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Research Article

Broad-Spectrum Trypanocidal Activity of the Natural Citrus Flavanone Glycosides Hesperidin and Neohesperidin Dihydrochalcone

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Abstract

Background and Objective: Hesperidin and neohesperidin are naturally occurring citrus flavanone glycosides that have a wide range of therapeutic uses. There is an increasing preference for natural products as chemotherapeutic agents. In this study, comprehensive research presented on the inhibitory effects of hesperidin and neohesperidin dihydrochalcone against 6 different species of *Trypanosoma*, as well as an analysis of their potential mechanism of action. **Materials and Methods:** The trypanocidal activity was evaluated using six trypanosome species cultivated in HMI-9 medium. Molecular docking procedures were conducted focusing on the *Trypanosoma brucei* Dihydrofolate Reductase (TbDHFR), with compound structures sourced from PubChem and optimized for physiological pH. Additionally, molecular dynamics (MD) simulations were carried out over 50 ns, assessing parameters such as RMSD, RMSF and hydrogen bonds to provide insights into the compound's stability and interactions. Data analysis utilized descriptive statistics to summarize the variations observed in the trypanocidal effects and molecular interactions. **Results:** The findings indicated that hesperidin lacked antitrypanosomal effects. On the other hand, neohesperidin dihydrochalcone showed significant trypanocidal activity across a wide range of *Trypanosoma* strains, including *Trypanosoma brucei brucei* GUT at 3.1, *T. b. rhodesiense* IL1501, *T. b. gambiense* IL1922, *T. evansi* Tansui, *T. equiperdum* IVM-t1 and *T. congolense* IL3000. The estimated IC₅₀ values ranged from 8.88 to 22.53 µg/mL, indicating low micromolar inhibition of *Trypanosoma*. Through docking and MD simulations, it is confirmed that neohesperidin dihydrochalcone binds to TbDHFR. The results of MD simulation support stable complex of neohesperidin dihydrochalcone with TbDHFR. **Conclusion:** The demonstrated efficacy of neohesperidin dihydrochalcone across various *Trypanosoma* species endorses its potential as a secure, natural agent against trypanosomiasis. It is strongly advised to further investigate the creation of analogues of neohesperidin dihydrochalcone and consider its application for the treatment of additional protozoan infections.

Key words: Neohesperidin, *Trypanosoma*, pathogenic, antiprotozoal, flavanone, natural

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Diseases caused by microbes and protozoa are widespread throughout the globe^{1,2}. While more and more sophisticated medicines are being developed every day to combat these infections, antimicrobial resistance is reducing the efficacy of even the newest of them³. Therefore, scientists are increasingly exploring natural substances as potential sources for antibacterial and antiprotozoal therapies^{4,5}. In this regard, neohesperidin seems to be a potential compound due to its antimicrobial properties. Neohesperidin is a flavanone glycoside compound found in several citrus fruits⁶. The compounds belonging to flavonoids have been well explored for their antimicrobial, anti-fungal, antioxidant, anti-tumor, anti-viral and anti-inflammatory roles^{7,8}. Therefore, neohesperidin may be considered a promising candidate for future pharmaceutical development.

Bergamot (*Citrus bergamia*) powder was explored for its antimicrobial and anti-inflammatory properties⁹. The neohesperidin, hesperidin and neohesperidin-rich extracts not only boosted gut-beneficial bacteria but also demonstrated potent antibacterial action against pathogenic bacteria. With the minimum inhibitory concentration (MIC) values, the extract's antibacterial potential was comparable to that of conventional antibiotics like gentamicin and vancomycin. The result indicated that the neohesperidin enriched extract had significant antimicrobial activity against both Gram-positive and Gram-negative bacteria at low concentrations. Similar results were also reported by Mandalari *et al.*¹⁰ using neohesperidin extract in a concentration range of 200 to 800 µg/mL.

