In Silico Analysis of Cucurbitacin IIa and Cucurbitacin IIb as Potential Modulators of Oxidative Stress Regulatory Proteins

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Abstract

Oxidative stress, resulting from imbalance between rective oxygen species (ROS) production and antioxidant defenses, contributes significantly to numerous pathological conditions, including inflammation, cancer and neurodegeneration. Natural compounds with antioxidant properties offer promising therapeutic potential. This study aims to investigate the potential of Cucurbitacin IIa and IIb to modulate oxidative stress regulatory proteins (NOS2, Lipoxygenase, KEAP1, and Xanthine oxidase) using molecular docking approaches. Target protein structures were retrieved from the RSCB Protein Data Bank. Ligand geometries were constructed and optimized using density functional theory. Molecular docking was performed using AutoDock 1.5.6 with a validated docking protocol (RPMS<2Å). Our results demonstrated favorable binding energies for both compounds with NOS2 (-9.75 and -9.57 kcal/mol) and KEAP1 (-8.52 and -9.34 kcal/mol), approaching the affinities of their respective native ligands. Moderate binding was observed with Lipoxygenase (-6.14 and -5.54 kcal/mol), while both compounds showed incompatibility with Xanthine oxidase, as evidenced by highly positive binding energies. The interaction between Cucurbitacins with NOS2, KEAP1 and Lipoxygenase was mediated through hydrogen and hydrophobic interaction. These findings provide mechanistic insight into their bioactivity and support further experimental studies for therapeutic development in oxidative stress-related disorders.

Keywords: *Cucurbitacin, Molecular docking, Oxidative stress, NOS2, KEAP1.*

INTRODUCTION

Oxidative stress represents a fundamental imbalance between the production of reactive oxygen species (ROS) and a biological system's ability to detoxify these reactive intermediates or repair the resulting damage (Sies, Berndt, & Jones, 2017). This state of redox disequilibrium has been implicated in the pathogenesis of numerous diseases, including cardiovascular disorders, neurodegenerative conditions, cancer, and inflammatory diseases (Pizzino, *et al*., 2017). At the

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molecular level, oxidative stress manifests through several damaging mechanisms, including lipid peroxidation, mitochondrial dysfunction, DNA damage, protein carbonylation, ion leakage across cellular membranes, and ultimately, apoptotic cell death (Liguori, *et al*., 2018). These processes collectively contribute to tissue damage and organ dysfunction, highlighting the critical importance of regulating oxidative stress for maintaining health and preventing disease progression.

The human body employs several strategies to counteract oxidative stress, primarily through antioxidant mechanisms that operate at various levels of cellular organization. These mechanisms include direct scavenging of free radicals through electron donation or acceptance, inhibition of enzymes that generate ROS, and enhancement of endogenous antioxidant enzyme systems. Particularly important are the latter two approaches, as they address the sources and regulatory systems of oxidative stress rather than merely neutralizing already-formed reactive species. This systematic regulation of oxidative stress pathways represents a promising target for therapeutic intervention in numerous pathological conditions.

Natural products have historically served as rich sources of bioactive compounds with therapeutic potential, including those with antioxidant properties (Newman & Cragg, 2020). Among these, cucurbitacins have emerged as a particularly interesting class of compounds. Cucurbitacins are a group of tetracyclic triterpenoids primarily found in the Cucurbitaceae plant family, characterized by their bitter taste and diverse biological activities (Zieniuk & Pawełkowicz, 2023). These compounds are classified alphabetically (Cucurbitacin A-T) based on their structural features and substitution patterns, and they exist in both free form and as glycosides in various plant tissues. Extensive research has demonstrated that cucurbitacins possess a wide range of biological activities, including anti-inflammatory, antidiabetic, anticancer, antiviral, and neuroprotective effects (Dai, *et al*., 2023).

Cucurbitacin IIa and Cucurbitacin IIb represent two closely related members of this family that have attracted particular attention due to their potent biological activities (Li, *et al*., 2023; Zeng, *et al*., 2021). These compounds share a common tetracyclic triterpenoid backbone but differ in specific functional group substitutions, which may influence their biological properties and target interactions (National Center for Biotechnology, 2025; National Center for Biotechnology Information, 2025). Previous studies have documented various therapeutic effects of these compounds, including anti-inflammatory actions in models of colitis, liver injury (Liu, *et al*., 2025), and arthritis (Peng, *et al*., 2020), as well as anticancer activities against multiple tumor types (Li, *et al*., 2024; Torres-Moreno, *et al*., 2020). While these effects have been partially attributed to modulation of inflammatory signaling pathways such as JAK/STAT and NF-κB, the potential direct interactions of these compounds with oxidative stress regulatory proteins remain largely unexplored (Kung, *et al*., 2025; Kusagawa, *et al*., 2022).

The regulation of oxidative stress involves several key proteins, including Nitric oxide synthase 2 (NOS2), also known as inducible nitric oxide synthase (iNOS), Lipoxygenase and Kelch-like ECH-associated protein 1 (KEAP1). NOS2, catalyzes the production of nitric oxide radicals that contribute to inflammatory responses and oxidative damage (Król & Kepinska, 2020). Lipoxygenase enzymes catalyze the formation of hydroperoxides from polyunsaturated fatty acids, contributing to the inflammatory cascade and oxidative stress (Mashima & Okuyama, 2015). KEAP1 functions as a sensor of oxidative stress and negatively regulates the nuclear factor erythroid 2-related factor 2 (Nrf2), a transcription factor that controls the expression of numerous antioxidant proteins (Suzuki & Yamamoto, 2015). Xanthine oxidase generates superoxide radicals during the metabolism of purines, representing another important source of ROS (Aziz & Jamil, 2023). Understanding how

225

natural compounds like cucurbitacins interact with these proteins could provide valuable insights into their mechanisms of action and potential therapeutic applications.

