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Research Paper

Anti-tyrosinase Activity of 3',4',5'-Trimethoxychalcones: Experimental and Computational Studies

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Abstract
Tyrosinase inhibitors are utilized as preservatives in the food industry and skin-lightening agents in the medical and cosmetic sectors. However, there has been little progress in clinical trials owing to challenges such as low bioavailability, significant skin irritation, and instability. Hence, the objective of this study was to evaluate the inhibitory activity of 3',4',5'-trimethoxychalcones through *in vitro*, molecular docking and molecular dynamics studies targeting tyrosinase. Five 3',4',5'-trimethoxychalcones (1-5) were evaluated their biological activity against tyrosinase for the first time. Compounds 4 and 5 were excellent inhibitory activity against tyrosinase with IC_{50} values of 1.9 and 1.7 μM compared with kojic acid and ascorbic acid. Isovanillin and catechol moieties are vital in this present study. This result was supported with molecular docking by shaping interaction in the catalytic site with histidine residues and the stability evaluation of the inhibitor-protein complexes using molecular dynamics simulation. The lipinski's rules showed a fit with two potential inhibitors (4, 5). Therefore, 3',4',5'-trimethoxychalcones possessing isovanillin and catechol parts in the B ring are promising candidate for further study as tyrosinase inhibitors by evaluating their efficacy *in vitro* and *in vivo*.

Keywords
Anti-tyronase, Trimethoxychalcones, Molecular Docking, Molecular Dinamics

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1. INTRODUCTION

Tyrosinase (EC 1.14.18.1, TYR) is a type 3 copper-incorporati ng oxidoreductase enzyme widely disseminated among microorganisms, plants, and animals (Parvez et al., 2007; Sanchez-Ferrer et al., 1995; Zolghadri et al., 2019). This enzyme performs two catalytic functions on phenolic compounds: monoph enolase and diphenolase activities and plays a pivotal role in melanogenesis. In insects, melanin is used for exoskeleton pigmentation, cuticle hardening, and wound healing (Barrett, 1984; Kramer and Hopkins, 1987; Sugumaran, 1991; Sugumaran and Barek, 2016). In contrast, in mammals, melanin affects skin pigmentation and protects against the detrimental effects of ultraviolet (UV) radiation, oxidative stress, and harmful drugs (Kumari et al., 2018; Lin and Fisher, 2007; Widyastuti et al., 2024). Furthermore, research has shown that melanin enhances fitness and cell survival in fungi, offers protection against UV radiation, and facilitates metal chelation in bacteria (Casadevall et al., 2017; Yuan et al., 2020). Recently, tyrosinase has attracted considerable interest and has sparked active discussion across various disciplines, including biology, agronomy, chemistry, and medicine.

Tyrosinase is a pivotal rate-limiting enzyme that catalyzes melanin production (Chen et al., 2015; Poungcho et al., 2025; Widyastuti et al., 2025). Abnormal expressions of tyrosinase can lead to excessive melanin accumulation, which is closely associated with various skin pigmentation disorders including freckles, age spots, melasma, and malignant melanoma tumors (Oetting, 2000). Furthermore, tyrosinase significantly influences the enzymatic browning of fruits and vegetables (Friedman, 1996). Tyrosinase inhibitors are utilized as preservatives in the food industry and skin-lightening agents in the medical and cosmetic sectors (McEvily, 1991; Wu et al., 2016). A recent report by Grand View Research, Inc., projects that the global market for skin-whitening products will reach 16.14 billion dollars by 2030 (Grand View Research, 2022).

Although numerous tyrosinase inhibitors have shown efficacy *in vitro*, there has been little progress in clinical trials owing to challenges such as low bioavailability, significant skin irritation, and instability. Clinically utilized tyrosinase inhibitors,

including hydroquinone (Arndt and Fitzpatrick, 1965), arbutin (Boo, 2021), azelaic acid (Breathnach et al., 1989), l-ascorbic acid (Li et al., 2013), ellagic acid (Son and Heo, 2013), and kojic acid (Gonçalez et al., 2013), have been proven effective in reducing skin pigmentation and treating skin disorders (Figure 1). These inhibitors are predominantly used in cosmetics but are associated with limitations such as inadequate skin penetration, instability, high cytotoxicity, and potential carcinogenic effects (Feng et al., 2021; Huang et al., 2013; Xue et al., 2022; Yu et al., 2019). Consequently, their application is limited. Therefore, there is an urgent need to develop new inhibitors that are more effective, with reduced toxicity and enhanced skin permeability, for the treatment of skin hyperpigmentation.

