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Anti-cancer potential of Cyperus tegetum: GC-MS & LC-MS Profiling, in-vitro and in silico studies targeting DHODH and DDX3X

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Abstract

Cancer is still a major global health challenge, underlining the urgent requirement for safer and more effective treatments. In this present work, the methanolic extract of *Cyperus tegetum* was extensively analysed using both biological and computational methods. Many bioactive compounds were found in phytochemical profiling of the extract using GC-MS and LC-MS. The anti-cancer potential of the plant extract was studied over B16F10 melanoma cell line, which displayed a moderate cytotoxic potential with an IC_{50} value of 267.4 µg/ml. To elucidate the molecular mechanisms underlying the observed potency, molecular docking was performed over two anti-cancer targets, dihydroorotate dehydrogenase (DHODH) and DEAD-box RNA helicase (DDX3X). Luteolin, kaempferol-7-neohesperidoside, and quercetin showed a superior binding profile compared to BG45. For luteolin and quercetin, ADME and toxicity predictions revealed good pharmacokinetic properties and safety profiles. Molecular dynamics simulations confirmed the structural stability and strong interaction patterns of luteolin with both proteins. Overall, this study highlights luteolin and quercetin as potential lead candidates for further anti-cancer drug development.

1. Introduction

Cancer is still one of the life threatening disease worldwide, posing a tremendous global health burden, responsible for millions of new cases and deaths every year (Ghufran et al. 2023). Researchers are focusing on a new and safer drug for treatment of cancer. Cancer is a complex disorder and it is coupled with drug resistance, systemic toxicity, and inadequate selectivity of many standard chemotherapeutic drugs, making it more dangerous (Eslami et al. 2024; Mengistu et al. 2024). Despite great advancements in cancer detection and therapy, the occurrence and cure rate are increasing, and the efficiency of conventional chemotherapeutic drugs is generally restricted by drug resistance, systemic toxicity, and poor selectivity for cancer cells (Gu et al. 2025; Song et al. 2025). In recent years, increased focus has been given toward phytochemicals as alternative and complementary sources of anticancer drugs, due to their structural diversity, biological compatibility, and potential to affect many cellular pathways simultaneously (Aljabali et al. 2025; Asma et al. 2022; Naeem et al. 2022). Numerous molecules isolated from plants have been demonstrated to inhibit cancer cells and are being investigated as potential anti-cancer molecules (Chatterjee et al. 2022).

Since immemorial times, plants have been employed for medicinal purposes to cure different diseases, including cancer (Gaobotse et al. 2023; Regassa et al. 2022). Phytochemicals such as in plant part demonstrate a wide variety of pharmacological characteristics, including anti-inflammatory, anticancer, antidiabetic, antioxidant, and antimicrobial properties (Mohammadi-Cheraghabadi and Hazrati 2023; Nistor et al. 2023; Ogbuagu et al. 2022; Wani et al. 2023). These chemicals show effectiveness over several cancer cell lines through various methods, such as regulation of oxidative stress, interact with several cancer cell signalling methods, and induce cell apoptosis, which are responsible for cancer development and metastasis (Almatroodi et al. 2022; Bou Antoun and Chioni 2023; Zhao et al. 2023). There are several anti-cancer drugs (paclitaxel, vincristine, and camptothecin derivatives) obtained from

natural sources, underscoring the relevance of phytochemicals in oncology (Dogra and Kumar 2023; Sharma et al. 2025).

Cyperus tegetum Roxb., a perennial plant belonging to the family *Cyperaceae*, is commonly used in herbal medicines for the treatment of several health issues (Chatterjee et al. 2022; Zhang et al. 2022). This plaint contains several phytochemicals like flavonoids, triterpenoids, saponins, phenolic compounds, and essential oils (Chatterjee et al. 2022). However, comprehensive studies that along with integrated advanced phytochemical characterization *in-vitro* anticancer study, and molecular level mechanistic insights remain limited, especially in the context of melanoma.

Recent advances in molecular oncology have identified various key metabolic and regulatory proteins that serve vital roles for drug development. (Pei et al. 2022; Xiao et al. 2023). DHODH, a mitochondrial enzyme important for de novo pyrimidine biosynthesis, is commonly increased in rapidly growing cancer cells and plays a critical role in supporting tumor development, mitochondrial respiration, and redox equilibrium (Curtabbi et al. 2024; Lee and Roh 2025; Peeters et al. 2023). Inhibition of DHODH impairs de novo pyrimidine biosynthesis, resulting in metabolic stress and supporting its potential as a promising therapeutic target (Mullen and Singh 2023). Similarly, the DDX3X has emerged as a crucial regulator of RNA metabolism and carcinogenic translation (Park and Oh 2022; Sharma et al. 2026). In melanoma, DDX3X enhances the translation of microphthalmia-associated transcription factor (MITF), a critical driver of tumor proliferation, metastasis, and therapeutic resistance (Gelmi et al. 2023; Shen et al. 2024). Dysregulation of DDX3X has been related to aggressive tumor characteristics and poor clinical outcomes (Secchi et al. 2022).