Molecular docking represents a powerful computational approach for predicting the binding orientations and affinities of small molecules to protein targets (Agu, *et al*., 2023). This method allows for the efficient screening of compounds against multiple targets and provides detailed insights into the molecular interactions that govern binding specificity. By employing molecular docking, we can explore the potential of Cucurbitacin IIa and IIb to interact with key oxidative stress regulatory proteins and generate hypotheses regarding their mechanisms of action that can guide subsequent experimental studies.

In this study, we aimed to investigate the binding potential of Cucurbitacin IIa and IIb to four key oxidative stress regulatory proteins—NOS2, lipoxygenase, KEAP1, and xanthine oxidase—using molecular docking techniques. We hypothesized that these cucurbitacins might directly interact with one or more of these proteins, potentially explaining their documented anti-inflammatory and protective effects. Through detailed analysis of binding energies and interaction patterns, we sought to identify the most promising protein targets for these compounds and gain insights into the molecular mechanisms underlying their biological activities. The findings from this study could contribute to a better understanding of cucurbitacin pharmacology and guide future experimental studies aimed at developing optimized derivatives for the treatment of oxidative stress-related disorders.

METHODS

Target Protein Selection

We selected four human oxidative stress regulatory proteins from the RCSB Protein Data Bank (PDB): nitric oxide synthase 2 (NOS2, PDB ID: 4NOS), lipoxygenase (bound with competitive inhibitor PDB ID: 6N2W and allosteric inhibitor PDB ID: 6NCF), Kelch-like ECH-associated protein 1 (KEAP1, PDB ID: 6HWS), and xanthine oxidase (PDB ID: 6N2W). Selection criteria included co-crystallization with inhibitors or native ligand, and absence of mutations in binding sites.

Ligand Preparation

Three-dimensional structures of Cucurbitacin IIa and IIb were constructed using Avogadro software and optimized using Density Functional Theory implemented in Gaussian 16, Revision D.01. This optimization ensured accurate representation of bioactive conformations for docking simulations.

Docking Protocol Validation

We validated our docking protocol using Autodock 1.5.6 by re-docking native ligands into their respective protein binding sites. RMSD values below 2 Å confirmed reliable prediction of binding modes. The same grid box parameters were maintained for subsequent docking with cucurbitacins.

Docking Simulation

For each protein-ligand complex, we performed 100 independent docking runs using the Lamarckian Genetic Algorithm in Autodock. Grid boxes centered on binding sites identified from co-crystallized ligands encompassed the entire binding pocket. Binding orientations were ranked by calculated binding energies, with lower values indicating stronger predicted affinities.

Data Analysis

Protein-ligand interactions were analyzed using Biovia Discovery Studio (BIOVIA, 2021). We identified key interacting residues, characterized bond types (hydrogen bonds, hydrophobic interactions, π-stacking), and measured interaction distances. Binding energy values were compiled for comparative analysis across protein-ligand complexes.

226

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RESULTS

Validation of Molecular Docking Protocol

Our investigation began with rigorous validation of the molecular docking protocol to ensure reliable predictions of Cucurbitacin IIa and IIb binding to oxidative stress regulatory proteins. Re-docking of native ligands to their respective protein targets yielded RMSD values consistently below 2 Å (Table 1), indicating that our docking methodology could accurately reproduce experimentally determined binding modes. The overlap between the redocked and native ligand conformations is shown in Figure 1. This validation was crucial for establishing confidence in subsequent docking experiments with our test compounds. The successful reproduction of crystallographic binding poses confirmed that the selected grid box parameters appropriately encompassed the binding sites and that the search algorithm effectively identified favorable binding conformations.