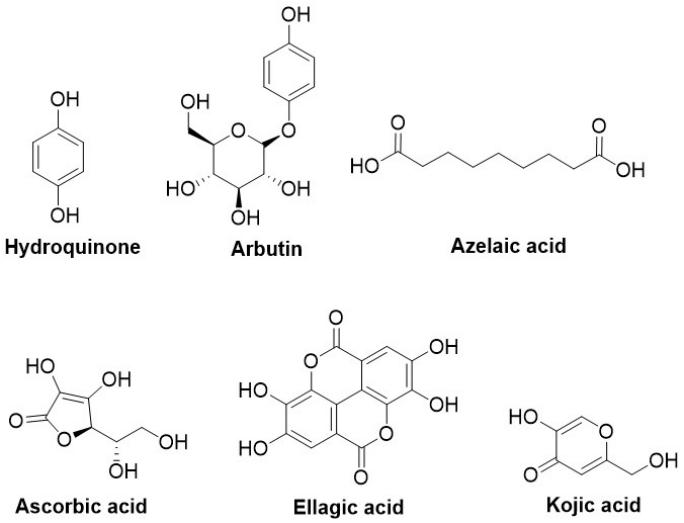

Figure 1. Structures of Commercially Available Tyrosinase Inhibitors

Chalcones represent a fundamental structural motif among privileged frameworks, characterized by two aromatic rings connected via α- and β-unsaturated ketones (Zhuang et al., 2017). This class of natural compounds exhibits anti-tyrosinase activity *in vitro*, which is attributed to a competitive mechanism owing to its structural resemblance to the substrate (Radhakrishnan et al., 2015b; Seo et al., 2010). Furthermore, chalcone interacts favorably with the copper atom or the hydrophobic protein pocket surrounding the binuclear copper active site of tyrosinase (Akhtar et al., 2015; Cai et al., 2019). Chalcone derivatives have been identified as potent tyrosinase inhibitors (G. et al., 2023; Liu et al., 2013; Najafi et al., 2023; Niu et al., 2016; Ranjbar et al., 2018). Moreover, studies have suggested that hydroxyl and methoxy substitutions positively influence tyrosinase inhibition (Choi et al., 2022; Chumkaew et al., 2024; Gunia-Krzyżak et al., 2023; Jun et al., 2007; Khatib et al., 2005; Klinngam et al., 2022; Kobkeatthawin et al., 2021; Li et al., 2021; Loizzo et al., 2012; Nerya et al., 2004; Obaid et al., 2021; Radhakrishnan et al., 2015a; Tuerxuntayi et al., 2022; Xue et al., 2023). The presence of an electron-donating group on atoms adjacent to the π system is crucial, as it enhances the electron density on the aromatic ring through a resonance-donating effect, facilitating binding to the Cu atoms at the tyrosinase active site (Radhakrishnan et al., 2015c). Consequently, hydroxy and methoxy substitutions were promising candidates for this study. Based on this literature review, our objective was to investigate the impact of chalcones with 3',4',5'-trimethoxyphenyl groups on the A ring and hydroxy/methoxyphenyl groups on the B ring on tyrosinase inhibition through both *in vitro* and *in silico* studies. This research will deepen our understanding of the role of hydroxy and methoxy groups in tyrosinase inhibition. These findings could lay the groundwork for the development of anti-tyrosinase agents that target mushroom tyrosinase.

2. EXPERIMENTAL SECTION

2.1 Materials

Tyrosinase from mushrooms (EC 1.14.18.1), L-tyrosine, and kojic acid (positive control) were purchased from Sigma-Aldrich. The chalcone derivatives were obtained from previous work (Danova et al., 2023b).