Apart from its effectiveness against bacteria, neohesperidin has also been studied for its antiprotozoal activity. Even though the literature available in this regard is quite limited, studies have reported that neohesperidin has shown potential against several protozoa, like *Plasmodium falciparum*¹¹. The study indicated a high binding affinity of neohesperidin with different proteins of *plasmodium*, especially the ones regulating different growth phases of the pathogen. Toxoplasmosis, caused by *Toxoplasma gondii*, is another critical protozoal disease. Toxoplasmosis is generally an opportunistic infection and it is more common in immunocompromised patients, where it can cause severe encephalitis, brain damage and even blindness¹². The antiprotozoal activity of neohesperidin against toxoplasmosis was compared to that of other flavonoids and conventional medicines¹³. The findings suggest that neohesperidin was efficacious, yet not to the extent of other

conventional medications or flavonoid compounds in terms of effectiveness. Neohesperidin was included in the extensive list of flavonoids compiled for their antiprotozoal properties¹⁴.

Plant extracts containing bioactive compounds as neohesperidin have been associated with antibacterial activity¹⁵. The precise mechanism of neohesperidin's antimicrobial activity is unknown, but it has been proposed that flavonoids interfere with many bacterial mechanisms such as bacterial motility, cytoplasmic permeability and metalloenzyme inhibition¹⁶. In addition, it has been found that citrus flavonoids block the cell-to-cell signaling activity of bacteria¹⁷. Regarding the antiprotozoal activity, previous research suggests that the antiprotozoal property of neohesperidin stands on its ability to bind with the pocket of phosphate of regenerating liver-protein tyrosine phosphatase (PfPRL-PTP)¹¹. The PfPRL is involved in the host invasion and is generally secreted by plasmodium-infected cells. The PfPRL also co-localizes with AMA-1 and their combination is important for Red Blood Cells (RBCs) invasion¹⁸. Due to the importance of PfPRL in malarial infection, the protein is an important target for neohesperidin therapy. Based on a previous report on the potential antiprotozoal action of neohesperidin and on ongoing work by targeting TbDHFR, this study was performed to screen the antitrypanosomal actions of neohesperidin on six *Trypanosoma* species.

MATERIALS AND METHODS

Study duration and location: At King Faisal University, Saudi Arabia, the molecular modeling was carried out from April, 2018-January, 2019, while the molecular dynamics studies were from October, 2019 to March, 2020. At Obihiro University of Agriculture and Veterinary Medicine, Japan, the trypanocidal assays were carried out during September, 2020 to July, 2021.

In vitro of trypanocidal activity of neohesperidin dihydrochalcone: Six trypanosome species namely *T. b. brucei* (Tbb) GuTat3.1, *T. b. rhodesiense* (Tbr) IL1501, *T. b. gambiense* (Tbg) IL1922, *T. evansi* (Tev) Tansui, *T. equiperdum* (Teq) IVM-t1 and *T. congolense* (Tc) IL3000 were cultivated using HMI-9 medium and used in the study. Following the procedures reported by Sukanuma *et al.*¹⁹ and Kandeel and Sukanuma²⁰, the trypanocidal activity of hesperidin and neohesperidin dihydrochalcone (Fig. 1) was assessed at 25 or 0.25 µg/mL. Neohesperidin dihydrochalcone showed substantial trypanocidal activity at 25 µg/mL, while no activity was noticed for hesperidin. The IC₅₀ of neohesperidin dihydrochalcone against six trypanosome species was

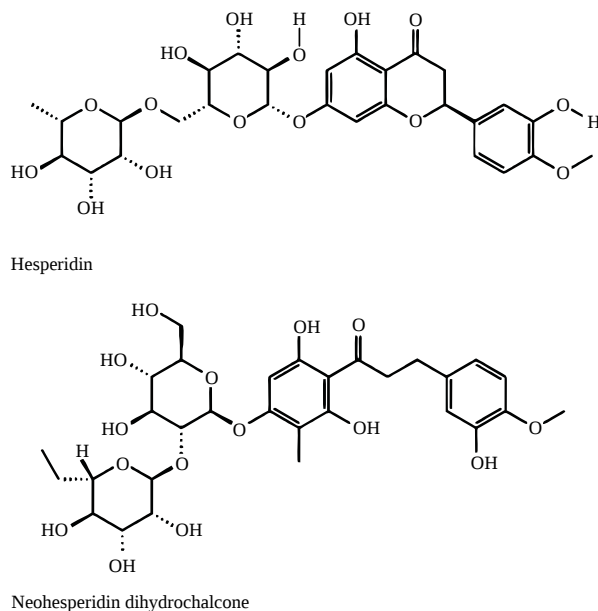


Fig. 1: Chemical structure of hesperidin and neohesperidin dihydrochalcone

determined using serial dilution in a 96-well plate (Optical bottom plate, ThermoFisher Scientific, Massachusetts, USA). After 3 days, 25 μ L of CellTiter-Glo Luminescent cell viability reagent (Promega Corporation, Wisconsin, USA) was aliquoted into each well and luminosity was measured with a GloMax plate reader (Promega Corporation, Wisconsin, USA).