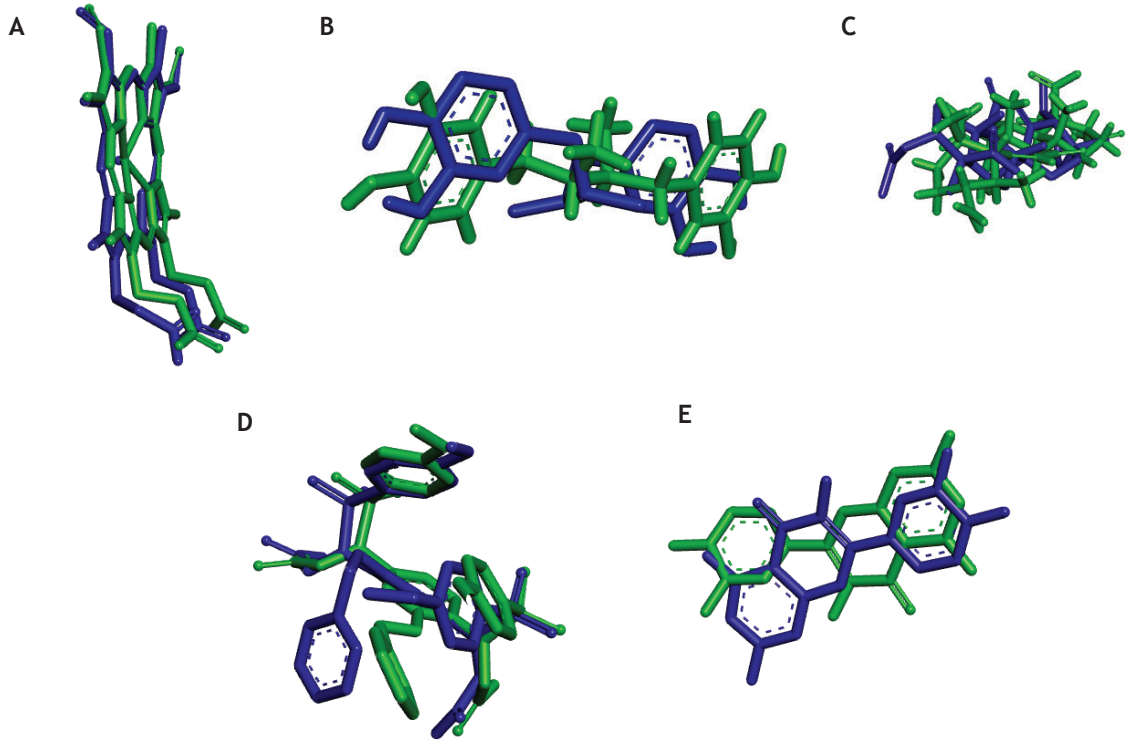

Figure 1. Overlay visualization of protein targets with each native ligands : A. NOS2 (4NOS) with native ligand NOS Cofactor Protoporphyrin IX Containing Fe (HEM), B. Lipoxygenase (6N2W) with native ligand Competitive inhibitor nordihydroguaiaretic acid (NDGA), C. Lipoxygenase (6NCF) with native ligand allosteric inhibitor 3-acetyl-11-keto-beta-boswellic acid (AKBA), D. KEAP1 (6HWS) with native ligand competitive inhibitor - 2-[[4-[2-hydroxy-2-oxoethyl-(4-methoxyphenyl)sulfonyl-amino]-3-phenylmethoxy-phenyl]-(4-methoxyphenyl)sulfonyl-amino]ethanoic acid (GX8), E. Xanthin oxidase (3NVY) with native ligand Quercetin.

227

Table 1. Resolution and RMSD values of docking validations.

Target Protein (PDB ID)	Resolution	Native Ligand	RMSD	References
NOS2 (4NOS)	2.25 Å	NOS Cofactor Protoporphyrin IX Containing Fe (HEM)	0,63	(Fischmann, *et al*., 1999)
Lipoxygenase (6N2W)	2.71 Å	Competitive inhibitor nordihydroguaiaretic acid (NDGA)	1.40	(Gilbert, *et al*., 2020)
Lipoxygenase (6NCF)	2.87 Å	Allosteric inhibitor 3-acetyl-11-keto-beta-boswellic acid (AKBA)	1.01	Gilbert, *et al*., 2020)
KEAP1 (6HWS)	1.75 Å	Competitive inhibitor - 2-[[4-[2-hydroxy-2-oxoethyl-(4-methoxyphenyl)sulfonyl-amino]-3-phenylmethoxy-phenyl]-(4-methoxyphenyl)sulfonyl-amino]ethanoic acid (GX8)	1.79	(Georgakopoulos, *et al*., 2022)
Xanthin oxidase (3NVY)	2.00 Å	Quercertin	1.51	(Cao, Pauff, & Hille, 2014)

Comparative Binding Energy Analysis

Analysis of binding energies revealed distinct patterns of interaction between the cucurbitacins and the selected target proteins. As shown in Table 2, both Cucurbitacin IIa and IIb demonstrated favorable binding energies toward NOS2, Lipoxygenase, and KEAP1, while unexpectedly yielding highly positive values with Xanthine oxidase, indicating steric clashes and unfavorable interactions. For NOS2 (PDB ID: 4NOS), Cucurbitacin IIa and IIb exhibited binding energies of -9.75 kcal/mol and -9.57 kcal/mol, respectively, approaching the binding affinity of the native ligand (-14.13 kcal/mol). This suggested strong potential for inhibitory interactions, with Cucurbitacin IIa showing slightly more favorable binding than Cucurbitacin IIb.

Table 2. The binding energy of native ligand, cucurbitacin IIa and cucurbitacin IIb to oxidative regulatory proteins.

Target Protein (PDB ID)	Native Ligand	Ligand Name	Binding Energy (kcal/mol)
NOS2 (4NOS)	NOS Cofactor Protoporphyrin IX Containing Fe (HEM)	Native ligand (HEM)	-14.13
		Cucurbitacin IIa	-9.75
		Cucurbitacin IIb	-9.57
Lipoxygenase (6N2W)	Competitive inhibitor nordihydroguaiaretic acid (NDGA)	Native ligand (NDGA)	-4.65
		Cucurbitacin IIa	+16951.64
		Cucurbitacin IIb	+20554.23
Lipoxygenase (6NCF)	Allosteric inhibitor 3-acetyl-11-keto-beta-boswellic acid (AKBA)	Native ligand (AKBA)	-8.36
		Cucurbitacin IIa	-6.14
		Cucurbitacin IIb	-5.54
KEAP1 (6HWS)	Competitive inhibitor - 2-[[4-[2-hydroxy-2-oxoethyl-(4-methoxyphenyl) sulfonyl-amino]-3-phenylmethoxy-phenyl]-(4-methoxyphenyl) sulfonyl-amino]ethanoic acid (GX8)	Native ligand (GX8)	-10.21
		Cucurbitacin IIa	-8.52
		Cucurbitacin IIb	-9.34
Xanthin oxidase (3NVY)	Quercertin	Native ligand (FAD)	-9.77
		Cucurbitacin IIa	+7620
		Cucurbitacin IIb	+3431