The present study intended to evaluate the anticancer property of C. tegetum using an integrated experimental and computational methodology. Comprehensive phytochemical assay of the methanolic extract was done utilizing GC-MS and LC-MS methods. The cytotoxic activity was investigated against B16F10 melanoma cells using the MTT assay. To validate the potential molecular docking, ADMET prediction, toxicity assessment, and molecular dynamics simulations were applied to investigate the binding behavior, stability, and drug-like features of important phytoconstituents against DHODH and DDX3X targets. By combining experimental validation with molecular insights, this work provides a rigorous scientific basis for the anticancer potential of *C. tegetum* and suggests prospective flavonoid-based lead molecules for future therapeutic development.

2. Results and Discussion

2.1 Phytochemical screening

The identification and isolation of phytoconstituents from *C. tegetum* were detailed in studies by Chatterjee et al. (2019) and Chatterjee et al. (2022) published in separate journals. High-performance liquid chromatography (HPLC) analysis of the methanol extract revealed several flavonoids and phenolic acids. The compounds identified were rutin (Rt: 3.00), myricetin (Rt: 3.9), and quercetin (Rt: 5.6), along

with gallic acid (Rt: 4.0), caffeic acid (Rt: 5.4), chlorogenic acid (Rt: 7.3), and ferulic acid (Rt: 8.8), based on retention times and UV spectra compared to standards. In addition, high-performance thin-layer chromatography (HPTLC) analysis of extract is also employed to quantify various compounds. In the extract, the amounts were as follows: chlorogenic acid (19.80 ± 0.94 mg/g), t-cinnamic acid (9.3 ± 0.31 mg/g), apigenin (8.95 ± 0.16 mg/g), quercetin (29.59 ± 1.19 mg/g), rutin (7.68 ± 0.36 mg/g), naringin (17.36 ± 0.72 mg/g) and stigmasterol (20.54 ± 1.34 mg/g).

2.1.1 Phytochemical screening: GC-MS

GCMS Different substances, primarily sesquiterpenic hydrocarbon, essential oil, sesquiterpenoids, and fatty acids, were detected by GC-MS analysis (Table S1). Fig. S1 shows the chromatogram and a list of the detected chemicals. Cyperene (a sesquiterpenic hydrocarbon), -bulnesene (an essential oil), cyperenone (a sesquiterpenoid), hexadecanoic acid methyl ester (a fatty acid), and 9-octadecanoic acid methyl ester (a fatty acid) are significant among the found chemicals. GC-MS analysis identified various compounds in the *C. tegetum* methanol extract, including sesquiterpenic hydrocarbons, essential oils, sesquiterpenoids, and fatty acids. Major constituents identified included cyperene, a sesquiterpenic hydrocarbon; β-bulnesene, an essential oil component; cyperenone, a sesquiterpenoid; and the fatty acid derivatives hexadecanoic acid methyl ester and 9-octadecanoic acid methyl ester. Figure S1 and Table S1 present the GC-MS chromatogram and the corresponding list of detected compounds, respectively, emphasizing their prominence among the identified constituents.

2.1.2 Phytochemical screening: LC-MS

The LC-MS analysis of the plant extract successfully identified several phytochemical constituents. The analysis was conducted in positive ion mode using a Q-TOF mass spectrometer interfaced with HPLC in Fig. S2. The results, including the name of the identified compounds, their molecular weights, m/z values, and their respective intensities, are tabulated in Table S2.

The LC-MS analysis revealed a diverse array of phytochemicals present in the extract, showcasing the plant's bioactive potential. The identified compounds included terpenoids, flavonoids, and other bioactive molecules, each associated with various pharmacological activities. Terpenoids Compounds like α-campholenal, cyperene, cyperone, and β-santal were identified. These are known for their antioxidant, anti-inflammatory, and antimicrobial properties, supporting the plant's traditional use in herbal medicine. Key flavonoids, including luteolin, catechin, naringenin and quercetin, were detected. Flavonoids are well-documented for their antioxidant and anti-cancer properties. Quercetin, with a high intensity (4.31×1044.31 \times 10^44.31×104), stands out as a potent antioxidant that may contribute significantly to the plant's therapeutic potential. Phenolic Glycosides Compounds like apigenin 8-C glycoside and kaempferol-7-neohesperidosode are glycosylated forms of flavonoids, known for their enhanced solubility and potential therapeutic effects in cancer and inflammatory diseases. The presence of these compounds underscores the plant extract's pharmacological diversity and supports its use in traditional and modern therapeutic contexts. The identification of specific bioactives such as quercetin, luteolin, and catechin highlights the potential for further investigation into their individual and synergistic

effects. This phytochemical profile provides a basis for targeted pharmacological studies to explore the therapeutic applications of the extract in antioxidant, antimicrobial, and anticancer domains.