Molecular docking: Docking runs were performed to check the binding potency with TbDHFR, which is an important antimicrobial and antiprotozoal target. Minimal changes were made to the protocols for protein expression, ligand design and docking that were previously described by Burayk *et al.*²¹ and Kandeel *et al.*²². Schrodinger Maestro suite (Schrodinger LLC, New York, USA) was used in all docking procedures. The compounds' 2D structures were downloaded from PubChem, processed by Ligprep and finally, 3D optimized at the physiologic pH. The structure of TbDHFR complexed with WR99210 at 2.00 Å resolution was retrieved from the Protein Data Bank (PDB, 3RG9)²³.

Using the protein preparation module, the docking of the PDB 3RG9 structure optimised. Detrimental crystallographic chemicals and surplus water molecules were removed from the solution. The protein was made protonated by the addition of polar hydrogens and the OPLS2005 force field was used to optimise the structures and reduce the overall energy. For docking grid generation, WR99210 was employed as the center of a 20 Å grid box.

The standard SP glide docking approach was applied and docking scores were used to rank the final findings. For verification, we redocked WR99210 and when compared to the bound ligand, that the docking position found was fully complementary and had a small Root-Mean-Square Deviation (RMSD).

Molecular dynamics simulation: Simulations of molecular dynamics for 50 ns were run using the desmond program by Schrödinger LLC. The System Builder software that was used to build up the system. The simulation utilized the OPLS 2005 force field. The simulation was run in the NPT ensemble (Isothermal-Isobaric: Moles (N), pressure (P) and temperature (T) are conserved) at a constant 300 K and 1 atm. The orthorhombic box of the Solvent Model was chosen for use with TIP3P. When necessary, counterions were added to the models to bring them into a neutral state. This was accomplished by supplementing the environment with 0.15 M NaCl, which is physiologically relevant. The Root Mean Square Deviation (RMSD) of the protein and ligand was measured over time by saving their trajectories from the simulations at 100 ps intervals. Root mean square fluctuations (RMSF), the radius of gyration (ROG) and the total number of hydrogen bonds were all recorded as simulation outputs.

Statistical analysis: In this research, the management and presentation of data were accomplished using MS Excel and

GraphPad Prism software. The results were expressed as Mean $\pm$ SD (standard deviation). Descriptive statistical methods were applied to represent the variations observed across each parameter of isolation.

RESULTS AND DISCUSSION

Antitrypanosomal assay: At first, hesperidin and neohesperidin dihydrochalcone were assayed at high and low concentrations of 25 or 0.25 $\mu\text{g/mL}$, respectively (Table 1). Hesperidin had inadequate antitrypanosomal activity, as evidenced by the lack or low rate of trypanocidal activity. In contrast, neohesperidin dihydrochalcone showed a broad-spectrum and strong inhibition of trypanosomal growth. At 25 $\mu\text{g/mL}$, neohesperidin dihydrochalcone suppressed all of the test strains. For TcIL3000, the inhibition rate was $82.07 \pm 6.4 \mu\text{g/mL}$, while stronger action was noticed on TbbGUTat3.1, TbrIL1501, TbgIL1922, Tev Tansui and Teq IVM-t1 by showing more than 98.8% inhibition rate (Table 1). At a low concentration of 0.25 $\mu\text{g/mL}$, the inhibition rate was 0-3.95%.

