Unraveling molecular mechanisms of β-glucuronidase inhibition by flavonoids from Centaurea scoparia: integrated in silico and in vitro insights†

Maha A. Alwalli, a Faris F. Aba Alkhayl, b Hassan A. Rudayni, c Ahmed A. Allam,cd Naif G. Altoom, e Al Mokhtar Lamsabhi f and Emadeldin M. Kamel *h

Investigating the detailed molecular mechanisms of β-glucuronidase inhibition is critical for pioneering new therapeutic solutions and driving progress in pharmaceutical research. The inhibitory potential of four flavonoid aglycones derived from Centaurea scoparia against β-glucuronidase was thoroughly examined using a combination of in vitro and in silico methodologies. The in vitro assays revealed that compounds 1 and 3 had the strongest inhibitory effects, demonstrated by their low IC50 values of 3.16 ± 0.34 and 3.82 ± 0.1 μM, respectively. The results of the enzyme kinetics assay revealed that compounds 2 and 3, and the reference drug EGCG displayed a mixed inhibition mode. Conversely, compound 1 was found to operate via a noncompetitive inhibition mechanism, as evidenced by the intersection patterns in the Lineweaver–Burk plots. The outcomes from the docking analysis are consistent with the in vitro inhibitory efficacy assays, showing that compounds 1 and 3 possess the lowest binding affinities (−8.6 and −9.0 kcal mol⁻¹, respectively). Isolated phytochemicals demonstrated substantial polar and hydrophobic interactions with the residues within the enzyme’s binding site. We investigated the interaction dynamics of isolated compounds with β-glucuronidase using a 100 ns molecular dynamics (MD) simulation. The analysis of various MD parameters indicated that compounds 1 and 3 showed stable trajectories and significant energy stabilization when bound to β-glucuronidase. Furthermore, compounds 1 and 3 exhibited the most favorable average Coulombic short-range interaction energies, recorded at −86.53 ± 11 kJ mol⁻¹ and −98.04 ± 17 kJ mol⁻¹, respectively. These compounds also demonstrated the lowest average Lennard–Jones short-range interaction energies, measured at −84.56 ± 14 kJ mol⁻¹ and −106.02 ± 4.6 kJ mol⁻¹, respectively. The results of MM/PBSA calculations revealed binding free energies of −10.99 ± 2.07, −11.39 ± 2.71, −26.77 ± 3.83, and 0.20 ± 0.32 kJ mol⁻¹ for isolated compounds 1–4 with the target enzyme, respectively. These computational results support the experimental data, suggesting that compounds 1 and 3 could be potent inhibitors of β-glucuronidase.

Introduction

β-Glucuronidase is an enzyme widely present in mammalian tissues, where it plays a pivotal role in the metabolism and detoxification of many endogenous and exogenous drugs.¹ The hydrolysis of glucuronide conjugates is mainly catalyzed by this enzyme, facilitating the excretion of these substances from the body.² However, aberrant β-glucuronidase activity has been implicated in a number of diseases, including tumors, inflammatory diseases, microbial infections, and metabolic disorders.² Elevated activity of this enzyme can lead to the reactivation of carcinogens, contributing to tumor progression and the development of drug resistance in chemotherapy.³ Additionally, in inflammatory conditions, increased β-glucuronidase levels can exacerbate tissue damage...
by releasing pro-inflammatory and cytotoxic metabolites. Given the enzyme’s significant role in these diseases, the search for effective β-glucuronidase inhibitors has gained considerable attention. Natural compounds, particularly those isolated from plants, have emerged as promising candidates due to their diverse and potent bioactivities. Among these, flavonoids have shown notable potential, owing to their polyphenolic structures, which confer strong antioxidant, anti-inflammatory, and anticancer properties. Moreover, structural modifications such as prenylation and geranylation can enhance the pharmacological profiles of these compounds, increasing their efficacy and selectivity for specific molecular targets. Consequently, identifying potent β-glucuronidase inhibitors is considered a promising therapeutic strategy for addressing a variety of diseases.

Centaurea scoparia (C. scoparia), a species belonging to the Asteraceae family, is a herbaceous plant with a long history of medicinal use and is known for its rich chemical composition and diverse biological activities. This plant is a source of numerous bioactive compounds, including chlorinated and non-chlorinated guaianolides, flavonoids, sesquiterpene lactones, phenolic acids, and essential oils. The intricate chemical profile of C. scoparia underpins its wide range of pharmacological effects, which include antioxidant, anti-inflammatory, antimicrobial, and anticancer properties. Flavonoids and sesquiterpene lactones, in particular, are notable for their potent bioactivities, contributing to the plant’s therapeutic potential. The comprehensive investigation of C. scoparia’s chemical constituents and their biological activities not only highlights its medicinal value but also opens avenues for the development of novel natural health products and pharmaceutical agents.

The combined utilization of in vitro experimental assays and computational investigations, such as docking and molecular dynamics (MD) simulations, represents a powerful approach in evaluating the inhibitory activity of natural compounds against enzymes. In vitro assays provide essential empirical data, revealing the potency and efficacy of these natural inhibitors in a controlled environment. Complementing these experiments, computational techniques offer detailed insights into the molecular interactions between the inhibitors and the enzyme. Molecular docking predicts the binding affinities and interaction sites, while MD simulations further elucidate the dynamic profile and stability of these interactions over time. This integrative methodology not only enhances the understanding of the mechanistic aspects of inhibition but also streamlines the drug discovery process, enabling a more efficient identification and optimization of potential therapeutic agents targeting β-glucuronidase.

