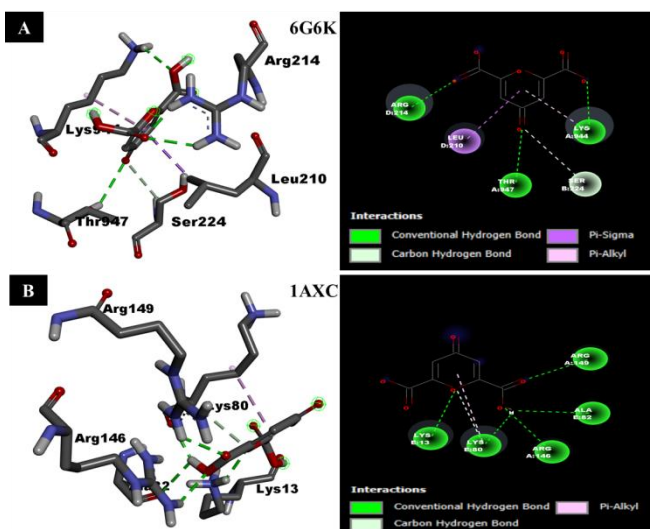


Figure 5: Binding interactions of CA with proliferation and cell-cycle regulatory proteins. 3D (left) and 2D (right) interaction profiles of CA with (A) c-Myc/Max (6G6K) and (B) PCNA (1AXC).

Table 4: Binding interactions profile and ligand efficiency of CA with intracellular signaling proteins.

Protein target	PDB ID	Predicted docking score (kcal/mol)	Ligand efficiency (kcal/mol/HA)	AA residues	Major contacts
PI3K α	4JPS	-6.5	-0.500	His670, Phe666, Arg818, Met811, Cys838	H; π -cation; π - π T-shaped
AKT1	3O96	-6.3	-0.485	Trp80, Ser205, Leu210, Thr211, Ala212, Leu213	H; C-H; π - π stacked; π -alkyl
Keap1	4IQK	-6.3	-0.485	Val606, Gly367, Gly558, Ala366, Val465	H; C-H; π -sigma
mTOR	4JSV	-5.9	-0.454	Ile2237, Leu2185, Ile2356, Gly2238, Val2240, Tyr2225.	H; π -alkyl; π - π T-shaped

Binding interaction with inflammatory mediators: CA showed predicted interactions with inflammatory mediators, with the predicted docking scores between -6.6 and -5.6 kcal/mol. COX-2 showed the docking score -6.6 kcal/mol, interacting with Arg333, Gln241 and Trp139. IL-6 showed -5.9 with Thr43, Glu106, Arg104, Thr163 and Gln156 residues. TNF- α contacts were observed through Ser60, Leu120 (2), Tyr151, and Gly121 residues, with a score of -5.8. NF- κ B p52 showed a score of -5.6 and interacted with Lys221, Lys252, Tyr285, and Ser226. iNOS showed -5.6 and interactions via Ala262, Trp496, Arg266, Asn283, Phe286, Ala282 and Glu285. The protein-ligand complexes were stabilized through various contacts, including hydrogen, van der Waals, amide- π stacking, π - π T-shaped, and π -alkyl ([Figure 7](#) and [Table 5](#)).

Interaction with matrix remodeling and angiogenesis-associated proteins: CA showed predicted interactions with proteins involved in matrix remodeling, invasion, and angiogenesis, ranging from -6.6 to -6.1 kcal/mol. MMP-9 showed the most negative predicted docking score (-6.6) with Arg143, Leu212, Lys214, and Gly213 (2) amino acid interactions. MMP-2 showed a score of -6.2 and interacted with Arg67(2), Phe65, and Asn64(2). VEGFR2 showed a predicted docking score of -6.1 and interacted with Asp998, His1004, Asn1040, Ser1037, Lys1039, and Phe921. The protein-ligand complexes were stabilized through various contacts, including hydrogen, carbon-hydrogen, π -anion, π -cation, π -alkyl, and π - π T-shaped ([Figure 8](#) & [Table 6](#)).