Given the promising initial antitrypanosomal action of neohesperidin dihydrochalcone, the IC_{50} was measured in the presence of several commutations of the compound. Neohesperidin dihydrochalcone showed broad-spectrum antitrypanosomal action with IC_{50} range of 8.88-22.53 $\mu\text{g/mL}$ (Table 2). The strongest trypanocidal activity was found on TbgIL1922 with $\text{IC}_{50} = 8.88 \pm 3.84 \mu\text{g/mL}$, while the TcIL3000 showed the highest resistance to the trypanocidal activity of neohesperidin dihydrochalcone with $\text{IC}_{50} = 22.53 \pm 2.3 \mu\text{g/mL}$. The strength of trypanocidal actions of neohesperidin dihydrochalcone was in the following order TbgIL1922 > TbrIL1501 > TeqIVM-t1 > TbbGUTat3.1 > Tev Tansui > TcIL3000.

While scarce reports are available on the antiprotozoal actions of neohesperidin, several evidences support the efficiency of selected protozoa. Neohesperidin showed

inhibitory activity against *T. brucei*¹⁴. The broad-spectrum action of neohesperidin dihydrochalcone was found on six *Trypanosoma* species. This is the first report to demonstrate this action. Further studies are requested to elucidate the mechanisms of actions as well as the structure-activity relationship and chemical modifications to deliver stronger derivatives.

Docking studies: During current study survey to investigate new compounds effective against TbDHFR, neohesperidin dihydrochalcone was raised as a potential binding agent.

Neohesperidin dihydrochalcone exhibited a docking score of -3.9, compared to -4.6 for the co-crystallized ligand WR99210, which accounts for approximately 85% of the crystallized ligand's score (Table 3). The binding of neohesperidin dihydrochalcone was supported by both favourable hydrogen bonds and lipophilic interaction scores. The HBond score for neohesperidin dihydrochalcone was -0.3, compared to -0.1 for the co-crystallized ligand. Furthermore, neohesperidin dihydrochalcone and compound WR99210 have favourable lipo and evdw values. These computational factors point to the favorable binding conditions associated with neohesperidin dihydrochalcone recognition.

Inspecting the mode of binding of neohesperidin dihydrochalcone with TbDHFR reveals that it has a favorable binding mode, which is supported by hydrogen bonds with the side chain of ILE160 and stacking interactions with PHE58. These interactions help in orienting and fixing the molecule into the active site of TbDHFR (Fig. 2a and b).

Molecular dynamics simulations: The RMSD was computed for the neohesperidin dihydrochalcone-TbDHFR complex and then compared to the value for Apo TbDHFR. The root mean square deviation graph, shown in Fig. 3a, demonstrates that the structure showed minor changes during the simulation. After the beginning of the simulation, the neohesperidin

Table 1: Inhibition rate of hesperidin and neohesperidin dihydrochalcone (Mean $\pm$ SD) at 0.25 or 25 $\mu\text{g/mL}$ against 6 *Trypanosoma* species

Trypanosome	Hesperidin concentration		Neohesperidin dihydrochalcone concentration	
	Inhibition rate		Inhibition rate	
	25 $\mu\text{g/mL}$	0.25 $\mu\text{g/mL}$	25 $\mu\text{g/mL}$	0.25 $\mu\text{g/mL}$
TcIL3000	0	0	82.07 ± 6.4	0
TbbGUTat3.1	1.05 ± 0.99	0	99.65 ± 0.2	2.89 ± 1.69
TbrIL1501	0	7.17 ± 3.1	99.63 ± 0.25	3.95 ± 5.59
TbgIL1922	0	1.56 ± 2.2	99.63 ± 0.28	1.09 ± 0.64
Tev Tansui	0	7.67 ± 5.9	99.18 ± 0.22	3.59 ± 5.5
Teq IVM-t1	0.97 ± 1.37	0.015 ± 0.022	98.8 ± 0.17	0.59 ± 0.84