2.2 Cytotoxic assay

Percent cell viability was determined by the MTT assay following treatment of B16F10 cells with preselected concentrations of the extract (50–1000 µg/ml) in complete culture medium, performed in duplicate for 48 h. The inset graph represents dose–response data of the positive control BG45 (0.041–42.844 µg/ml; IC_{50} = 6.947 µg/ml). Results are expressed as mean ± SD (n = 2) and normalized to vehicle-treated cells (100% viability). The *C. tegetum* extract exhibited an IC_{50} value of 267.4 µg/ml (Fig. S3).

2.3 *In-silico* analysis

2.3.1 Pocket Analysis

A distinct primary binding cavity of the targeted protein (P_0) with much better geometry and druggability properties than all secondary pockets was found by pocket analysis of both targets. P_0 has the greatest cavity volume (1597.57 Å³) and surface area (1438.87 Å²) for 6ET4 protein, along with the highest drug score of (0.81) and simple score (0.66) (Table S3, Fig. S4). These measurements validate P_0 as the most prevalent and pharmacophore-accommodating site, supporting ligands with significant electronic and steric complementarity. The remaining pockets (P_1–P_8) demonstrated a sharp decrease in drug score (0.73 − 0.15) and volume (497.66-104.83 $Å^3$), suggesting restricted ligand accommodation and somewhat decreased druggability.

A similar trend was seen in 2I4I protein, where pocket P_0 once more became the primary binding site with a high drug score of (0.80), a large cavity volume (1560.61 $Å^3$), along with a large surface area of (2071.11 $Å^2$) (Table S4, Fig. S5). Two sub-pockets, P_1 (473.10 $Å^3$) and P_2 (449.82 $Å^3$), in particular, had moderate drug scores (0.69 each), indicating the existence of auxiliary accessible areas surrounding the main cavity that might facilitate longer interactions for larger ligands. The remaining pockets (P_3-P_12) had low drug scores (≤ 0.57) and smaller diameters, which were consistent with minimal druggability.

All of these findings pocket binding analysis, the P_0 as a main and most pharmacologically significant binding sites in both 6ET4 and 2I4I protein, which provides the best steric volume, surface exposure, and druggability criteria for ligand accommodation.

2.3.2 Docking Results

In order to confirm and clarify the *in vitro* anticancer activity against melanoma (B16F10) and murine myeloma cell lines, the two proteins 2I4I (DDX3X) and 6ET4 (DHODH) were selected as a molecular target in the current study. The de novo pyrimidine synthesis requires the mitochondrial enzyme DHODH, which is frequently overexpressed in cancer. It emboldens the growth of tumors and uncontrolled nucleic acid production. In addition to its metabolic function, DHODH controls mitochondrial respiration and promotes the production of ROS, both of which have an impact on cancer cells' ability to survive. Its

function in the development of tumors is further highlighted by its context-dependent action on ROS, which can either increase or decrease oxidative stress. All things considered, these features make DHODH an attractive and mechanistically target for anticancer therapy. Melanoma and other cancers frequently have mutations in the ATP-dependent DEAD-box RNA helicase DDX3X. Specifically, it facilitates the translation of MITF, an essential oncogenic driver of melanoma cell proliferation and metastasis. Translation and splicing are also impacted. Tumor-promoting mRNAs, which help in the development of melanoma and have been connected to treatment resistance and a poor prognosis, are produced more frequently when DDX3X is present.

Docking studies with 2I4I protein revealed binding affinities from − 3.9 to -8.6 Kcal/mol, which indicates the moderate to strong interactions across the tested phytoconstituents. From the molecular docking study results, the most potent ligand was Kaempferol-7-neohesperidoside (**26**) (-8.6 Kcal/mol), followed by Quercetin (**35**) (-7.5 Kcal/mol) and Luteolin (**28**) (-7.2 Kcal/mol). Additional compounds, including Eleutherol (**25**) (-6.8 Kcal/mol), Catechin (**17**) (-6.7 Kcal/mol), and Vitexin (**40**) (-7.6 Kcal/mol), also displayed favorable interactions with targeted proteins. These findings highlight the preference of 2I4I for oxygen-rich, polyphenolic frameworks with multiple hydrogen-bonding functionalities. In contrast, long-chain aliphatic molecules such as Methionine (**29**) (-3.9 Kcal/mol) and Palmitic acid (**33**) (-5.0 Kcal/mol) exhibited weaker binding, consistent with limited polar interactions. Notably, the standard drug BG45 showed a binding score of -6.7 Kcal/mol, which is lower than the top-scoring polyphenolic phytoconstituents.