The aim of this work is to assess the inhibitory activity of our previously isolated flavonoid aglycones from C. scoparia on β-glucuronidase. By investigating the bioactivity of these plant-derived substances, we targeted the identification of potential therapeutic agents that could mitigate the adverse effects associated with dysregulated β-glucuronidase activity and take part in the development of novel treatments for related diseases.

Experimental

Phytochemical investigation

General. The Bruker AV-500 spectrometer was the spectrometer utilized for calculating the NMR spectral data (1H NMR at 500 MHz and 13C NMR at 125 MHz). The ultraviolet (UV) spectra were obtained using a Shimadzu UV-vis 160i spectrophotometer. The mass spectrometric analysis (HREIMS and EIMS) was performed using a Finnigan MAT TSQ 700 mass spectrometer. A Shimadzu FTIR-8400 spectrometer was employed to calculate the IR spectral data.

Plant material. Specimens of C. scoparia aerial parts were gathered from the Eastern desert in close proximity to Helwan governorate on February 2017. Expert taxonomic analysis was performed by a specialist from the Department of Botany at the University of Beni-Suef, Egypt. A verified sample was carefully stored within the Natural Product Laboratory at Beni-Suef University for future analyses and as a reference specimen.

Extraction and isolation of flavonoids. The procedure for extracting flavonoids from C. scoparia has been thoroughly outlined in a prior publication authored by our research team. These previously isolated compounds are utilized in the current study to evaluate their effectiveness in inhibiting β-glucuronidase activity.

In vitro enzyme inhibition assays

Chemicals and reagents. The inhibitory activity of flavonoids from C. scoparia against β-glucuronidase was assessed using an in vitro assay. p-Nitrophenyl β-D-glucuronide (PNPG) and the β-glucuronidase enzyme derived from Escherichia coli were sourced from Sigma-Aldrich, with (−)-epigallocatechin gallate (EGCG) acting as the positive control. All chemicals and reagents used in this study were of high quality, meeting HPLC grade standards. The β-glucuronidase preparation followed a previously established protocol. A 100 mM solution of PNPG was prepared in DMSO and kept at −20 °C until needed for the assay. Dimethyl sulfoxide (DMSO) and Millipore water were used throughout the experiments. The phosphate buffer was adjusted to a pH of 7.4 at a 0.1 M concentration.

β-Glucuronidase inhibitory activity assay. In this study, PNPG was employed as the β-glucuronidase substrate to assess the inhibitory effectiveness of flavonoids from C. scoparia. The procedure for assessing the inhibitory effects of these flavonoids followed established protocols from the literature. The experiments were carried out in a 96-well plate, with each well containing a 100 μL total volume. Each well included 10 μL of enzyme solution at a concentration of 2 μg mL⁻¹, 10 μL at a concentration of 10 μM of the investigated flavonoid inhibitors or the standard drug, 10 μL of the substrate (PNPG) at 250 μM concentration, and a volume of 70 μL of PBS buffer at a pH of 7.4. The experiments were performed in triplicate, and the activity of β-glucuronidase was measured by assessing the production of PNP at an OD of 410 nm following a 30-minute incubation at 37 °C. The relative activity percentage for each experimental group was calculated by monitoring the PNP concentration with respect to the blank control.
(-)-Epigallocatechin gallate (EGCG) served as the positive control in this study.

**Determination of the IC$_{50}$ values.** The IC$_{50}$ represents the concentration of an inhibitor needed to decrease the enzyme's activity by 50%. To determine the IC$_{50}$ values of the tested inhibitors against the target enzyme, an *in vitro* incubation was performed at a temperature of 37 °C for a duration of 30 minutes. The assay utilized 10 μL of enzyme solution at a 2.0 μg mL$^{-1}$ final concentration, a volume of 70 μL of PBS buffer at pH 7.4, and varying concentrations (from 0.001 to 1000 μM) of the tested flavonoids or the standard drug EGCG (10 μL).

**Enzyme kinetics analysis.** To explore how different tested flavonoids interact with β-glucuronidase and modify its catalytic activity, enzyme kinetics analyses were conducted to identify the modes of inhibition of these inhibitors. A comprehensive examination of the enzyme kinetics and inhibition constant ($K_i$) values was performed for the isolated flavonoids demonstrating the lowest IC$_{50}$ values. The hydrolysis of the substrate was observed at PNPG concentrations ranging from 200 to 1000 μM, adjusting the concentrations of the investigated inhibitors and the standard drug accordingly. Following this, Michaelis–Menten and Lineweaver–Burk plots were constructed to interpret the outcomes. The mode of inhibition, whether noncompetitive, mixed, uncompetitive, or competitive, was determined by locating the intersection point on the Lineweaver–Burk plots. The methodology for identifying the mode of inhibition and calculating the inhibition constant ($K_i$) was previously detailed in prior literature.$^{20,21}$

**Statistical analysis.** The outcomes were expressed as mean ± standard deviation (SD), and all data points reflect the average of three independent experiments. IC$_{50}$ Values were determined as the concentration of the drug needed to elicit a response halfway between the highest and lowest points of the curve. Nonlinear regression analysis with GraphPad Prism 9.0 software was utilized to compute IC$_{50}$ and $K_i$ values.