Instrumental: Tyrosinase inhibition was measured using an ALLSHENG AMR-100 microplate reader. Molecular docking study was operated using a Lenovo Yoga 7 computer with AMD Ryzen AI 7-8840HS w/ Radeon 780 M graphics, 3301 Mhz, 8 Core(s), and 16.0 GB RAM. Molecular dynamics simulation was performed using AMD Ryzen 9 5950× 16-core processor 32 Threads @3.4 GHz, 128 GB RAM, Ubuntu 20.04.5 LTS 64-bit, and graphics processing unit (GPU) NVidia GeForce RTX 4070.

2.2 Methods

2.2.1 Mushroom Tyrosinase Inhibition Assay

In vitro test for tyrosinase inhibition had been described in our previously report (Danova et al., 2023a). In summary, samples were dissolved in 10% DMSO in buffer. Furthermore, a sample solution in buffer was placed in 96 well plate, mushroom tyrosinase (250 u/mL) was added, and incubated for 5 min at 25°C. 5 mM l-tyrosine as a substrate was added and was incubated for 30 min at 25°C. The absorbance was measured at 492 nm. Kojic acid was used as positive control.

2.2.2 Molecular Docking and Dynamics Simulation

Molecular docking was conducted based on our previous method (Danova et al., 2023a). Protein structure of the mushroom tyrosinase (PDB ID: 2y9x) was dowloaded in the Protein Data Bank (`https://www.rcsb.org/`) (Ismaya et al., 2011). The AutoDock Vina tool in PyRx V.1.1 software was implemented for molecular docking with an exhaustiveness of 32 and 9 poses for each docked ligand (Dallakyan and Olson, 2015; Trott and Olson, 2010). DockThor is a free molecular docking web tool (`https://dockthor.lncc.br/v2/`) (Guedes et al., 2024). The 2D shape was imagined using BIOVIA Discovery Studio. Molecular dynamics (MD) simulation was operated using YASARA software version 21.16.17, and the AMBER14 forcefield (Duan et al., 2003; Krieger et al., 2004; Krieger and

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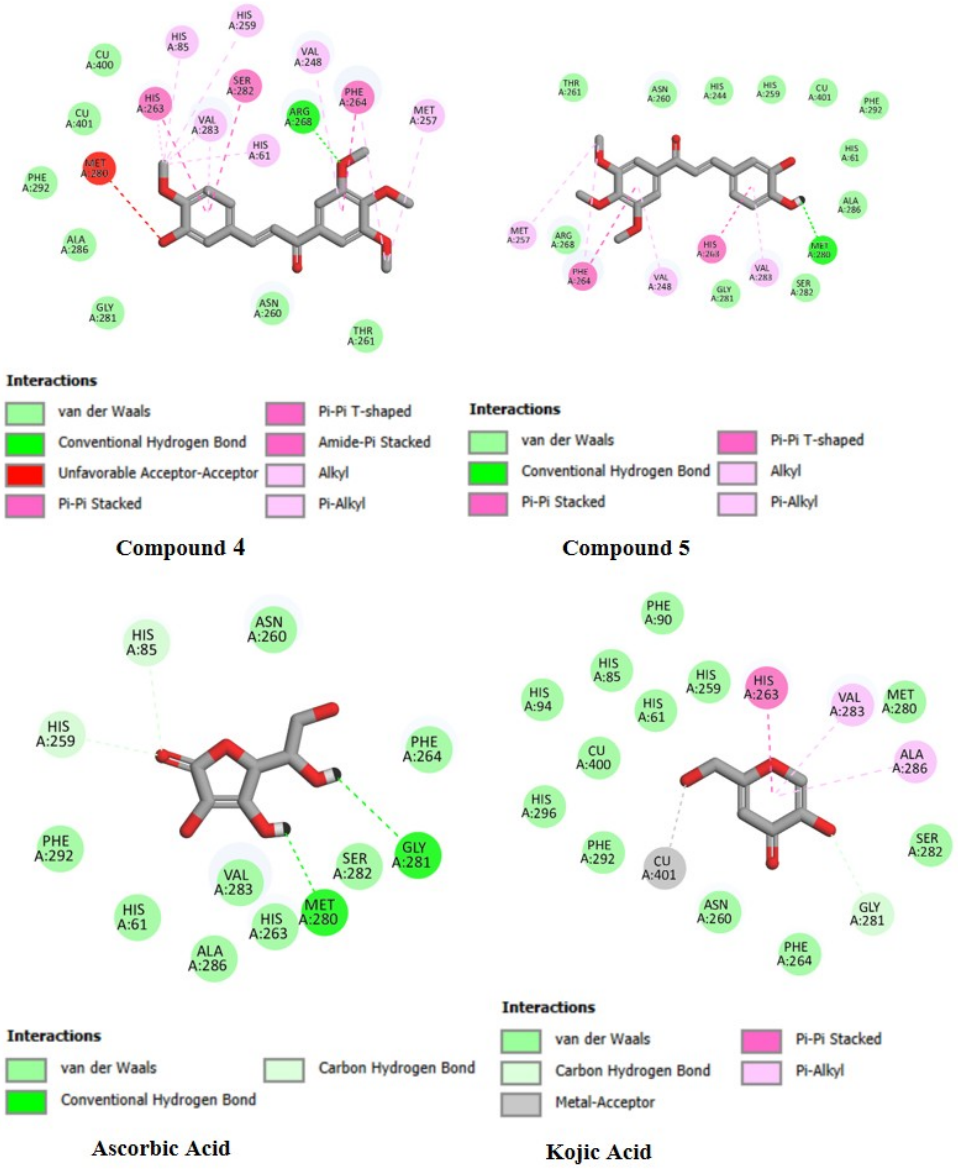