Molecular docking against 6ET4 generated comparatively stronger binding energies, between − 4.7 to -10.2 Kcal/mol. The most noticeable interaction was achieved for compound (**28**) (-10.2 Kcal/mol), closely followed by (**26**) (-10.1 Kcal/mol) and (**35**) (-9.8 Kcal/mol). Compounds such as (**25**) (-9.0 Kcal/mol), (**3**) (-9.1 Kcal/mol), and (**17**) (-7.9 Kcal/mol) also demonstrated strong affinity with targeted protein. The docking background suggested that the presence of a well-defined hydrogen-bond-rich cavity in 6ET4, capable of accommodating planar polyphenolic ligands with multiple hydroxyl groups. Meanwhile, aliphatic and fatty-acid derivatives showed markedly lower affinities (e.g., Methionine (**29**) -4.7 Kcal/mol; Pyranone (**34**) -5.2 Kcal/mol), reinforcing the binding selectivity of this target. The standard drug BG45 exhibited a binding energy of − 7.6 Kcal/mol, again lower than the leading flavonoids, indicating that these polyphenolic compounds display superior complementarity to the binding pockets of both proteins (Table S5).

When combined, the computational results clearly showed that compounds (**26)** (Kaempferol-7-neohesperidoside), (**28**) (Luteolin), and (**35)** (Quercetin) are the most effective ligands for both protein targets, exhibiting better docking scores and stronger binding complementarity than the typical reference molecule BG45. These flavonoid-based phytochemicals consistently greater affinities are compatible with the class of natural compounds proven anticancer effects in *in vitro* systems. All the 2D and 3D binding orientations of all top docked compounds and standard drugs are represented in Figs. S6 and S7.

2.3.3 ADMET & Physicochemical Properties

An integrated assessment of physicochemical parameters, ADMET predictions, and toxicity profiles further consolidates the superiority of the top-performing phytochemicals—particularly compounds (**26)**, (**28**), and (**35**) when compared against the reference molecule BG45. Compound (**26**) with a markedly higher molecular weight 594.52 g/mol, negative ClogP (-1.05) value, higher number of hydrogen bond donors and acceptors (9 and 15) and extremely large TPSA of 249.2 $Å^2$ showed limited passive permeability. Whereas compounds (**28**) and (**35**) showed acceptable physicochemical properties, such as molecular weight 286.24 g/mol and 302.24 g/mol, balanced lipophilicity (28: ClogP = 1.75 and 35: ClogP = 1.23), moderate polarity (TPSA = 111.13 $Å^2$ & 131.36 $Å^2$) and acceptable number of hydrogen-bonding capacity. These characteristics indicate that both compounds are ideal for rapid permeability and drug-like properties. Compound (**35**) is the most structurally balanced, with moderate flexibility (nRB = 1) and good solubility (LogS = -3.16) (Table S6).

ADME screening showed that low GI absorption for compound (**26**), consistent with its excessive polarity and high molecular weight. Compound (**26**) is a P-gp substrate with likely reduced bioavailability but showed no major inhibition for CYP, suggesting minimal risk of metabolic drug-drug interaction. Compounds (**28**) and (**35**) demonstrated high GI absorption and no BBB permeability, which is desirable for non-CNS anticancer agents (Table S7). Also, both the compounds were identified as P-gp substrates and predicted to inhibit CYP2D6 and CYP3A4. Additionally, according to the toxicity prediction data, all three compounds (**26**, **28** and **35**) were inactive for hepatotoxicity, carcinogenicity, immunotoxicity, mutagenicity and cytotoxicity, suggesting a favourable preliminary safety profile.

Additionally, drug-likeness property of BG45 was supported by its acceptable physicochemical characteristics, which included moderate polarity (TPSA 80.9 $Å^2$) and good Lipinski parameter compliance. The compound's observed systemic action was supported by its strong gastrointestinal absorption and lack of BBB permeability (Fig. S8). BG45 did not present major CYP inhibition liabilities, suggesting a low risk of metabolic interactions. Toxicity predictions indicated moderate safety with an LD_{50} of 850 mg/kg, aligning with its profile as a potent synthetic anticancer agent (Table S8).

Overall, the investigation shows that the safety, ADME, and physicochemical profiles of Compounds (**28**) and (**35**) are quite similar to those of the standard medication BG45. They have a great potential to be attractive lead candidates for further anticancer medication development because of their balanced characteristics and anticipated safety.