**Molecular docking.** To achieve the most energetically favorable geometrical conformation of each drug (Fig. S1, ESI†), density functional theory (DFT) was employed, utilizing the B3LYP method at the exchange–correlation functional level, without imposing constraints.$^{22,23}$ These calculations utilized the 6-311G** basis set.$^{24}$ Additionally, frequency calculations were conducted at the same level to confirm the absence of imaginary frequencies. The DFT calculations for the flavonoids investigated in this study were executed using the Gaussian 16 package.$^{25}$ For the docking calculations, AutoDock Vina v1.5.6 and Autodock Tools (ADT) v1.5.6 were utilized.$^{26}$ The crystal structure of β-glucuronidase (PDB ID: 3K4D) was retrieved from the protein data bank (PDB). ADT was utilized to prepare β-glucuronidase for the docking process, involving the addition of polar hydrogens, removal of all nonstandard residues, adjustment of the grid box to match the native ligand position, and generation of the pdbqt files essential for the docking simulations.

**Molecular dynamics simulations.** After molecular docking analysis aimed at pinpointing complexes with the lowest binding affinities, we selected those formed between flavonoids from *C. scoparia* and β-glucuronidase (PDB ID: 3K4D) for further MD simulations. By means of the GROMACS 2022.4 suite, we performed a 100 ns MD simulation.$^{27,28}$ Initially, we dissociated various flavonoids from the β-glucuronidase PDB complexes for further processing with pdb2gmx. We selected the CHARMM36 force field alongside the CHARMM-modified TIP3P water model for the simulations,$^{29,30}$ and the CGenFF server (https://cgenff.com/) was used to generate topologies and geometrical parameters of the studied flavonoids, which were then integrated into the processed target enzyme's topology. The free β-glucuronidase and various flavonoid-β-glucuronidase complexes were positioned within a dodecahedron box, adjusting the box volume to 842.77 nm$^3$, and electrical neutrality was attained by using 24 sodium counterions.$^{31}$ To relieve steric clashes or unfavorable contacts, energy minimization was performed using the steepest descent algorithm until the maximum force on any atom was less than 1000 kJ mol$^{-1}$ nm$^{-1}$ or until a maximum of 10 000 steps was reached. Subsequently, NVT equilibration was conducted for 100 ps at 300 K using the velocity rescaling thermostat, followed by NPT equilibration for 100 ps at 300 K and 1 bar using the Parrinello–Rahman barostat.$^{31}$ This allowed the system to stabilize thermodynamically and adjust its density. Finally, a 100 ns production MD simulation was conducted under conditions of 300 K and 1 bar, maintaining temperature and pressure using the velocity rescaling thermostat and Parrinello–Rahman barostat, respectively. Periodic boundary conditions were applied in all directions, the LINCS algorithm was used to constrain bond lengths involving hydrogen atoms, allowing a time step of 2 fs, and long-range electrostatic interactions were calculated using the particle mesh Ewald (PME) method with a cutoff of 1.2 nm for both Coulomb and van der Waals interactions.

The molecular mechanics/Poisson–Boltzmann surface area (MM/PBSA) method was employed to provide detailed insights into the binding interactions among the ligands and the receptor. This method combines molecular mechanics (MM), Poisson–Boltzmann (PB) electrostatics, and solvent accessibility (SA) models to estimate binding energies. It is recognized for its efficiency and reliability in calculating the binding free energies of noncovalently bound complexes, as noted in various studies.$^{32,33}$ For this study, the gmx_MMPBSA tool was utilized to compute the binding free energies using the MD simulation outputs.$^{34}$

**Results and discussion**

**Phytochemical investigation**

The exhaustive phytochemical separation of the dichloromethane extract derived from the aerial components of *C. scoparia* resulted in the isolation of four isoprenylated flavonoids, as detailed in our earlier publication.$^6$ Spectroscopic analyses, notably $^1$H and $^{13}$C NMR spectroscopy, formed the cornerstone of compound identification from the isolated samples. Preliminary identification steps included TLC.
Subsequently, the acquired spectral data, particularly the NMR spectra, underwent meticulous scrutiny to deduce the structural attributes of the compounds obtained. To enhance the structural elucidation, the data obtained were compared with existing literature on known compounds, providing further validation. Thus, the chemical structures of the isolated compounds were elucidated to be 2′-hydroxychrysin (1), 35,36 jaceosidin (2), 37 5,7,4′,5′-tetrahydroxy-6,3′-dimethoxyflavone (3), 38 and 5,4′-dihydroxy-6,7-dimethoxyflavone (4). 39 The ESI† for this study, which includes spectral data and Cartesian coordinates of the isolated flavonoids, has been documented in our previous work (Fig. 1). 6