Cucurbitacin IIa and IIb were docked to lipoxygenase at two distinct binding sites to explore their potential mechanisms of inhibition—either through direct interaction with the catalytic (active) site or modulation via an allosteric site. This dual-site docking approach is crucial for understanding the full therapeutic potential of the compounds, as inhibitors may exert their effects through different binding modes. The availability of two lipoxygenase crystal structures in the PDB—one bound to a competitive inhibitor (NDGA, PDB ID: 6N2W) and another to an allosteric inhibitor (AKBA, PDB ID: 6NCF)—provides a unique opportunity to model ligand interactions at both functional sites. Docking to 6N2W was performed to evaluate binding at the enzyme's active site, while docking to 6NCF targeted the allosteric pocket. Investigating both sites allows us to distinguish whether Cucurbitacins act via classical inhibition or through allosteric modulation, which may offer advantages such as reduced off-target effects and improved selectivity (Ma, *et al*., 2025).

Both Cucurbitacins exhibited negative binding energies at the allosteric site (PDB ID: 6NCF), suggesting potential as allosteric inhibitor. However, they showed positive binding energies at the active site (PDB ID: 6N2W), indicating limited affinity as competitive inhibitors. Specifically, Cucurbitacin IIa and IIb showed moderate binding affinities at the allosteric site with energies of -6.14 kcal/mol and and -5.54 kcal/mol, respectively. Although these values are less negative than the native ligand AKBA (-8.36 kcal/mol), they still indicate meaningful interactions capable of modulating lipoxygenase activity.

Interestingly, docking studies with KEAP1 (PDB ID: 6HWS), Cucurbitacin IIb (-9.34 kcal/mol) showed more favorable binding than Cucurbitacin IIa (-8.52 kcal/mol), both approaching the binding energy of the native ligand (-10.21 kcal/mol). This indicated that structural differences between the two cucurbitacins might influence their binding preferences for different protein targets.

The most striking observation was the exceptionally high positive binding energies obtained with Xanthine oxidase (PDB ID: 3NVY), showing values of +16971.47 kcal/mol for Cucurbitacin IIa and +20646.92 kcal/mol for Cucurbitacin IIb. These values contrasted sharply with the negative binding energy of the native ligand (-4.82 kcal/mol) and suggested severe steric hindrance or conformational incompatibility within the binding pocket, rendering both cucurbitacins unsuitable as Xanthine oxidase modulators.

NOS2 Interactions

Using the binding site derived from Heme (PDB ID : 4NOS), both cucurbitacins demonstrated promising docking affinities. The site is located in a narrow groove within the larger active-site cavity that accommodates both heme and BH4 cofactor. The key amino acid residues of iNOS that interact with the heme and are likely critical for enzymatic activity include Phe369, which is also involved in binding the SEITU inhibitor, indicating their dual role in substrate and inhibitor recognition (Fischmann, *et al*., 1999). In addition, Cys200 plays an essential role as the axial ligand to the heme iron and has been reported to be important for the binding of heme, the natural substrate nitroarginine, and the cofactor BH_4 (Cubberley, *et al*., 1997). Cucurbitacin IIa formed hydrogen bonds with Cys200, Ile201, and Trp37, along with hydrophobic interactions involving Trp194, Leu209, Val352, and Phe369 (Figure 2). The interactions with Cys200 and Phe369 suggest that Cucurbitacin IIa may inhibit iNOS activity by occupying the active site and interfering with substrate or cofactor binding. In comparison, Cucurbitacin IIb formed hydrogen bonds with Ile201, Pro350, and Trp372, and hydrophobic interactions with Val352 and Met355 (Figure 2), which may account for its slightly different binding affinity. While its interaction with Cys200 appears to be mediated through a weaker C–H bond rather than a conventional hydrogen bond, this proximity may still contribute

to inhibitory activity by disrupting substrate or cofactor association with the heme center. Notably, both compounds positioned their tetracyclic cores near the heme group, suggesting possible inhibition through blocking substrate access.