2.3.4 MD Simulation:

The MDS study was performed on both proteins (6ET4 and 2I4I), displayed that compound (**28**) (Luteolin) and BG45 maintained stable interactions over the 100-ns trajectory, although compound (**28**) consistently demonstrated a more adaptable and strongly interacting binding property. For the 6ET4 systems, the protein backbone RMSD displayed rapid attainment of equilibrium. The BG45-6ET4 complex stabilized by 10–12 ns and thereafter remained within a range of 0.22–0.30 nm. The 28-6ET4 system exhibited a similar stabilization pattern, though with slightly higher fluctuation of 0.24–0.32 nm, suggesting modest conformational flexibility without any indication of overall instability (Fig. S9A). The

2I4I system exhibited a similar stabilization trend, with compound (**28**) causing significantly smaller structural deviations (0.55–0.85 nm) compared to BG45, which showed wider fluctuations between 0.90–1.40 nm, suggesting a stronger stabilizing effect of compound (**28**) on the protein backbone (Fig. S10A).

The 2 molecules have discrete binding affinities as further evidenced by the ligand RMSD analysis. The standard molecule that is BG45 maintained a rigid and well-positioned binding positioning in the target protein that is (PDB ID: 6ET4) complex, with variations restricted to 0.10–0.15 nm. Although there were infrequent spikes (0.18–0.22 nm) around 50, 70, and 90 ns. Molecule (**28**) remained mostly constant around at (0.06–0.12 nm), which suggests a small, transient conformational variation inside the active site (Fig. S9B). The standard molecule that is BG45 showed a minute less ligand mobility (0.20–0.22 nm) in the 2I4I system than compound (**28**) (0.28–0.34 nm); nonetheless, these differences didn't result in any apparent protein backbone instability (Fig. S10B).

Radius of gyration (Rg) investigation established the permanency of 2 target proteins during the ligand binding within the binding site. The 6ET4 complexes endured stable, with the Rg values of 1.95 and 2.00 nm. In contrast, to the compound (**28**) which demonstrated a slightly more uniform mean Rg (≈ 1.98 nm) than the standard molecule BG45 (≈ 1.96 nm) (Fig. S9C). In place of the 2I4I protein, ligand (**28**) formed a complex and maintained a more compact conformation (2.45–2.65 nm; mean ≈ 2.55 nm), whereas BG45 showed higher fluctuations (2.55–2.85 nm; mean ≈ 2.70 nm), which indicates a lower structural stability in the BG45-protein complex (Fig. S10C).

Further, these results were confirmed using RMSF exploration. The common residues in the 6ET4 protein diverse between 0.06 and 0.15 nm, which represents a general backbone stiffness. Although compound (**28**) showed better flexibility around residues 180 and 230 (0.25–0.33 nm), whereas BG45 presented mobility inside the 55–85 loop (0.40–0.43 nm) (Fig. S9D). Even though BG45 caused wider residue fluctuations (0.25–0.70 nm) with fatal regions exceeding 0.80 nm, Compound (**28**) partial residue changes to 0.15–0.60 nm in the 2I4I simulations, which indicates better stability of local structural characteristics (Fig. S10D).

In complexes with 2I4I and 6ET4, the SAS profiles of BG45 and compound (**28**) closely match, with the majority of residues displaying SAS values below ~ 1.5 nm² and occasional peaks reaching ~ 2.1–2.2 nm². Localized conformational changes are indicated by slight variations in peak intensity that are distinct to individual residues. Overall, compound (**28**) retains structural stability and solvent exposure comparable with BG45 (Figs. S9E and S10E). Throughout the simulations, time-dependent SASA values stayed constant. The 28-6ET4 occupied a greater range of 160–170 nm² (≈ 165–166 nm²) for 6ET4, whereas BG45 occupied 156–162 nm² (≈ 160 nm²) (Fig. S9F). Compound (**28**), on the other hand, had lower SASA values (195–205 nm²) for 2I4I than BG45 (205–210 nm²), and displayed better binding of the 28-2I4I complex (Fig. S10F).

Compound (**28**) showed a stronger interaction profile over the 6ET4 protein, sustaining around 2–6 hydrogen bonds and 8–18 van der Waals interactions throughout the course of the 100 ns simulation.

BG45 displayed relatively lower interactions, with around 1–4 hydrogen bonds and 5–12 van der Waals bonds, suggesting weaker and less stable binding (Figs. S9G, S9H).