The inhibitory activity of the investigated flavonoids against β-glucuronidase

Given the crucial involvement of β-glucuronidase in a wide range of physiological functions and its connection to several pathological conditions, including drug-induced side effects, the discovery of effective inhibitors to regulate its enzymatic activity is of great therapeutic significance. The inhibitory activity of four flavonoid aglycones isolated from C. scoparia against β-glucuronidase was extensively studied, with findings summarized in Fig. 2 and Table 1. Among the flavonoids tested, compound 1 demonstrated the strongest inhibitory effect, with an IC₅₀ value of 3.16 ± 0.34 μM and an inhibition rate of 97.12 ± 1.74%. This level of activity is quite comparable to that of the reference drug EGCG, which has an IC₅₀ of 3.67 ± 0.4 μM and an inhibition rate of 95.95 ± 1.28%. These results suggest that compound 1 is almost as effective as EGCG in inhibiting β-glucuronidase. Compound 3 also showed notable inhibitory activity, with an IC₅₀ of 3.82 ± 0.1 μM, placing it in the same efficacy range as both compound 1 and EGCG. On the other hand, compound 4 displayed the highest IC₅₀ value at 9.95 ± 0.46 μM, indicating that it was the least potent inhibitor among the tested flavonoids. Compound 2 had an intermediate level of inhibitory activity with an IC₅₀ of 5.70 ± 0.08 μM. Fig. 2A represents the dose-dependent inhibition of the target enzyme-mediated PNPG hydrolysis using the proposed inhibitors. The dose–response curves depicted in this figure offer valuable insights regarding the potency and efficacy of the flavonoids. The curves clearly show that as the concentration of isolated phytochemicals increases, the inhibition of β-glucuronidase activity also increases, confirming the effectiveness of these compounds as enzyme inhibitors. Additionally, Fig. 2B presents the IC₅₀ values of the investigated flavonoids compared to the reference drug EGCG. This comparison highlights the superior inhibitory potency of compounds 1 and 3. The relative inhibitory potencies of these compounds are visually evident, with compound 1 showing the closest IC₅₀ value to that of EGCG. Furthermore, Fig. 2C demonstrates the use of nine different concentration gradients to measure the relative activity of the tested flavonoids and the positive control (EGCG) against β-glucuronidase. This comprehensive analysis underscores the robust inhibitory activity of compounds 1 and 3, suggesting their potential as effective β-glucuronidase inhibitors.

In summary, the data indicate that among the flavonoid aglycones isolated from C. scoparia, compounds 1 and 3 exhibit significant inhibitory activity against β-glucuronidase, comparable to the well-known inhibitor EGCG. These findings provide a basis for more exploration of these compounds as potential therapeutic agents targeting β-glucuronidase-related pathologies. Compound 4, with the highest IC₅₀ value, appears to be the least effective, whereas compound 2 holds moderate...
inhibitory potential. The concentration-dependent inhibition and relative potency highlighted in Fig. 2A–C offer a detailed understanding of the inhibitory dynamics of these flavonoids, reinforcing the potential therapeutic efficacies of compounds 1 and 3.

Both synthetic and naturally derived compounds have been studied as inhibitors of \( \beta \)-glucuronidase.\(^4\) Noteworthy natural inhibitors include flavonoids, phenolic acids, coumarins, and terpenoids, which are commonly found in fruits, vegetables, and medicinal plants.\(^{4,40}\) For instance, compounds such as scutellarein, luteolin, catechin, apigenin, kaempferol, sanggenon C, kuwanon G, and scumoniline have shown strong \( \beta \)-glucuronidase inhibitory activity in preclinical studies.\(^ {4,20,41,42}\) These natural inhibitors present a promising alternative to synthetic counterparts, with potential benefits in enhancing drug absorption, reducing enterohepatic recirculation, and modulating the gut microbiome. Further research is needed to fully understand the mechanisms and therapeutic potential of these natural \( \beta \)-glucuronidase inhibitors.

### Enzyme kinetics analysis

To further elucidate the inhibitory potential of the flavonoids, detailed enzyme kinetics calculations of \( \beta \)-glucuronidase were performed. This analysis focused on compounds 1–3, and the positive control EGCG, given their high potency and significant inhibition rates. To clarify the dose-dependent inhibition patterns of these compounds against \( \beta \)-glucuronidase, inhibition kinetics analyses were performed, incorporating alteration in both the substrate and investigated flavonoid concentrations. The Michaelis–Menten plot in Fig. 3A demonstrated that the tested inhibitors interact with the catalytic site of \( \beta \)-
glucuronidase. This plot illustrates a clear relationship between flavonoid concentration and the degree of enzymatic inhibition, highlighting the potential of these flavonoids as effective β-glucuronidase inhibitors. These results emphasize the effectiveness of compounds 1, 2, and 3 in regulating β-glucuronidase activity. To gain deeper insight into the inhibitory mechanisms and ascertain the mode of inhibition of the tested flavonoids, Lineweaver–Burk plots were generated and are shown in Fig. 3B. Examination of these plots unveiled that compounds 2, 3, and EGCG displayed a mixed inhibition mechanism. This was evident from their intersections in the second quadrant, positioned away from the y-axis, which indicates a combination of competitive and noncompetitive inhibition. In contrast, compound 1 revealed a noncompetitive inhibition mode. This was apparent from its line intersections occurring at a specific point on the y-axis (1/V_{max}) and a shared point on the x-axis. This behavior suggests that compound 1 interacts with the enzyme at a binding site different from the PNPG binding pocket, affecting enzyme function without affecting substrate binding. The differences in inhibition mechanisms observed can be attributed to variations in the geometrical structures of the flavonoids. The $K_i$ values, which
represent the inhibition constants, were calculated as 3.07, 6.18, 3.55, and 1.81 μM for compounds 1, 2, 3, and EGCG, respectively. These values further underscore the diverse inhibitory profiles of the tested compounds, with compound 1 and EGCG demonstrating the strongest binding affinities for β-glucuronidase. The derived $K_i$ values align closely with the measured IC$_{50}$ values, thus reinforcing the effectiveness of the tested flavonoids as inhibitors. In essence, the enzyme kinetic investigations offer comprehensive insights into the inhibitory mechanisms employed by compounds 1, 2, 3, and EGCG against β-glucuronidase. These findings, corroborated by IC$_{50}$ and $K_i$ values, highlight the significant potential of these flavonoids as effective β-glucuronidase inhibitors. This dual confirmation of inhibitory efficacy underscores their promise for therapeutic applications, suggesting that these compounds could be developed into potent treatments targeting conditions associated with β-glucuronidase activity.