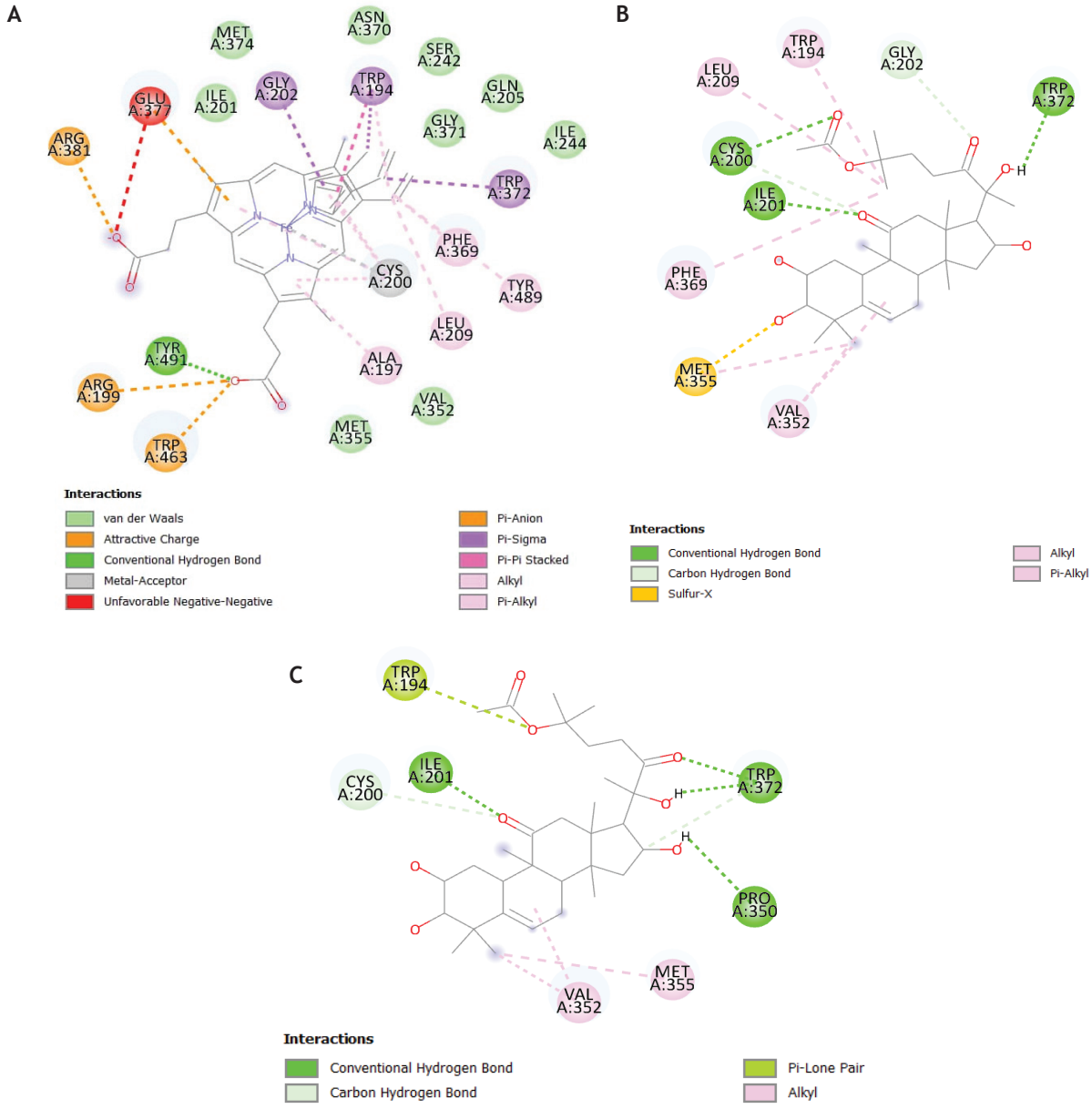

Figure 2. Two-dimensional visualization of protein-ligand interaction between nitric oxide synthase 2 (PDB ID : 4NOS) to native ligand NOS Cofactor Protoporphyrin IX Containing Fe (Heme) (A), Cucurbitacin IIa (B) and Cucurbitacin IIb (C). Heme interaction to NOS2 key residues Phe 369 and Cys200 are mediated through alkyl and metal acceptor interaction. Cucurbitacin IIa interaction to NOS2 key residues Phe 369 and Cys200 are mediated through alkyl and hydrogen interaction. Cucurbitacin IIb interaction to NOS2 key residue Cys200 are mediated through carbon-hydrogen interaction.

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Lipoxygenase Interactions

The allosteric inhibitor 3-acetyl-11-keto-β-boswellic acid (AKBA) binds to 5-lipoxygenase at a site located between the membrane-binding and catalytic domains, approximately 30 Å from the catalytic iron. Polar interactions with Arg101, Thr137, Arg138, and Val110, along with close contact with His130, help anchor AKBA precisely within this interdomain groove (Gilbert, *et al.*, 2020). Similarly, Cucurbitacin IIa interacts with Arg101, Val110, and His130, while Cucurbitacin IIb binds to Val110, Arg138, and His130, suggesting that both compounds also target the same groove between the membrane-binding and catalytic domains (Figure 3).

Importantly, AKBA disrupts a conserved cation–π interaction between Trp102 and Arg165, and destabilizes the interdomain charge pair at Arg101 and Asp166, leading to increased flexibility of the catalytic domain—particularly the region containing Phe177 and Tyr181, which are critical for sealing the active site, thus impairing 5-LOX enzymatic activity. Mutation of His130 or Arg101 to alanine reduces 5-LOX activity under AKBA treatment, indicating their key role in AKBA binding (Gilbert, *et al.*, 2020). The interaction of Cucurbitacin IIa with Arg101 and His130, and of Cucurbitacin IIb with His130, suggests that both compounds not only bind to 5-lipoxygenase but may also inhibit its activity through a similar mechanism (Figure 3).