Figure 2. 2D Interaction Poses of 4, 5, Ascorbic Acid, and Kojic Acid with Mushroom Tyrosinase

Vriend, 2015). These simulations used md_run.mcr macros along with 2 × 1.25 fs timestep for 25 ns. The parameters of MD simulations included temperature at 298 K, pressure of 1 bar, Coulomb electrostatics with a 7.86 cut off, solvent density of 0.997, pH of 7.0, and periodic boundaries within a single simulation box containing 0.9% NaCl.

2.2.3 Drug-Likeness Properties

The assessment of drug-likeness profile was performed using SwissADME web tool (`http://www.swissadme.ch/`) (Daina et al., 2017).

3. RESULTS AND DISCUSSION

Five 3',4',5'-trimethoxychalcones (1-5) was synthesized in the previous study (Danova et al., 2023b). These compounds were tested in their inhibition against tyrosinase. As presented in Table 1, two compounds (4, 5) showed better inhibition than kojic acid and ascorbic acid as positive control. Compound 1 possessing hydroxy group at position 3 on the B ring inhibited tyrosinase with IC_{50} value of 66.5 μM, but compound 2 with hydroxy group at position 4 was more than 200 μM. Furthermore, compound 3 with vanillin moiety showed IC_{50} value more than 200 μM, but compound 4 with isovanillin moiety exhibited strong inhibition against tyrosinase with IC_{50} value of 1.9 μM compared with positive controls. Moreover,

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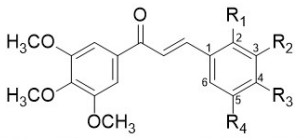
Table 1. IC_{50} Values of 3′,4′,5′-Trimethoxychalcones (1–5), Ascorbic Acid, and Kojic Acid Against Mushroom Tyrosinase



1: $R_{1,3,4}$ = H, R_2= OH
2: $R_{1,2,4}$ = H, R_3= OH
3: $R_{1,4}$ = H, R_2= OCH_3, R_3= OH
4: $R_{1,4}$ = H, R_2= OCH_3, R_3= OH
5: $R_{1,4}$ = H, R_2= OH, R_3= OH

3',4',5' -Trimethoxychalcones	Binding Energy (kcal/mol) Autodock Vina	DockThor	Tyrosinase Inhibition IC_{50} (μM)[a]
1	-7.0	-8.372	66.5 ± 0.37
2	-7.0	-7.729	>200
3	-7.5	-9.052	>200
4	-7.0	-9.092	1.9 ± 0.17
5	-7.3	-7.608	1.7 ± 0.5
Ascorbic Acid	-5.5	-6.18	17.6 ± 1.12
Kojic Acid	-5.6	-6.606	36.1 ± 1.07

[a] Data were obtained from triplicate experiments.

compound 5 with catechol moiety displayed strong inhibitory activity the same as compound 4 with IC_{50} value of 1.7 μM. This finding shows that isovanillin and catechol moieties on the B ring are crucial to maintain tyrosinase inhibitory activity (Choi et al., 2022; Zolghadri et al., 2019).