**Molecular docking analysis**

To gain deep insights into the binding interactions between the tested flavonoids and β-glucuronidase, docking studies were performed. This approach provided detailed insights into how these compounds interact with the enzyme at the molecular level, supporting their potential as effective inhibitors. The findings, detailed in Table 2 and Fig. 4 and 5, offer significant insights into these interactions. Compounds 1 and 3 displayed the lowest binding affinities among the flavonoids tested, aligning with their low IC$_{50}$ values and indicating strong inhibitory potential. Conversely, compound 4 exhibited the highest binding affinity but also the highest IC$_{50}$ value, suggesting that it binds to β-glucuronidase but is less effective at inhibiting its activity compared to compounds 1 and 3. Compounds 2 and 3 showed the greatest extent of polar interactions with the enzyme, which are crucial for stabilizing the inhibitor-enzyme complex and enhancing inhibitory effects. In contrast, compound 1 exhibited the most significant hydrophobic interactions, contributing to its strong binding affinity and effective inhibition. Key residues such as Phe554 and Phe448 were involved in the binding mechanisms of several flavonoids. These residues facilitate thermodynamically favorable π–π interactions, which further stabilize the binding of the flavonoids to the enzyme. This presence supports the experimental enzyme inhibitory activity findings. In summary, the molecular docking analysis provides a detailed understanding of the interactions between the flavonoids and β-glucuronidase. Compounds 1 and 3, with their low binding affinities and extensive hydrophobic interactions, emerge as potent inhibitors. The significant polar interactions observed in compounds 2 and 3, along with the binding affinity but higher IC$_{50}$ of compound 4, highlight the diverse mechanisms by which these flavonoids inhibit the enzyme. Thus, the investigated flavonoids exhibited binding affinities lower than that of the native ligand, (2S,3R,4S,5R)-3,4,5-trihydroxy-6-oxopiperidine-2-carboxylic acid (EVA), which had a binding energy of $-6.7$ kcal mol$^{-1}$. However, EVA demonstrated a high degree of hydrogen bonding interactions with the target enzyme. The results of the molecular docking analysis for EGCG and EVA with the target enzyme are presented in Fig. S2 and S3 (ESI†), respectively, in the ESI.† The involvement of key residues Phe554 and Phe448 in favorable π–π interactions contributes to the stability and efficacy of these inhibitor-enzyme complexes, underscoring the therapeutic potential of these flavonoids.

**Molecular dynamics simulation**

Building upon the initial docking analyses that prioritized complexes with the most favorable binding free energies, this section delves into the findings obtained from 100 ns MD simulations investigating the interactions between isolated flavonoids and β-glucuronidase. These simulations aimed to elucidate the dynamic behavior of these complexes at the atomic level. The analysis focuses on key parameters that shed light on the intermolecular interactions between the flavonoids and the enzyme. These parameters include binding energies, root mean square deviations (RMSD), radius of gyration ($R_g$), root mean square fluctuations (RMSF), hydrogen bond occupancy, and solvent accessible surface area (SASA). By examining these parameters, we aim to gain an intensive understanding of the binding modes, conformational flexibility, and potential inhibitory mechanisms of the studied flavonoids against β-glucuronidase.

The RMSD analysis highlights the relationship between enzyme conformational flexibility and the binding efficiency of the tested flavonoids. The results of backbone RMSD calculations of the unbound enzyme and different flavonoid complexes are represented in Fig. 6A. Initially, all systems exhibited an increasing RMSD trend during the first quarter...

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<tr>
<th>Compound</th>
<th>Binding affinity (kcal mol$^{-1}$)</th>
<th>Polar interactions</th>
<th>Hydrophobic interactions</th>
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<tr>
<td>Compound 1</td>
<td>$-8.6$</td>
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<td>His162, Asp163, His330, Val355, Glu413, Met447, Tyr468, Tyr472, and Phe554</td>
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<td>Compound 2</td>
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</tr>
<tr>
<td>Compound 3</td>
<td>$-9.0$</td>
<td>Lys568, Trp549, Asn566, and Met447</td>
<td>Asp163, His330, Leu361, Glu413, Val446, Phe448, Tyr468, and Tyr472</td>
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<tr>
<td>Compound 4</td>
<td>$-8.4$</td>
<td>Glu504, Arg562, and Glu413</td>
<td>Val446, Met447, Leu361, Phe448, Tyr468, Tyr472, and Asp163</td>
</tr>
<tr>
<td>EGCG</td>
<td>$-8.9$</td>
<td>Phe448, Asp163, Trp549, Tyr472, Asn566, Arg562, and Lys568</td>
<td>Tyr468, His330, Glu504, Leu361, Glu413, Met447, and Leu561, and Val473</td>
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of the simulation span. Then, the simulated systems reached equilibrium and fluctuated around a stable mean, indicating that the enzyme-flavonoid complexes had settled into a stable conformation. The average RMSD values of the free enzyme and complexes of flavonoids 1–4 are 0.295 ± 0.046, 0.335 ± 0.059, 0.255 ± 0.022, 0.272 ± 0.050, and 0.273 ± 0.036 nm, respectively. Notably, compound 1, which has the lowest IC₅₀, displayed the highest average RMSD value after equilibration. This suggests that compound 1 induces considerable structural changes in the enzyme, potentially correlating with its higher binding affinity and inhibitory efficiency.