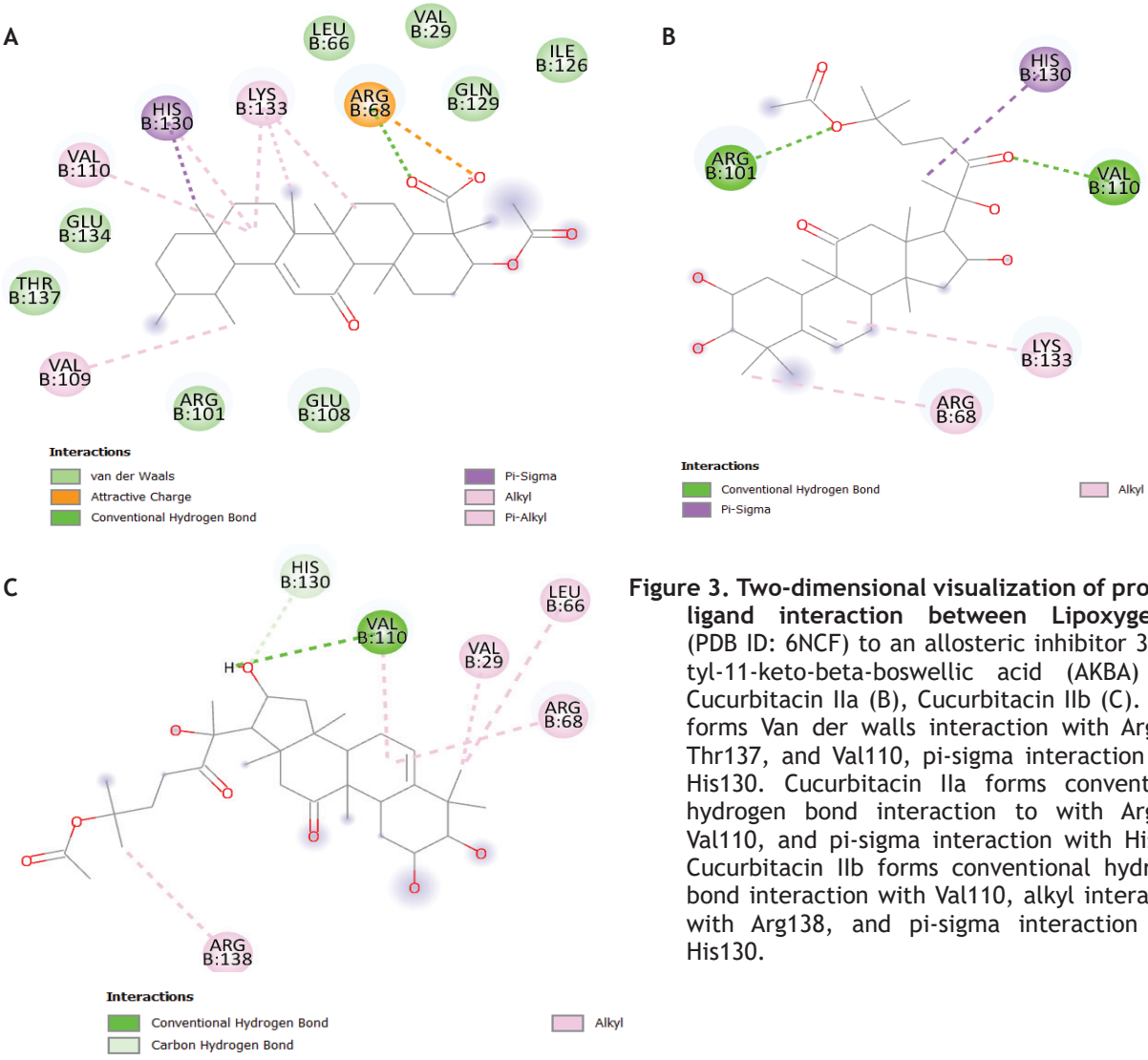

Figure 3. Two-dimensional visualization of protein-ligand interaction between Lipoxygenase (PDB ID: 6NCF) to an allosteric inhibitor 3-acetyl-11-keto-beta-boswellic acid (AKBA) (A), Cucurbitacin IIa (B), Cucurbitacin IIb (C). AKBA forms Van der walls interaction with Arg101, Thr137, and Val110, pi-sigma interaction with His130. Cucurbitacin IIa forms conventional hydrogen bond interaction to with Arg101, Val110, and pi-sigma interaction with His130. Cucurbitacin IIb forms conventional hydrogen bond interaction with Val110, alkyl interaction with Arg138, and pi-sigma interaction with His130.

KEAP1 Interactions

GX8 occupies the Nrf2 binding site of KEAP1 sub-pockets, through interaction with Arg415, Gly462, Ser363, Arg380, Asn414, Gly364, Gly509, Ala556, Ser602, Gly603, Tyr572 and Phe577 (Georgakopoulos, *et al.*, 2022). Cucurbitacin IIa also binds Keap1 sub-pockets of Nrf2 binding site, through interaction with the Arg415, Phe478, Arg483, Arg380, Tyr334 and Ser555. Similarly, Cucurbitacin 2b also binds Keap1 sub-pockets of Nrf2 binding site, through interaction with the Arg415, Arg380, Asn414, Ala556, Tyr334, Tyr572 and Tyr525 (Figure 4). Both compounds positioned in the Nrf2-binding region, suggesting potential disruption of KEAP1-Nrf2 interaction, thus indicating their inhibition activity of KEAP1.