Docking validation-RMSD: Co-crystal/native-ligand redocking reproduced the crystallographic binding poses with RMSD values ranging from 0.436 to 1.898 Å across the 10 validated targets. All RMSD values were below the predefined acceptance criterion of 2.0 Å, supporting the docking protocol's reproducibility for the validated targets. Supplementary Table 5 provides the resulting docking scores and RMSD values.

Reference-ligand benchmarking: The reference/co-crystallized ligand docking scores and binding-site interactions were determined using the same docking protocol and are provided in Supplementary Table 5 alongside the corresponding CA docking results. Supplementary Table 6 provides a residue-level comparison of the CA docking sites with co-crystallized/native ligand-associated residues and experimentally established catalytic or functional residues. For targets with suitable native/co-crystallized ligands, common residues between the native ligand-associated site and the CA-interacting residues were identified. For targets without suitable conventional native-ligand reference structures, we compared CA-interacting residues with experimentally established catalytic or functional residues, where applicable.

Ligand efficiency of CA against selected proteins: The LE values of CA ranged from -0.577 to -0.415 kcal/mol/HA, as shown in [Tables 1-6](#).

Table 5: Binding interactions profile and ligand efficiency of CA with inflammatory mediator proteins

Protein target	PDB ID	Predicted docking score (kcal/mol)	Ligand efficiency (kcal/mol/HA)	AA residues	Major contacts
COX-2	5KIR	-6.6	-0.508	Arg333, Gln241, Trp139	H bond
IL-6	1ALU	-5.9	-0.454	Thr43, Glu106, Arg104, Thr163, Gln156	H; π -alkyl
TNF- α	2AZ5	-5.8	-0.446	Ser60, Leu120 (2), Tyr151, Gly121	Van der Waals; H; amide π -stacked
NF- κ B p52	1A3Q	-5.6	-0.431	Lys221, Lys252, Tyr285, Ser226	H; π -alkyl
iNOS	3E7G	-5.6	-0.431	Ala262, Trp496, Arg266, Asn283, Phe286, Ala282, Glu285	H; π - π T-shaped; π -alkyl

Table 6: Binding interactions profile and ligand efficiency of CA with matrix remodeling and angiogenesis -associated proteins

Protein target	PDB ID	Predicted docking score (kcal/mol)	Ligand efficiency (kcal/mol/HA)	AA residues	Major contacts
MMP-9	1GKC	-6.6	-0.508	Arg143, Leu212, Lys214, Gly213 (2)	H; C-H; π -cation; π -alkyl
MMP-2	7XJO	-6.2	-0.477	Arg67(2), Phe65, Asn64(2)	H; C-H; π -alkyl
VEGFR2	3VO3	-6.1	-0.469	Asp998, His1004, Asn1040, Ser1037, Lys1039, Phe921	H; π - π T-shaped