To examine the intrinsic movement and conformational changes of flavonoid molecules independently from their interaction with β-glucuronidase, we calculated the RMSD values of the inhibitors (Fig. 6B). These values were obtained by aligning the trajectory frames to the initial structure of the drug and then computing the RMSD of the flavonoid coordinates relative to its geometry as a reference. Consequently, one can evaluate the deviation of the inhibitor from its initial conformational structure over the course of the 100 ns simulation period, which is essential in drug design studies for evaluating the stability and dynamics behavior of the inhibitor molecules. As depicted in Fig. 6B, the RMSD analysis for different flavonoids showed significant variations in their profiles. All the flavonoids investigated displayed high fluctuation in their RMSD values. These high fluctuations indicate considerable conformational flexibility, which could be attributed to the inherent structural dynamics of the flavonoid molecules. Such flexibility is important for understanding the potential adaptive behavior of the drugs when binding to various targets, highlighting their potential efficacy in dynamic biological environments.

The RMSD profiles of various flavonoids-β-glucuronidase complexes, as shown in Fig. 7A, provide a detailed view of the conformational stability and dynamics of these complexes during a 100 ns MD simulation. Compounds 1 and 3, which demonstrated the highest inhibitory activity, exhibited the lowest average complex RMSD value among the tested inhibitors (RMSD values of 0.77 ± 0.36 and 1.19 ± 0.27 nm,
respectively). These low RMSD values indicate that these compounds form stable complexes with the enzyme, maintaining consistent conformational integrity throughout the simulation. The stability of these complexes is further highlighted by the minimal fluctuation pattern observed in their RMSD profiles. These findings suggest that these compounds achieve an optimal fit within the binding site of the target enzyme, leading to a stable binding interaction. This stability is likely a key factor contributing to their high inhibitory activity, as reflected by their low IC₅₀ values. The average complex RMSD values of compounds 2 and 4 are 2.2 ± 1.37 and 1.35 ± 0.4 nm, respectively. In addition, compounds 2 and 4 displayed highly fluctuating RMSD profiles.

Fig. 7B illustrates the hydrogen bonding profiles of isolated flavonoid-β-glucuronidase complexes over a 100 ns MD simulation. Notably, compounds 1 and 3, identified by their high inhibitory potency and low IC₅₀ value, demonstrated the most extensive and intense hydrogen bonding interactions throughout the simulation. This significant hydrogen bonding activity suggests that these compounds establish a strong and stable interaction with β-glucuronidase. The multitude of hydrogen bonds likely contributes to the stability and effectiveness of the enzyme-inhibitor complex, underscoring the compound’s potent inhibitory effect.²⁰,⁴⁶ The extensive hydrogen bonding indicates that compounds 1 and 3 interact robustly with key residues in the enzyme’s active site, stabilizing the complex and reducing conformational flexibility.

The RMSF patterns offer insights into the dynamic characteristics of various regions within the enzyme’s structure. In Fig. 8A, the time-averaged RMSF values for unbound β-glucuronidase and different flavonoids-β-glucuronidase complexes are presented. Interestingly, all analyzed complexes exhibited RMSF profiles akin to that of the free β-glucuronidase.⁴⁷ This similarity in the RMSF profiles suggests that the binding of flavonoids to β-glucuronidase does not trigger notable changes in protein flexibility or induce substantial...
conformational alterations. Despite the formation of enzyme-inhibitor complexes, the enzyme’s flexibility remains comparable to its unbound state.

The $R_g$ values provide insights into both the compactness and the overall structural integrity of both the target enzyme and different inhibitor complexes. In Fig. 8B, the outcomes of computing the $R_g$ values for both the unbound enzyme and different enzyme complexes over a 100 ns MD simulation are depicted. Intriguingly, all complexes with flavonoids exhibited $R_g$ behavior akin to that of the unbound enzyme. This similarity suggests that the presence of flavonoids does not significantly modify the enzyme’s overall conformation or compactness. The average $R_g$ values of the free enzyme and the flavonoid complexes are $2.55 \pm 0.016$, $2.53 \pm 0.017$, $2.54 \pm 0.017$, $2.54 \pm 0.016$, and $2.55 \pm 0.018$, respectively.

The SASA values are indicators of how accessible the enzyme’s surface is to solvent molecules, providing insights into potential changes in its shape and movement. In Fig. 8C, the SASA values for both the enzyme alone and different enzyme complexes during a 100 ns MD simulation are displayed. The average SASA values of the unbound enzyme and the complexes of flavonoids 1–4 are $274.6 \pm 5.4$, $284.4 \pm 8.5$, $267.2 \pm 4.9$, $274.5 \pm 7.2$, and $270.2 \pm 6.3$ nm, respectively. Interestingly, all complexes involving flavonoids showed SASA behavior similar to that of the enzyme without any inhibitors. This similarity in SASA behavior suggests that the presence of flavonoids doesn’t
notably affect the accessible enzyme’s surface area.\(^5\,\text{,}^{49}\) Of particular interest is compound 1, known for exerting the highest potency among investigated flavonoids. Compound 1 exhibited the highest average SASA value among all inhibitors, indicating that it increases the enzyme’s surface accessibility more than the other inhibitors. This increased surface accessibility could potentially facilitate better interaction with surrounding solvent molecules and other compounds, impacting the inhibitor’s ability to inhibit the enzyme.