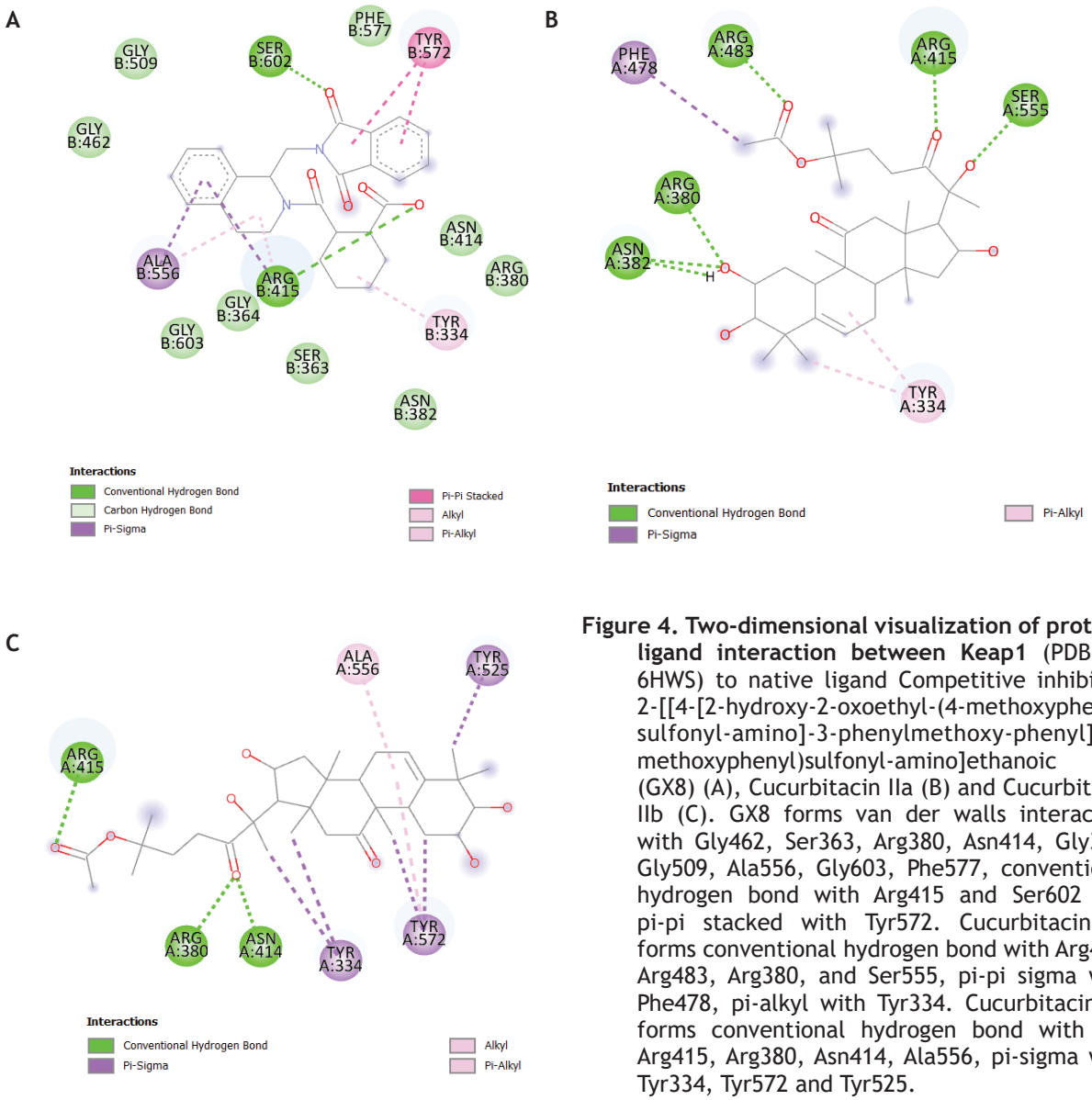

Figure 4. Two-dimensional visualization of protein-ligand interaction between Keap1 (PDB ID: 6HWS) to native ligand Competitive inhibitor-2-[[4-[2-hydroxy-2-oxoethyl-(4-methoxyphenyl) sulfonyl-amino]-3-phenylmethoxy-phenyl]-(4-methoxyphenyl)sulfonyl-amino]ethanoic acid (GX8) (A), Cucurbitacin IIa (B) and Cucurbitacin IIb (C). GX8 forms van der walls interaction with Gly462, Ser363, Arg380, Asn414, Gly364, Gly509, Ala556, Gly603, Phe577, conventional hydrogen bond with Arg415 and Ser602 and pi-pi stacked with Tyr572. Cucurbitacin IIa forms conventional hydrogen bond with Arg415, Arg483, Arg380, and Ser555, pi-pi sigma with Phe478, pi-alkyl with Tyr334. Cucurbitacin IIb forms conventional hydrogen bond with the Arg415, Arg380, Asn414, Ala556, pi-sigma with Tyr334, Tyr572 and Tyr525.

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DISCUSSION

This study demonstrates that Cucurbitacin IIa and IIb have potential as modulators of key oxidative stress regulatory proteins, particularly NOS2 (binding energies: –9.75 kcal/mol for IIa and –9.57 kcal/mol for IIb), KEAP1 (–8.52 kcal/mol for IIa and –9.34 kcal/mol for IIb) and Lipoxygenase (–6.14 kcal/mol for IIa and –5.54 kcal/mol for IIb). In contrast, marked incompatibility is shown with Xanthine oxidase, as reflected by highly positive binding energies (+7620 kcal/mol for IIa and +3431 kcal/mol for IIb). These findings suggest that cucurbitacins may exert their documented anti-inflammatory and cytoprotective effects, at least in part, through direct interaction with oxidative stress-related proteins.

The strong binding affinity of both cucurbitacins for NOS2 is particularly noteworthy, as this enzyme plays a critical role in generating nitric oxide radicals that contribute to inflammatory responses and oxidative damage. The binding poses and interaction profiles suggest that these compounds may inhibit NOS2 activity by interfering with substrate access to the catalytic site. This finding aligns with previous studies demonstrating the anti-inflammatory effects of cucurbitacins in various disease models. For instance, Cucurbitacin IIb mitigates acute liver injury by suppressing M1 macrophage polarization through modulation of NF-κB and MAPK signaling pathways. Since NOS2 expression is regulated by NF-κB, our results provide a potential molecular mechanism for this observed effect, suggesting that direct inhibition of NOS2 by Cucurbitacin IIb may complement its effects on upstream signaling pathways.