Similarly, throughout the simulation, BG45 sustains stable connections with around 3–6 hydrogen bonds and 4–7 van der Waals bonds over the 2I4I protein. On the other hand, compound (**28**) exhibited an improved binding profile with 3–8 hydrogen bonds and a much greater number of van der Waals bonds (~ 10–25), indicating stronger protein-ligand binding (Figs. S10G, S10H).Overall, the integrated MD descriptors RMSD, RMSF, Rg, SAS/SASA behavior, hydrogen bond, van der Waals bonding, and non-bonded energies showed that while both ligands form stable complexes, compound (**28**) induces a markedly high compact, less flexible, and more strongly stabilized binding mode in both 6ET4 and 2I4I, highlighting its superior conformational stabilization and potential as a more effective ligand.

3. Experimental

3.1 Plant material

In May–June 2019, rhizomes of *Cyperus tegetum* Roxb. were collected from West Midnapur, West Bengal, India (22.4254 °N, 87.3199 °E). The plant material was taxonomically identified and authenticated by Dr. Vijay Ranjan, Scientist 'D', Central National Herbarium, Botanical Survey of India (BSI), Howrah, West Bengal, India. The voucher specimen was deposited at the Central National Herbarium, BSI, Howrah-711103, India, under reference number CNH/Tech.II/2019/75 (Specimen No. AC-01). The authenticated specimens have been preserved in our laboratory for future reference and research purposes.

3.2 Preparation of extract and phytochemical screening

The dried rhizomes were pulverized into a coarse powder using a mechanical grinder. Approximately 1.0 kg of the powdered material was subjected to maceration for five days with methanol (MeOH) in a ratio of 1:2.5 (w/v) for an appropriate duration with occasional agitation to ensure efficient extraction of phytoconstituents. The resulting extract was filtered, and the solvent was removed under reduced pressure using a rotary vacuum evaporator operated at 100–200 rpm and maintained at 40–50°C. The concentrated methanolic extract was collected, transferred to an airtight container, and stored at 4°C following the method reported by Chatterjee *et al.* (Chatterjee et al. 2019) and further purified by silica gel (60–120 mesh) column chromatography. RP-HPLC analysis confirmed flavonoids (rutin, quercetin, myricetin) and phenolic acids (gallic, ferulic, chlorogenic, caffeic). HPTLC profiling identified chlorogenic acid, *t*-cinnamic acid, apigenin, quercetin, rutin, naringin, and stigmasterol (Chatterjee et al. 2022).

3.2.1 Phytochemical screening: GC-MS

To identify additional phytoconstituents, GC–MS analysis of the rhizome of *C. tegetum* methanolic fraction was carried out following the methods described by Bhattacharjee et al. and Saha et al (Saha et al. 2024). Analysis was performed using an Agilent GC-MS system. Separation was achieved on an HP-

5MS capillary column (30 m × 0.25 mm, 0.25 µm film thickness). The oven temperature was programmed from 60.0°C (1 min) to 240.0°C at 4.0°C/min, with a 5 min hold. Helium was used as the carrier gas at 1 ml/min. Mass spectra (50–550 m/z) were recorded in EI mode (70 eV), and compounds were identified using the library.

3.2.2 Phytochemical screening: LC-MS

LC–MS analysis was carried out to further explore the phytochemical profile of the extract. The HPLC system was coupled to a Q-TOF mass spectrometer equipped with an ESI source and operated in full-scan mode (m/z 100–1200). Separation was achieved using a Phenomenex C8 (5 µm) column with methanol containing 0.3% formic acid as the isocratic mobile phase at 0.1 ml/min. Spectra were recorded in positive ion mode with optimized source conditions. The filtered extract (5 µL) was injected, and compounds were identified using spectral libraries (Elaloui et al. 2021; Kadam et al. 2018).

3.3 *In vitro* cell viability studies against B16F10, murine myeloma cell line

The MTT assay was performed with the B16F10, murine myeloma cancer cell line to calculate the cell viability effect of the extract. In brief, $1*10^3$ cells each well were contained in 96-well plate in the DMEM (Dulbecco's modified eagle high glucose media) enclosing 10.0% fetal bovine serum and 1% antibiotic (Pen strep: A001), incubated under a 5.0% CO_2 atmosphere at 37.0°C. The adherent cells were preserved with extract ^{o}f *C. tegetum* made up in complete cell culture growth media for 48 h after discarding the old media from the wells. The cells were treated with varying preselected concentrations of *C. tegetum* extract (50–1000 µg/ml) prepared in complete culture medium and incubated in duplicate for 48 h. Following the completion of the treatment period, the media was aspirated, and 50 µl of a 5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution in Dulbecco's phosphate-buffered saline (PBS) solution was added. The mixture was then incubated for 4 h to allow the formation of formazan crystals. After discarding the MTT solution, 150 µL of DMSO was added to each well, and the wells were gently shaken to dissolve the formazan crystals that had formed. Molecular Devices' Spectra max micro plate reader was used to measure the solution's absorbance at 570 and 650 nm. BG45 (CAS 926259-99-6 and Sigma Aldrich cat# SML0867) was used as a positive control for confirming the assay. Data represents mean ± SD, n = 2 and normalized with vehicle treated cells as 100% viable. The percentage of viable cells was calculated using the equation below, and the data was analysed using graph pad Prism 9.0.2 software to estimate the half-maximal inhibitory concentration (IC_{50}) (Paul et al. 2019).