We explored the interaction energies between various flavonoids and \(\beta\)-glucuronidase using 100 ns MD simulations (Fig. 9). Our focus in this section was primarily on examining the Coulombic short-range (Coul-SR) and Lennard-Jones short-range (LJ-SR) interaction energies within the enzyme-flavonoid complexes.\(^5\) Assessing these energies offers valuable insights into the nature and strength of intermolecular interactions within the system.\(^5\) Such energy calculations provide crucial information about the stability, binding affinity, and specificity of molecular interactions, facilitating the understanding of protein–ligand binding mechanisms, including the formation of enzyme-inhibitor complexes. Utilizing the assessments of both interaction energies can aid in identifying pivotal binding
Fig. 8  The dynamic properties of β-glucuronidase following 100 ns MD simulations, both in its free state and when complexed with various inhibitors. Panel (A) depicts the time-averaged profile of each amino acid’s fluctuation (RMSF) within the enzyme, comparing the free and inhibitor-bound states, panel (B) explores the $R_g$ for both unbound and complexed β-glucuronidase, providing insights into the enzyme’s overall compactness upon inhibitor binding, and panel (C) investigates the SASA of the enzyme, revealing changes in solvent exposure due to inhibitor interactions.
interactions, delineating binding pockets, and guiding the rational design of novel therapeutics with enhanced potency and selectivity.

Fig. 9A displays the results of the Coul-SR interaction energy calculations for the various enzyme-flavonoid complexes. Compounds 1 and 3, which demonstrated the highest inhibitory potency \textit{in vitro} as indicated by their low IC\textsubscript{50} values, showed the lowest value of average Coul-SR interaction energy (Table 3). Conversely, compounds 2 and 4, which had the highest IC\textsubscript{50} values, showed the highest value of average Coul-SR interaction energy. The Coul-SR interaction energy values are critical for understanding the strength of electrostatic interactions between the enzyme and flavonoid inhibitors. The lower Coul-SR interaction energy values observed for compounds 1 and 3 suggest stronger electrostatic interactions with β-glucuronidase, which likely enhances the binding affinity and

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<thead>
<tr>
<th>Compound</th>
<th>Coul-SR interaction energy (\text{kJ mol}^{-1})</th>
<th>RMSD (nm)</th>
<th>LJ-SR interaction energy (\text{kJ mol}^{-1})</th>
<th>RMSD (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>86.53 ± 11</td>
<td>36.35</td>
<td>84.56 ± 14</td>
<td>14.97</td>
</tr>
<tr>
<td>2</td>
<td>44.89 ± 13</td>
<td>45.23</td>
<td>67.28 ± 10</td>
<td>29.09</td>
</tr>
<tr>
<td>3</td>
<td>98.04 ± 17</td>
<td>50.28</td>
<td>106.02 ± 4.6</td>
<td>16.94</td>
</tr>
<tr>
<td>4</td>
<td>17.21 ± 1.6</td>
<td>14.93</td>
<td>74.99 ± 1.9</td>
<td>15.67</td>
</tr>
</tbody>
</table>
contributes to their greater inhibitory potency. This robust electrostatic attraction probably facilitates a more stable and effective binding conformation, thereby improving the inhibitor's ability to block the enzyme's activity effectively. In

Fig. 10 The free energy landscape of free β-glucuronidase and different drug–enzyme systems; (A) 2D-free energy landscape of the investigated systems, (B) a representation of the drugs within the binding site of the enzyme, showing the conformations with minimal energy and residues involved in the binding interactions are highlighted, and (C) a 3D representation of the free energy landscape.
contrast, the higher Coul-SR interaction energy observed for compounds 2 and 4 indicates weaker electrostatic interactions, which may result in less stable binding and lower affinity. This corresponds to its higher IC$_{50}$ value, reflecting reduced inhibitory potency. These findings highlight the significant role of electrostatic interactions in determining the efficacy of enzyme inhibitors. The clear correlation between low Coul-SR interaction energies and high inhibitory potency emphasizes the importance of these interactions in stabilizing the enzyme-inhibitor complex.

The outcomes of the LJ-SR interaction energy calculations for various enzyme-flavonoid complexes, based on 100 ns MD simulations, are shown in Fig. 9B and Table 3. Similar to the Coul-SR interaction energy findings, compounds 1 and 3, which have the lowest IC$_{50}$ values, exhibited the lowest average LJ-SR interaction energy values. In contrast, compounds 2 and 4 had higher, more positive LJ-SR interaction energy values. The LJ-SR interaction energy reflects the van der Waals forces and steric interactions between the enzyme and the flavonoid inhibitors. Lower LJ-SR interaction energy values for compounds 1 and 3 suggest stronger van der Waals attractions and better steric complementarity with the enzyme, likely contributing to their higher binding affinity and greater inhibitory potency. These strong LJ-SR interactions may promote a more stable and optimal binding conformation, enhancing the inhibitors’ ability to block the enzyme’s activity effectively. Conversely, the higher LJ-SR interaction energy values for compounds 2 and 4 indicate weaker van der Waals interactions and potentially suboptimal steric fit within the enzyme’s active site. This can lead to less stable binding and lower affinity, correlating with their higher IC$_{50}$ values and reduced inhibitory efficacy. The less favorable LJ-SR interactions suggest that these flavonoids may not interact as effectively with key non-polar residues essential for high-affinity binding.