To further our investigation into molecular interaction, molecular docking was carried out. This study utilized two molecular docking programs (Autodock Vina and DockThor) to calculate the binding energy between inhibitor and protein. Binding energies of five compounds (1-5) were -7.0 to -7.5 kcal/mol using Autodock Vina and -7.608 to -9.092 kcal/mol using DockThor. However, the binding energies of two positive controls (ascorbic acid and kojic acid) were greater than chalcones, as shown in Table 1. This result was in line with the *in vitro* test, where the IC_{50} values of compounds 4 and 5 were lower than positive controls. Therefore, compounds 4, 5 and positive controls were then visualized in two dimensions to highlight the intermolecular interactions within the inhibitor-protein complex. As shown in Figure 2, kojic acid presented hydrophobic and metal interactions with His263, Val283, Ala286, and Cu401, whereas ascorbic acid formed two H-bond interactions with Met280 and Gly281 in the active site of tyrosinase. Furthermore, compound 4 exhibited one H-bond interaction with Arg268 and several hydrophobic interactions with nine amino acid residues, such as His61, His85, Val248, Met257, His259, His263, Phe264, Ser282, and Val283. Compound 5 displayed one H-bond interaction with Met280 and hydrophobic interactions with five amino acid residues, such as His263, Phe264, Val248, Met257, and Val283. Hence, compounds 4 and 5 present interactions with His263 that are similar like kojic acid, but those compounds could not interact with Cu ions in the active site. This finding reveals that interaction with histidine residues (His61, His85, His94, His259, His263, and His296) in the catalytic site are vital to upholding the inhibitory activity of 3',4',5'-trimethoxychalcones against tyrosinase (Sabuakham et al., 2024; Xu et al., 2025). Moreover, both compounds (4 and 5) might behave like competitive inhibitors (Choi et al., 2022; Sabuakham et al., 2024).

Furthermore, molecular dynamics simulation was perform ed to evaluate the stability of the complexes of ligand and protein using root-mean-square displacement (RMSD), radius of gyration (Rg), number of atomic contacts (#contacts), and solvent accessible surface (SASA) (Afzal et al., 2024; Sabuakham et al., 2024), as presented in Figure 3. Compounds 4, 5, ascorbic acid, and kojic acid were selected to be investigated. The RMSD averages of compounds 4, 5, and kojic acid were stable with less than 2 Å from 0 to 25 ns. However, ascorbic acid showed a high fluctuation of more than 2 Å from 5 to 25 ns (Figure 3a) because there was no interaction with the catalytic sites of histidine residues, as shown in Figure 2. This result was associated with a radius of gyration (Rg), as presented in Figure 3b. Yet, atomic contacts and SASA were quite similar for all compounds during simulation (Figures 3c and 3d). Furthermore, the binding free energy averages of compounds 4, 5, ascorbic acid, and kojic acid that were calculated using MM/PBSA method during simulation from 0 to 25 ns were -11.43, -106.751, -17.352, and -232.625 kcal/mol, respectively. Hence, compounds 4 and 5 could be stable in aqueous condition and potent as tyrosinase inhibitors.