The binding pattern observed with KEAP1 presents another important mechanism through which cucurbitacins may modulate oxidative stress responses. KEAP1 functions as a sensor of oxidative stress and negatively regulates the Nrf2 transcription factor, which controls the expression of numerous antioxidant proteins. Our docking results suggest that both cucurbitacins, but particularly Cucurbitacin IIb, bind at the interface where KEAP1 interacts with Nrf2. Such binding could potentially disrupt the KEAP1-Nrf2 interaction, leading to increased Nrf2 stability and enhanced expression of antioxidant genes. This proposed mechanism offers an explanation for the observed protective effects of cucurbitacins against oxidative damage in various tissues. Similar mechanisms have been documented for other natural compounds such as sulforaphane, which activates Nrf2 signaling by modifying cysteine residues on KEAP1.

The binding affinity of both cucurbitacins for Lipoxygenase suggests that they may also influence inflammatory responses through modulation of eicosanoid production. Lipoxygenases catalyze the formation of leukotrienes and other inflammatory mediators from arachidonic acid, and their inhibition represents a strategy for mitigating inflammation. Although the binding energies for Lipoxygenase were less favorable than for NOS2 and KEAP1, they still indicate potential biological significance. Previous studies have shown that Cucurbitacin IIa alleviates colitis via promoting the release of extracellular vesicles containing microRNA-30b-5p, and Cucurbitacin IIb exhibits anti-inflammatory activity through modulating multiple cellular behaviors of lymphocytes. Our findings suggest that direct inhibition of lipoxygenase might represent an additional mechanism contributing to these observed anti-inflammatory effects.

The unexpected and highly unfavorable binding energies observed with xanthine oxidase highlight an important limitation in the potential therapeutic applications of cucurbitacins. The severe steric clashes and unfavorable interactions indicate that the current structural features of these compounds are incompatible with the xanthine oxidase binding site. This suggests that while cucurbitacins may modulate certain oxidative stress pathways, they likely do not exert substantial effects on the generation of superoxide radicals through the Xanthine oxidase pathway. This finding

underscores the selective nature of cucurbitacin interactions with oxidative stress regulatory proteins and suggests that their therapeutic effects may be pathway-specific rather than broadly applicable to all sources of ROS.

Visualization of interactions with amino acid residues provides valuable insights for the potential optimization of cucurbitacin derivatives by targeting key residues essential for the activity of each protein. The slight differences in binding energies and interaction patterns between Cucurbitacin IIa and IIb highlight how subtle structural variations can influence binding preferences. For instance, Cucurbitacin IIa showed marginally better binding to NOS2, while Cucurbitacin IIb exhibited stronger affinity for KEAP1. These differences could be exploited in the design of derivatives with enhanced specificity for particular protein targets. Previous work by Yu, *et al*., (2020) demonstrated that synthetic derivatives of Cucurbitacin IIa could induce apoptosis in cancer cells, suggesting that structural modifications of these compounds can indeed yield derivatives with altered biological activities.

Our computational findings align with and extend previous experimental observations of the anti-inflammatory and protective effects of cucurbitacins. For example, Peng, *et al*., (2020) reported that Cucurbitacin IIa enhances the expression of the anti-inflammatory regulator SIGIRR in human macrophages. Similarly, Zhao, Jiang, and Zuo (2025) demonstrated that showed that Cucurbitacin IIb reduces the levels of pro-inflammatory cytokines IL-6 and IL-1β in the colonic tissue of mice with dextran sulfate sodium (DSS)-induced colitis. The molecular interactions revealed in our study offer further insight into these effects by highlighting additional anti-inflammatory mechanisms, particularly the potential of cucurbitacins to directly modulate key oxidative stress-related proteins such as NOS2, 5-lipoxygenase, and KEAP1. This suggests that their therapeutic actions may, in part, be mediated through redox regulation.

It is important to acknowledge the limitations of our computational approach. Molecular docking provides predictions of binding orientations and affinities but does not account for the dynamic nature of protein-ligand interactions in physiological environments. Factors such as solvent effects, protein flexibility, and allosteric changes may influence actual binding *in vivo*. Additionally, the binding energies obtained through docking calculations represent estimates rather than absolute values and should be interpreted as indicative of relative binding preferences rather than precise measurements of binding strength. Therefore, while our results provide valuable insights into potential mechanisms, they require validation through experimental studies such as enzyme inhibition assays, cellular assays of oxidative stress markers, and structural studies using techniques like X-ray crystallography or nuclear magnetic resonance.

Despite these limitations, our findings contribute substantially to understanding the molecular basis of cucurbitacin activity and provide direction for future research. The strong binding affinities observed for NOS2 and KEAP1 suggest that these proteins represent promising targets for experimental validation. Enzyme inhibition assays could confirm whether the predicted binding translates to functional inhibition, while cellular studies could investigate whether treatment with cucurbitacins leads to changes in nitric oxide production or Nrf2 activation consistent with our computational predictions. Furthermore, our results highlight the potential for structure-based optimization of cucurbitacin derivatives to enhance their specificity and potency as modulators of oxidative stress pathways.

CONCLUSION

In conclusion, our molecular docking analyses reveal that Cucurbitacin IIa and IIb show promising potential as modulators of key

oxidative stress regulatory proteins, particularly NOS2 and KEAP1. These interactions may contribute to the documented anti-inflammatory and protective effects of cucurbitacins by inhibiting nitric oxide radical production and enhancing antioxidant responses through the Nrf2 pathway. While moderate binding to Lipoxygenase suggests additional anti-inflammatory mechanisms, the incompatibility with Xanthine oxidase indicates pathway selectivity. These findings not only provide mechanistic insights into the biological activities of cucurbitacins but also establish a foundation for future experimental studies and the development of optimized derivatives for therapeutic applications targeting oxidative stress-related disorders.

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