% Cell viability = Abs $_{Sample}$ / Abs $_{Control}$ * 100

3.4 *In silico* analysis

3.4.1 Binding Pocket Analysis

The main binding site of the GLUT4 protein (PDB ID: 7WSM) has been identified using the online server DoGSiteScorer (https://proteins.plus/#dogsite). The DoGSiteScorer estimated a protein's pockets and subpockets considering the coordinates of its heavy atoms. The volume or surface area of the predicted pocket was determined by multiplying the grid points that comprise the pocket by the volume or surface area of the grid box. To determine pocket depth, a breadth-first search strategy was employed, starting with the solvent-exposed parts of the pocket and working down to the most deeply buried parts. Pocket druggability was assessed using Support Vector Machines (SVMs), a supervised machine-learning technique. Greater druggability in the anticipated pocket is indicated by a higher druggability score ranging from 0 to 1 (Saha et al. 2024; Volkamer et al. 2012).

3.4.2 Molecular docking

Molecular docking was done in accordance with Saha S et al. (2024) and Gholam G et al. (2022) (Gholam et al. 2022; Paul et al. 2025; Saha et al. 2024). The method was performed using AutoDock Vina software (version 4.2); a higher negative score denotes a stronger and more beneficial interactions between protein and ligand. The Steepest Descent technique in Swiss-Pdb Viewer was used to minimize protein energy, and the grid box was specified by the location of the cocrystal ligand and its interactions with the protein. ChemDraw Professional (v16.0.1.4) was used to prepare the ligands, and Chem3D Extreme (v16.0.1.4) was used in combination with AutoDock-4 (AD4) to optimize and convert their structures to the PDBQT format. The binding site's molecular surface was analyzed and 2D and 3D docking interactions were visualized using Discovery Studio Visualizer (v21.1.0.20298).

3.4.3 Drug-Likeness Study and Molecular Properties

The SwissADME tool (http://www.swissadme.ch/) was utilized to study the isolated compounds' *in silico* physicochemical characteristics and drug-likeness. Molecular formula, molecular weight (MW), number of rotatable bonds, hydrogen bond donors (HBD) and acceptors (HBA), molar refractivity (MR), and topological polar surface area (TPSA) are some of the important physical characteristics that were found for the compounds in study. Drug-likeness was evaluated in accordance with accepted empirical standards. Lipinski's rule of five was specifically implemented, taking into account limitations of MW < 500 Da, MlogP ≤ 4.15, HBD ≤ 5, and HBA ≤ 10. Additionally, Veber's criteria were used, which highlight a TPSA value of no more than 140 Å², less than 10 rotatable bonds, and a total hydrogen bonding capacity (HBD + HBA) of less than 12 (Truong et al. 2021; Zekri et al. 2023).

The integrated ChemAxon Marvin JS molecular editor (http://www.chemaxon.com) was employed to construct molecular structures on the SwissADME platform, which was obtained using a web browser. After being automatically translated into SMILES (Simplified Molecular Input Line Entry System) notation, the generated structures were forwarded for analysis. As an alternative, the compounds' SMILES strings can be submitted straight to the server. Tables, graphical representations, and Excel files that could be downloaded were utilized to show the output data. SwissADME provides a particular output panel that summarizes all computed parameters for each compound, making data export and interpretation easier (Saha et al. 2024).

3.4.4 Investigation of *in silico* pharmacokinetic properties (ADMET)

The SwissADME web server was used to evaluate the ADME characteristics of the separated substances. Key pharmacokinetic characteristics, including gastrointestinal absorption (GI), blood–brain barrier (BBB) permeability, P-glycoprotein (P-gp) substrate status, cytochrome P450 (CYP450) isoenzyme interactions, and skin permeation coefficient (log Kp), were predicted using this web server. Additionally, the Brain or Intestinal Estimated Permeation (BOILED-Egg) model was used to assess the drugs' overall pharmacokinetic properties and oral bioavailability (Saha et al. 2024).