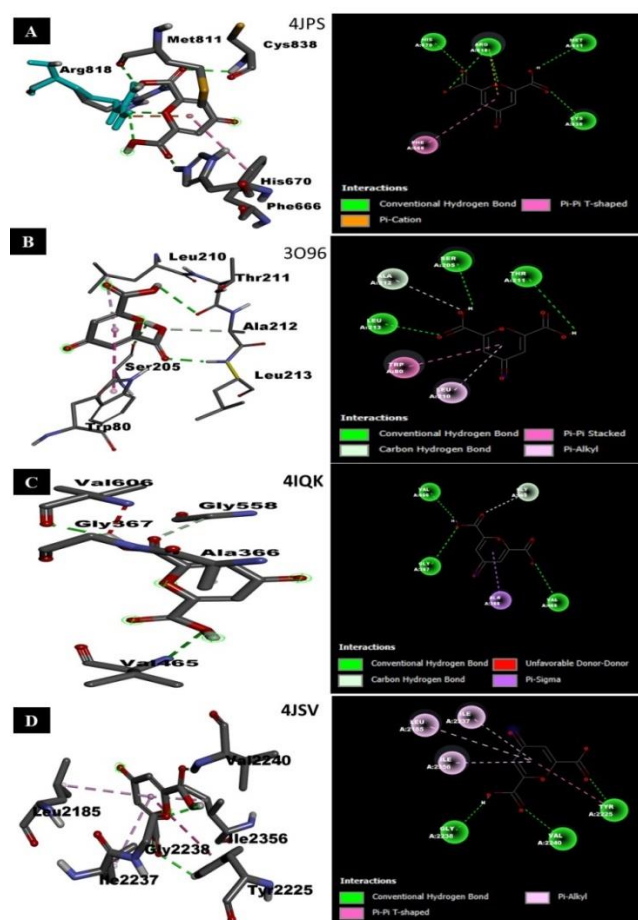


Figure 6: Binding interaction of CA with intracellular signaling proteins. 3D (left) and 2D (right) binding interactions between CA and (A) PI3K α (4JPS), (B) AKT1 (3O96), (C) Keap1 (4IQK) and (D) mTOR (4JSV).

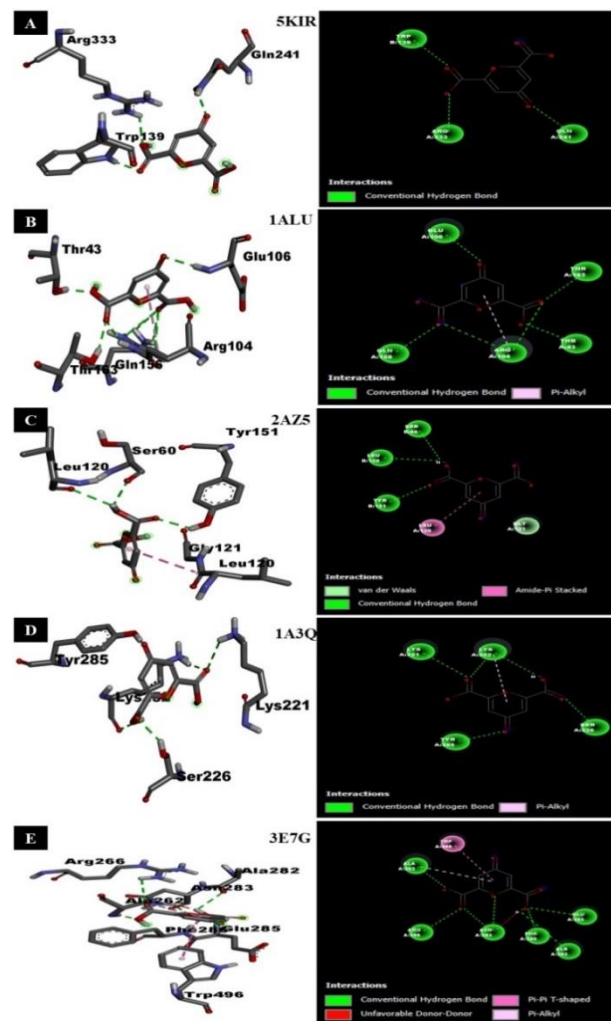


Figure 7: Binding interactions of CA with inflammatory mediator proteins. 3D (left) and 2D (right) interaction profiles of CA with

(A) COX-2 (5KIR), (B) IL-6 (1ALU), (C) TNF α (2AZ5), (D) NF- κ B p52 (1A3Q), and (E) iNOS (3E7G).