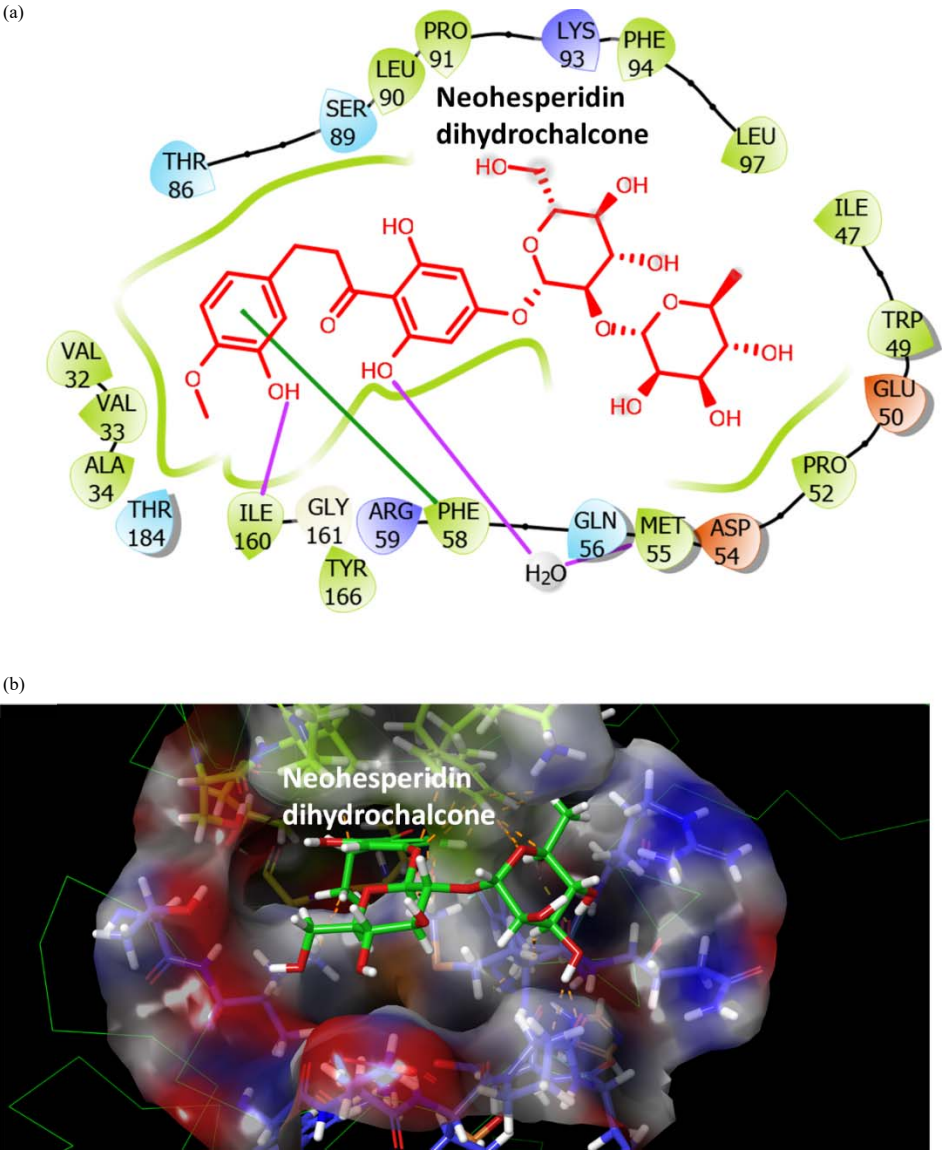


Fig. 2(a-b): Ligand interactions and docking site of neohesperidin dihydrochalcone, (a) Ligand interactions of neohesperidin dihydrochalcone with TbDHFR and (b) Docking site of neohesperidin dihydrochalcone with TbDHFR

Table 2: IC₅₀ of neohesperidin dihydrochalcone (Mean±SD) against 6 *Trypanosoma* species

Trypanosome	Neohesperidin dihydrochalcone
	IC ₅₀ (µg/mL)
TcIL3000	22.53±2.3
TbbGUTat3.1	11.97±0.026
TbrIL1501	9.47±2.73
TbglL1922	8.88±3.84
Tev Tansui	12.69±0.98
Teq IVM-t1	11.38±3.93

Table 3: Docking score and binding parameters for neohesperidin dihydrochalcone and the compound WR99210 with TbDHFR

Title	Docking score	Glide HBond	Glide lipo	Glide evdw
Neohesperidin dihydrochalcone	-3.9	-0.3	-1.01	-25.9
compound WR99210	-4.6	-0.1	-1.7	-20.8