ProTox-II (https://tox-new.charite.de/protox_II/), a virtual laboratory for *in silico* toxicity evaluation, was used to forecast the compounds' toxicity profiles. To predict various toxicity parameters, such as acute toxicity, hepatotoxicity, immunotoxicity, cytotoxicity, carcinogenicity, mutagenicity, and other adverse outcomes, ProTox-II combines molecular fragment analysis, frequent feature identification, structural similarity, and machine-learning techniques. The platform is built on 33 prediction models and requires the canonical SMILES of each molecule as input. The output findings, which show the chance of each drug being active or inactive for the assessed toxicity parameters, are displayed as probability scores (Banerjee and Ulker 2022).

3.4.5 MD simulation

MD simulation was performed utilizing GROMACS software. The procedure was followed as per Eastman P *et al*. (2010) and Martínez L (2015) with minor modification (Archna et al. 2023; Eastman and Pande 2010; Martínez 2015). To evaluate the conformational stability of the top-docked molecules and receptor complex, MD simulations were analyzed. To assay the simulations, it depends on 'Visual Dynamics for Macromolecular Simulations' (https://visualdynamics.fiocruz.br/login) 'Visual Dynamics for Macromolecular Simulations' (https://visualdynamics.fiocruz.br/login), which uses MD simulations, is required to examine the simulations (Vieira et al. 2023). To identify the most active molecule, the study ran a "protein with ligand simulation" against each of the selected receptors. The first step was using the PRODRG service to obtain the ligand topology files in ZIP format and the PyMOL reader to acquire the PDB format of the docked protein-ligand complex (Van Aalten et al. 1996). After choosing AMBER99 as the force field, the PDB file and ZIP folder were uploaded to the Visual Dynamics web server. The steepest descent method was used to minimize energy, force < 1000 steps. The MD simulation was run at a constant temperature of 310 K, constant volume and number of atoms (NVT), and constant pressure of 1.0 bar (NPT) for 100 ns, or 100,000 ps, using GROMACS equilibration settings. Leap-frog, an MD integrator, was used. The simulation produced about 1000 frames. MD trajectory analysis was carried out for the top compounds relative to a reference molecule in terms of RMSD protein and RMSD ligand, RMSF, Rg, and hydrogen bonding between the protein-ligand. The research revealed the length of the active site residues' contacts with the ligands, the residence time of those interactions, and the complex's overall stability throughout the 100-ns isothermal-isobaric simulation (Paul et al. 2025).

4. Conclusion

The combined *computational and experimental* studies provided a consolidated scientific perspective for the anticancer property of *C. tegetum*. Chemical profiling using GC-MS and LC-MS provided a diverse library of forty phytochemicals, several of them, particularly Kaempferol-7-neohesperidoside (**26**), Luteolin (**28**), and Quercetin (**35**) displayed strong binding affinity toward the selected targets 6ET4 and 2I4I, along with favourable ADMET properties. The isolated flavonoid derivatives showed a good docking scores as well as the pharmacokinetic predictions that are consistent with established anticancer scaffolds, according to comparative research against the reference compound BG45.

The crude C. tegetum extract demonstrated a significant anti-proliferative efficacy over the B16F10 melanoma cell line, with an IC_{50} of 267.4 µg/ml, according to in-vitro MTT experiments. The low concentration of active molecules in the unfractionated extract, the restricted cellular permeability of glycosylated flavonoids, and potential antagonistic effects from other non-active components are all consistent with this moderate activity. BG45, on the other hand, showed a significantly lower IC_{50} of 6.947 µg/ml, demonstrating the potency difference between a chemically complex extract and the reference molecule.

Overall, the data showed that although the extract contains truthfully active components with potential anticancer properties, the crude matrix dilutes their effects. The strong *binding property and* acceptable ADMET profiles of key flavonoids indicate that these molecules may yield substantially increased cytotoxic potency once isolated in pure form. Consequently, bioassay-guided fractionation and targeted isolation of the top-docking phytochemicals represent logical next steps to validate their mechanistic relevance and to develop potent, plant-derived melanoma inhibitors. This integrated approach provides a solid foundation for advancing *C. tegetum* as a source of promising anticancer leads.

Abbreviations

Abbreviation	Full Form
HPTLC	High-Performance thin-layer Chromatography
HPLC	High-Performance thin-layer Chromatography
DHODH	dihydroorotate dehydrogenase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
ROS	reactive oxygen species
FBS	fetal bovine serum
PDB	Protein Data Bank
DMEM	Dulbecco's modified eagle high glucose media
MITF	Microphthalmia-associated transcription factor
HP-5MS	5 percent diphenyl-95 percent dimethyl polysiloxane
DMEM	Dulbecco's modified eagle high glucose media

Declarations

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Author Contribution:

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Pronoy Kanti Das: Manuscript preparation, Docking, Figure preparation.

Ritu Khanra: Contributed to the writing of this manuscript.

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Santanu Ghosh: Primary editing, design, and supervision

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Fernando Berton Zanchi: MD simulation

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