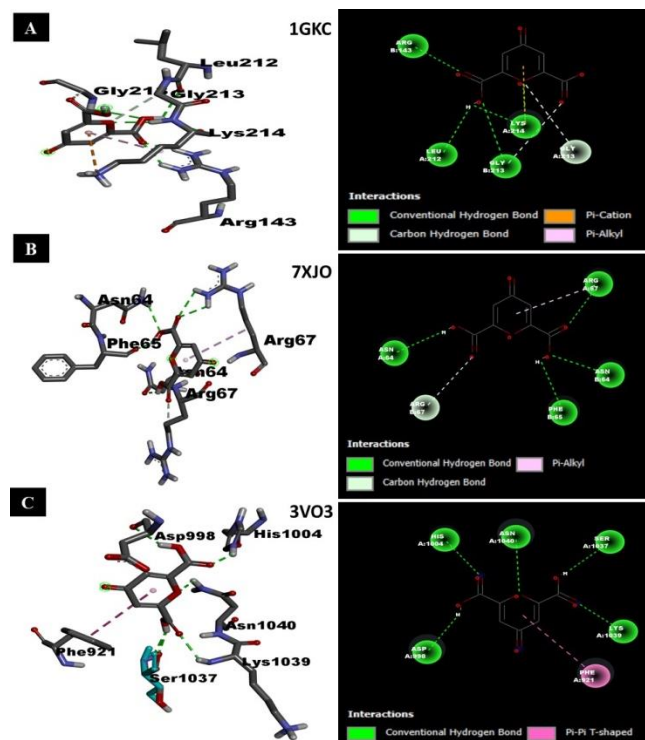


Figure 8: Binding interactions of CA with matrix remodeling and angiogenesis-associated proteins. 3D (left) and 2D (right) interaction profiles of CA with (A) MMP-9 (1GKC), (B) MMP-2(7XJO), and (C) VEGFR2 (3VO3).

4. DISCUSSION

This study predicted interactions between CA and various molecular targets associated with carcinogenesis. Oxidative stress is involved in initiating and promoting carcinogenesis. Excessive ROS production can lead to DNA damage, lipid peroxidation, genomic instability, and activation of tumor-promoting signaling pathways. [13] CAT and GR maintain cellular redox homeostasis by detoxifying hydrogen peroxide and maintaining the reduced glutathione pool, respectively. [14] In the current study, CA showed more negative predicted docking scores with CAT and GR. Furthermore, predicted interactions were observed with other antioxidant proteins like SOD, GPX4, GST, and HO-1, suggesting CA involvement in redox-regulated mechanisms; however, these predicted interactions require experimental validation.

The redox-inflammation interface provides a mechanistic link between oxidative stress and cancer progression.

Chronic ROS production can activate NF- κ B-mediated signaling pathways and increase pro-inflammatory mediators such as TNF- α , COX-2, IL-6, and iNOS, which contribute to a tumor-promoting microenvironment through cell proliferation, angiogenesis, and invasion. [15] The predicted interaction profile of CA with COX-2, IL-6, TNF- α , NF- κ B p52, and iNOS suggested a potential interaction of CA with inflammation-associated targets.

Cancer progression is marked by dysregulation of cell proliferation and programmed cell death. c-Myc is a transcription factor that combines with Max to form a heterodimeric complex and regulates the expression of many genes responsible for cell proliferation and survival. Increased c-Myc levels may lead to uncontrolled cell proliferation, resulting in cancer development. [16] PCNA plays a vital role in DNA synthesis and repair. The predicted interaction of CA with these proteins suggests that CA may help regulate these cellular processes. p53 is a central tumor suppressor involved in the response to DNA damage and controls cell-cycle progression and apoptosis. [17] Predicted interactions between CA and these proteins provide hypotheses about its possible involvement in these cellular processes.

PI3K α , AKT1, and mTOR are major intracellular signaling proteins that regulate proliferation, survival, metabolism, and resistance to apoptosis. Aberrant PI3K/AKT/mTOR signaling is associated with neoplastic transformation. [18,19] Predicted interactions among these interlinked signaling pathways may be more mechanistically relevant than interactions with a single signaling protein. In addition, the interaction between CA and Keap1 represents a possible molecular bridge connecting redox regulation and intracellular signaling. This association is especially important because oxidative stress can stimulate tumor-associated signaling pathways while impairing antioxidant defense systems. The predicted interactions between CA and these signaling proteins suggest their possible involvement in these pathways.