The drug-likeness properties of compounds 4, 5, including

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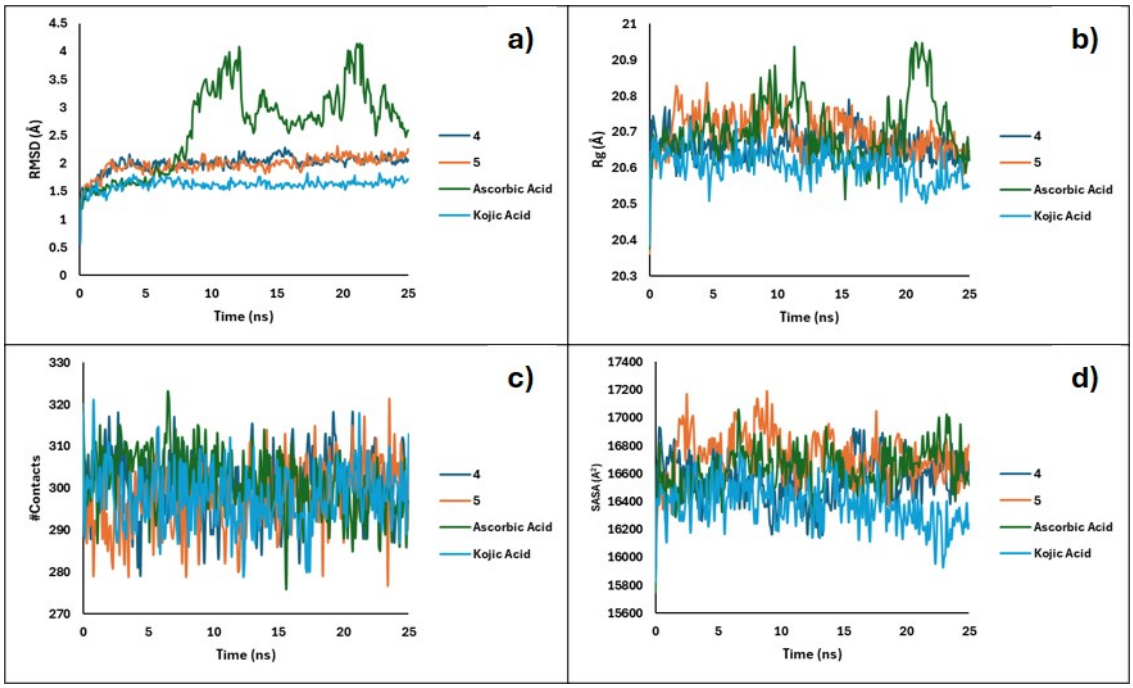

Figure 3. Analysis of a) Root-Mean-Square Displacement (RMSD), b) Radius of Gyration (Rg), c) Number of Contacts (#Contacts), and d) Solvent Accessible Surface (SASA) of Compounds 4, 5, Ascorbic Acid, and Kojic Acid in Complexes with Tyrosinase

Table 2. Drug-Likeness Prediction Values of Compounds 4, 5, Ascorbic Acid, and Kojic Acid Based on Lipinski's Rule

Compound	Rule of Five (Lipinski's)						Drug-likeness
	MW (≤500)	HBD (≤5)	HBA (≤10)	RB (≤10)	TPSA (≤140Å^2)	*Log P* (≤5)	
4	344.36	1	6	7	74.22	1.44	Yes
5	330.33	2	6	6	85.22	1.21	Yes
Ascorbic Acid	176.12	4	6	2	107.22	-2.60	Yes
Kojic Acid	142.11	2	4	1	70.67	-1.69	Yes

ascorbic acid and kojic acid for comparison, were predicted using SwissADME web tool (Daina et al., 2017) because this properties is very important in developing drugs (Hu et al., 2018). This study used several criteria, such as molecular weight (MW), H-bond donors and acceptors (HBD and HBA), rotatable bonds (RB), topological polar surface area (TPSA), and lipophilicity (log *P*) (Benet et al., 2016; Lipinski et al., 2012). The result suggested that two compounds (4 and 5) obeyed Lipinski's rules, as presented in Table 2. Therefore, these discoveries revealed that these compounds (4, 5) fit with drug-likeness profiles and further studies were required.

4. CONCLUSIONS

In summary, five 3',4',5'-trimethoxychalcones (1-5) were evaluated their biological activity against tyrosinase. Compounds 4 and 5 were excellent inhibitory activity against tyrosinase with IC_{50} values of 1.9 and 1.7 μM compared with kojic acid and ascorbic acid. Isovanillin and catechol moieties are vital in this present study. This result was supported with molecular docking by forming intermolecular interaction with histidine residues as an important amino acid residue as well as molecular dynamics simulation to calculate the stability of the inhibitor-protein complexes in aqueous condition. Two potent compounds were suitable with Lipinski's rules in developing drug targets. Therefore, 3',4',5'-trimethoxychalcones possessing isovanillin and catechol parts in the B ring are promising candidate for further study as tyrosinase inhibitors by evaluating their efficacy *in vitro* and *in vivo*.

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