The global minimum energy conformation of various enzyme–drug complexes was determined through free energy landscape (FEL) analysis. Principal component analysis (PCA) was conducted on the 100 ns trajectories of the unbound enzyme and the various flavonoid-bound systems, leading to the construction of 2D and 3D free energy landscape (FEL) diagrams, as shown in Fig. 10. The FEL analysis provided insights into the energy minima within each system, and the representative structures corresponding to these minima are depicted in Fig. 10B. In the 2D-FEL contour maps (Fig. 10A), the free enzyme system exhibited a broad minimal energy cluster, indicating a more open and flexible active site conformation conducive to substrate entry. Conversely, the systems containing the investigated inhibitors showed a more compact and localized energy minimum, suggesting a closed conformation of the active site that obstructs substrate access. This conformational change highlights the potential of these inhibitors to effectively block the enzyme’s active site. The 3D-FEL representations (Fig. 10C) further corroborate these findings, showing distinct energy wells for the flavonoid-bound systems compared to the free enzyme. The bound forms of β-glucuronidase demonstrated fewer and smaller energy minima, indicative of a more restricted and stable conformation upon inhibitor binding. This suggests that the binding of flavonoids to β-glucuronidase induces significant alterations in the structural dynamics of the active site, thereby modulating substrate accessibility. The conformational variance observed between the free and bound enzyme forms underscores the potency of the inhibition mechanism. The binding of the flavonoids not only stabilizes the enzyme in a closed conformation but also reduces the structural flexibility of the active site, which is critical for effective inhibition. The representative structures corresponding to energy minima (Fig. 10B) illustrate the specific interactions between the drugs and the enzyme, highlighting the key residues involved in binding. These interactions are crucial for the inhibitory activity and support the FEL analysis findings.

The results of MM/PBSA calculations are represented in Table 4. The MM/PBSA calculations revealed significant variations in binding free energies among the four compounds studied. The superior inhibitory activities of flavonoids 1 and 3 can be attributed to their strong van der Waals and electrostatic interactions, which effectively counterbalance the desolvation penalties upon binding to the enzyme. These findings provide valuable insights into the molecular interactions governing the binding affinities and can guide the design of more potent inhibitors.

**Table 4** The results of MM-PBSA (kJ mol$^{-1}$).

<table>
<thead>
<tr>
<th>System</th>
<th>Compound 1</th>
<th>Compound 2</th>
<th>Compound 3</th>
<th>Compound 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ$E_{dew}$</td>
<td>$-21.12 \pm 2.59$</td>
<td>$-20.82 \pm 3.34$</td>
<td>$-31.03 \pm 3.27$</td>
<td>$-0.26 \pm 0.69$</td>
</tr>
<tr>
<td>Δ$E_{ele}$</td>
<td>$-9.53 \pm 6.08$</td>
<td>$-6.22 \pm 4.48$</td>
<td>$-29.30 \pm 5.49$</td>
<td>$-0.26 \pm 0.19$</td>
</tr>
<tr>
<td>Δ$G_{ele}$</td>
<td>$19.66 \pm 6.07$</td>
<td>$15.66 \pm 3.86$</td>
<td>$33.57 \pm 3.93$</td>
<td>$0.72 \pm 2.36$</td>
</tr>
<tr>
<td>Δ$G_{total}$</td>
<td>$-30.65 \pm 7.11$</td>
<td>$-27.04 \pm 5.90$</td>
<td>$-60.34 \pm 6.57$</td>
<td>$-0.52 \pm 2.51$</td>
</tr>
</tbody>
</table>

**Conclusion**

Our previous research into the phytochemical constituents of *C. scoparia* identified four isoprenylated flavonoids and four flavonoid aglycones. In this study, we evaluated the inhibitory efficacy of the four flavonoid aglycones against β-glucuronidase using *in vitro* assays, docking, and molecular dynamics simulations. The findings of *in vitro* inhibition assays revealed that compounds 1 and 3 had the highest inhibitory activity, with IC$_{50}$ values of 3.16 ± 0.34 and 3.82 ± 0.1 μM, respectively. Enzyme kinetics analyses indicated that compounds 2 and 3, and the positive control drug EGCG displayed a mixed inhibition mode. In contrast, compound 1 demonstrated a noncompetitive inhibition mechanism, as shown by the line intersection patterns in the Lineweaver–Burk plots. The outcomes from the docking studies align with the results from the experimental inhibitory activity assays, indicating that compounds 1 and 3 have the lowest binding affinities. These compounds exhibited significant polar and hydrophobic interactions with the residues in the enzyme’s binding site. Furthermore, crucial residues involved in the binding mechanism with the flavonoids contribute to their higher binding affinity and greater inhibitory potency.
β-glucuronidase were identified, highlighting the potential of these flavonoids as effective inhibitors.

Our study utilized a 100 ns MD simulation to investigate the interactions between isolated flavonoids and β-glucuronidase. Analyzing various MD parameters, we found that compounds 1 and 3, which had the lowest IC₅₀ values, formed notably stable interactions with the enzyme. Compound 1 exhibited the highest average enzyme backbone RMSD values, indicating significant conformational changes during the simulation, which may reflect its ability to adapt and effectively interact with the enzyme’s dynamic binding site. Additionally, the lowest average drug-to-drug RMSD profile was detected for compound 1, indicating a more stable and consistent conformation throughout the simulation. Both compounds 1 and 3 showed minimal fluctuations in their complex RMSD profiles, suggesting that these complexes maintained a stable conformation throughout the simulation. Additionally, compound 1 showed the most extensive and intense hydrogen bonding throughout the simulation, indicating a strong and stable interaction with β-glucuronidase. It also had the minimum average R² values and the highest average SASA values, indicating the high compactness of the enzyme conformation and greater solvent accessibility. Furthermore, compounds 1 and 3 demonstrated the lowest average Coul-SR and LJ-SR interaction energies with the enzyme’s binding site. This indicates strong electrostatic attractions, robust van der Waals forces, and better steric complementarity with the enzyme, which likely enhances their binding affinity and contributes to their greater inhibitory potency.

Data availability
The data supporting this article have been included in the ESI.†

Conflicts of interest
There are no conflicts to declare.

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References