The activation of MMP-2 and MMP-9 results in ECM degradation and contributes to invasion and tissue remodeling. [20,21] Concurrently, angiogenesis is mediated by the activation of the VEGF pathway; dysregulation of these pathways leads to uncontrolled proliferation. [22,23] The observed interaction of CA with MMP-2, MMP-9, and VEGFR2 suggests a potential interaction with targets involved in angiogenesis and invasion pathways.

Multiple complementary non-covalent interactions stabilized the predicted structural complexes. These interactions contribute to ligand recognition and stabilization of the protein-ligand complexes. The more negative predicted docking scores for CAT, GR, caspase-9, COX-2, MMP-9, and PI3K α may indicate more negative predicted interactions within the applied docking protocol. Nevertheless, these scores should not be interpreted as direct measures of biological target preference or selectivity. Docking scores and interaction patterns are computational predictions that require experimental validation.

We validated the docking protocol by redocking available co-crystallized/native ligands using the same docking parameters applied for CA. Validated targets showed RMSD values below 2.0 Å, ranging from 0.436 to 1.898 Å, supporting the protocol's reproducibility for these targets.

The biological relevance is further corroborated by the recently reported pharmacological effects of CA. *In vitro* and *in vivo* studies have reported antioxidant, anti-inflammatory, and cytoprotective effects of CA, including improving SOD, CAT, and GSH-based defense mechanisms and regulating IL-6, COX-2, iNOS and NF- κ B-mediated inflammatory effects and Nrf2-associated signaling pathways in diseased animal models. Previous studies have also reported its effects on oxidative-stress-induced cellular senescence and p53-associated pathways. [24-29] The ligand efficiency (LE) calculations were used to normalize the predicted docking scores according to the size of CA. [30] We calculated LE by dividing the predicted docking score by the number of heavy atoms in CA (13). Because

the same ligand was assessed against all 23 target proteins, the calculated LE values are directly derived from the corresponding docking scores and therefore preserve the same target ranking. The LE values ranged from -0.577 to -0.415 kcal/mol/HA, with CAT showing the most negative value (-0.577), followed by GR (-0.531). Therefore, in this study, LE provides a normalized representation of predicted docking scores rather than an independent measure of target selectivity or experimentally validated binding efficiency.

5. CONCLUSION

In conclusion, CA showed predicted interactions with 23 target proteins associated with carcinogenesis. These predicted interactions suggest that CA may be involved in multiple molecular pathways associated with oxidative stress, inflammation, abnormal cell proliferation, apoptosis, angiogenesis, and invasion. However, docking predictions alone cannot establish whether CA activates or inhibits these targets or determine their biological effects. Therefore, the predicted multi-target interactions of CA provide hypotheses about its possible anticancer mechanisms of action. Further experimental investigations that specifically target these predicted interactions are needed to confirm their biological relevance.

Strengths and Limitations: The key strengths of the current study are the systematic analysis of CA against 23 functionally related proteins that cover various hallmarks and biological processes in carcinogenesis, providing a more comprehensive mechanistic perspective than single-target docking studies.

A limitation of the current study is that molecular docking predicts chelidonic acid interactions with target proteins but does not experimentally validate their functional effects. Therefore, the predicted interactions need validation through appropriate biochemical experiments and/or *in vitro* and/or *in vivo* approaches. We did not perform molecular dynamics simulations in the present study. Therefore, we could not evaluate the dynamic stability of the predicted protein-ligand complexes.

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Acknowledgements – The authors gratefully acknowledge the research advisory committee (RAC) members for their valuable suggestions.

Data Availability Statement: The docking data and computational files generated during the present study are available from the corresponding author upon reasonable request.

Ethical Statement: This study did not involve human participants or experimental animals; therefore, Institutional Ethics Committee approval and informed consent were not applicable.

Declaration of Generative AI: The authors declare this manuscript was written without the use of generative artificial intelligence tools. All the content, including text generation, data analysis and references was developed and reviewed by the authors without assistance from AI technologies.

Conflict of Interest – The authors declare no conflicts of interest.

Source of Support – The authors declare no source of support.

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