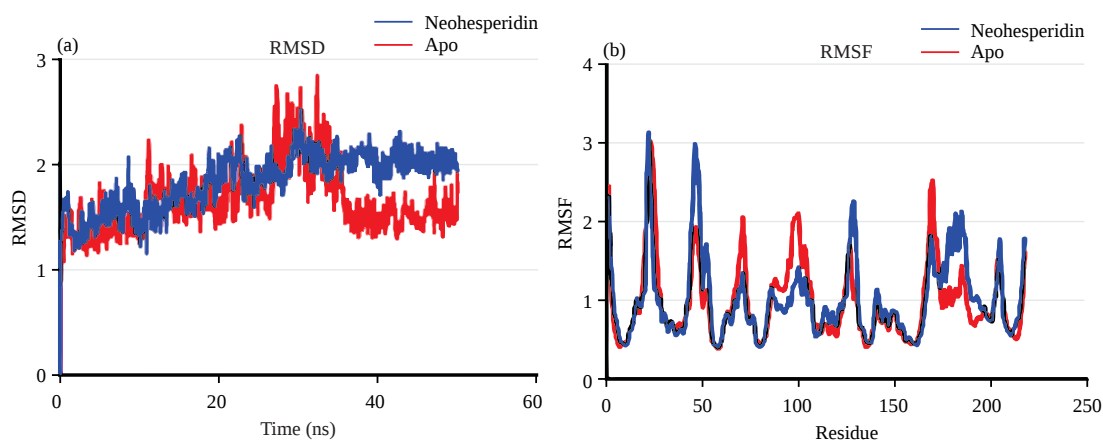


Fig. 3(a-b): Molecular dynamics simulation of Apo TbDHFR or bound with neohesperidin dihydrochalcone, (a) RMSD of Apo TbDHFR or bound with neohesperidin dihydrochalcone for 50 ns and (b) RMSF of Apo TbDHFR or bound with neohesperidin dihydrochalcone for 50 ns

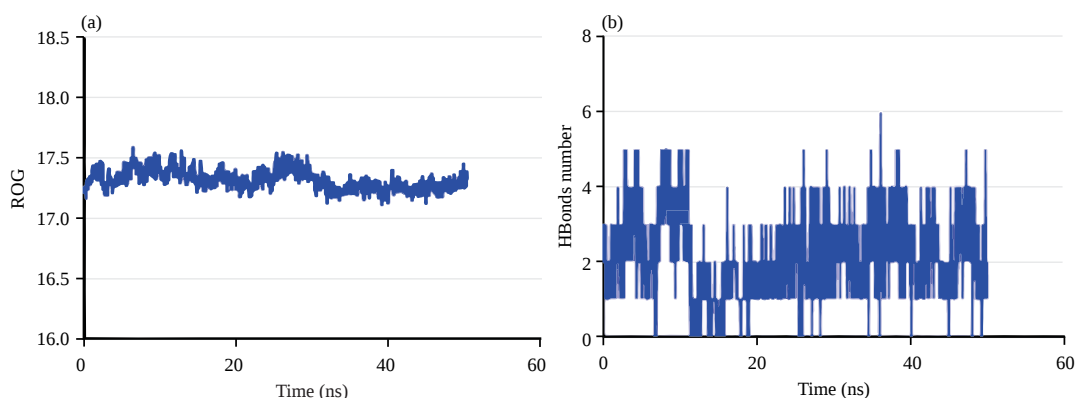


Fig. 4(a-b): Radius of gyration and number of hydrogen atoms during the interaction of neohesperidin dihydrochalcone with TbDHFR, (a) ROG of neohesperidin dihydrochalcone during simulation and (b) Number of HBonds formed between neohesperidin dihydrochalcone and TbDHFR during 50 ns simulation

dihydrochalcone-TbDHFR complex arrived at stability in a short amount of time (Fig. 3a). In comparison to the Apo structure, the RMSD analysis showed that the stability of TbDHFR was significantly improved when it was complexed with neohesperidin dihydrochalcone. The ApoDHFR went through a range of deviations that lasted between 25 and 30 ns before returning to its stable baseline.

The neohesperidin-TbDHFR complex residues showed low RMSF, which did not exceed 3 Å (Fig. 3b). In comparison with ApoTbDHFR, the neohesperidin dihydrochalcone complex lowered the amino acid fluctuations, especially at the active site within the range of residues 60-90.

The fact that there was just a marginal shift in ROG values demonstrates that the neohesperidin dihydrochalcone-

TbDHFR complex is compact (Fig. 4a). During a simulation lasting 50 ns, the total number of hydrogenbonds that were formed between neohesperidin dihydrochalcone and TbDHFR was traced (Fig. 4b). According to the statistics on hydrogen bonding, the number of hydrogen bonds can range anywhere from 0 to 6, with a mean value of 2.1 and a standard deviation of 1.1. This suggests that neohesperidin dihydrochalcone has established a stable binding relationship with TbDHFR.

The discovery of new anti-trypanosomal agents is a long and tedious process. However, it is highly recommended for the relief of the suffering of the affected population. New imidazopyridines class was discovered with potent activity²⁴. In the field of natural products, several natural derivatives were investigated²⁵. These compounds include phenolics,

quinones, alkaloids, terpenes, saponins and terpenoids²⁶⁻²⁹. These substances exhibit significant efficacy against *T. brucei*. In particular, phenolics, quinones and terpenoids have been found to hinder the growth of *Trypanosoma* by affecting various parasite drug targets, including trypanothione reductase, rhodesain, farnesyl diphosphate synthase and triosephosphate isomerase.

The research on *Artemisia elegantissima* revealed that crude fractions demonstrated effectiveness at a 20 µg/mL concentration. Within these effective fractions, 3 substances were discovered to exhibit significant antitrypanosomal activity: Scopoletin, 3',4'-dihydroxy bonanzin and bonanzin with MIC levels of 0.19, 6.25 and 20 µg/mL, respectively²⁶. The estimated inhibitory level of neohesperidin dihydrochalcone is positioned in the same range of these natural compounds by exhibiting antitrypanosomal activity in the low micromolar range. *Scrophularia lepidota* root compounds also showed moderate inhibition against *T. brucei* with IC₅₀ range 29.3-73.0 µg/mL³⁰. Low micromolar range of inhibition against *Trypanosoma* species was recorded from compounds from natural source comprising several quinoline alkaloids³¹, quinones³² and terpenoids³³. The presence of natural products like neohesperidin dihydrochalcone at low micromolar levels underscores the importance of ongoing research into chemical modifications. These modifications aim to enhance their potency to the nanomolar level, making them robust enough for use in *in vivo* applications.

The use of computational methods uncovered the possibility of neohesperidin dihydrochalcone forming stable complexes with TbDHFR that have a strong binding affinity. In addition, the effectiveness of neohesperidin dihydrochalcone against six different species of *Trypanosoma* was demonstrated by trypanocidal assays.

CONCLUSION

During our *in silico* studies on TbDHFR, neohesperidin dihydrochalcone has been raised as a potential strong binding agent. As part of this research project, we investigate its potential binding stability and characterize its effectiveness as an antitrypanosomal drug against six distinct species of *Trypanosoma*. The MD simulations and *in silico* studies were used to monitor the binding's stability and strength. The trypanocidal effects of neohesperidin dihydrochalcone were reported to be effective against a wide variety of parasites, such as TbbGUTat3.1, TbrIL1501, TbgIL1922, Tev Tansui and Teq IVM-t1. Studies done in computer simulations provided support for the conclusion

that neohesperidin dihydrochalcone has a strong affinity for TbDHFR. Future studies can now explore the production of neohesperidin derivatives and the drug's repurposing in the battle against other forms of protozoa because of neohesperidin dihydrochalcone's ability to expand its range of activity against numerous species of *Trypanosoma*.

SIGNIFICANCE STATEMENT

This study represents a significant advancement in the field of natural product-based chemotherapeutics, highlighting the potent anti-trypanosomal properties of neohesperidin dihydrochalcone, a citrus flavanone glycoside. Notably, neohesperidin dihydrochalcone demonstrated broad-spectrum trypanocidal activity against six *Trypanosoma* species, with low micromolar inhibition, distinguishing it from hesperidin, which showed no antitrypanosomal activity. These findings not only reinforce the potential of neohesperidin dihydrochalcone as a safe, natural antitrypanosomal agent but also pave the way for further research into its analogues and application against other protozoan diseases.

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