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Steroidal glycosides from *Mallotus philippensis* induce apoptosis in MCF-7 breast cancer cells via MTT and DAPI assays

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Abstract. *Manikyam HK, Joshi SK, Patil SB, Vakadi S, Patil AR. 2025. Steroidal glycosides from* Mallotus philippensis *induce apoptosis in MCF-7 breast cancer cells via MTT and DAPI assays. Asian J Nat Prod Biochem 23: 47-51. Mallotus philippensis* (Lam.) Müll.Arg., commonly referred to as the kamala tree, is a widely known ethnomedicinal plant possessing diverse pharmacological activities. Despite its traditional applications, modern research into its anticancer properties remains underexplored. The present study investigates the in vitro anticancer activity of steroidal glycoside-enriched fractions derived from the aerial parts of *M. philippensis* against the MCF-7 breast cancer cell line. Ethanolic extraction followed by acetone precipitation was employed to obtain the glycoside-rich fraction. Phytochemical screening confirmed the presence of steroidal glycosides using Liebermann–Burchard and Salkowski tests. To evaluate cytotoxicity and apoptosis induction, MTT cell viability assay and DAPI nuclear staining assay were performed. The MTT assay results revealed a concentration-dependent inhibition of cell growth, achieving complete (100%) growth inhibition at 200 µg/mL. DAPI staining further confirmed the pro-apoptotic activity of the extract, showing prominent nuclear fragmentation and chromatin condensation, with an apoptotic index of 67.87 ± 6.13%. These effects were comparable to the standard positive control, sodium Lauryl Sulfate (SLS), suggesting a potent anticancer potential. The findings support the hypothesis that steroidal glycosides present in *M. philippensis* can significantly induce apoptosis in hormone-dependent breast cancer cells. The anticancer effect is likely mediated through mitochondrial apoptotic pathways, characteristic of steroidal compounds. This study provides a strong rationale for further investigation into the bioactive constituents of *M. philippensis*, including isolation, molecular mechanism elucidation, and in vivo validation.

Keywords: Apoptosis, cytotoxicity, DAPI staining, *Mallotus philippensis*, MCF-7 cell line, MTT assay steroidal glycoside

INTRODUCTION

The kamala tree, which is also referred to as *Mallotus philippensis* (Lam.) Müll.Arg. is a medium-sized deciduous shrub of the family Euphorbiaceae. It is found in tropical and subtropical areas of the South and Southeast Asian regions and grows in dry and moist deciduous forests in countries such as India, Sri Lanka, Malaysia, and the Philippines (Ju et al. 2013; Tripathi et al. 2024). One of the distinguishing features of the species is its reddish-orange rind of the fruits is covered in a resinous exudate that possesses cultural, medicinal, and industrial significance. *Mallotus philippensis* has traditionally been a part of indigenous medicine that has been used for antiparasitic, hepatoprotective, anti-inflammatory, and antimicrobial purposes (Sumithira et al. 2022; Ali et al. 2024; Khadim et al. 2024). The red-colored powder on the fruit, a traditional natural dye and vermifuge, highlights the significance of the plant in traditional medicine and industry (Mao et al. 2019; Xiong and Long 2020). The medicinal potential of the plant is rooted in its rich phytochemical diversity. Secondary metabolites like polyphenols, flavonoids, chalcones, tannins, and steroidal glycosides have been separated from different parts of the plant (Khadim et al. 2024). Of them, rottlerin is one of the most investigated constituents. The polyphenolic compound has various pharmacological actions, like anticancer, antioxidant, and anti-inflammatory activity. It acts through blocking protein kinase C-delta (PKC-δ), disrupting mitochondrial function, and triggering autophagy and apoptosis in cancer cells (Manhas et al. 2021). Other significant compounds are iso-rottlerin, kamala chalcones, and mallotophilippens and β-sitosterol-D-glucoside, which is responsible for the plant's synergistic therapeutic action (Pal et al. 2022). These compounds collectively enhance wound healing, inhibit microbial growth, and improve cytoprotection in human as well as veterinary use (Bodas et al. 2022; Wani et al. 2022).

The pharmacological actions of *M. philippensis* have a wide range. Its long-standing use as an anthelmintic, especially for the treatment of intestinal worms in children, is justified by the availability of chalcones and related constituents with established antiparasitic activity (Pal et al. 2022). Furthermore, fruit resin extracts exhibit considerable antibacterial and antifungal action, justifying their application

in skin infection treatment and wound healing. Flavonoids and polyphenols are responsible for antioxidant and anti-inflammatory effects by stabilizing free radicals and lowering oxidative stress, which is pivotal in the management of inflammatory disorders and cell damage (Sumithira et al. 2022). The anticancer activity of the plant is especially significant—rottlerin exhibited cytotoxicity, anti-proliferative, and pro-apoptotic activities against multiple cancer cell lines by targeting mitochondrial pathways and blocking PKC-δ (Lee et al. 2020; Sakthidhasan et al. 2022). Steroidal glycosides complement this action by mimicking the action of endogenous hormones and interfering with hormone-receptor pathways, which are highly applicable in hormone-sensitive cancers like breast cancer. Recent evidence highlights the prospect of steroidal glycosides as anticancer agents against hormone-dependent cancers, such as estrogen receptor-positive (ER+) breast cancer (Sakthidhasan et al. 2022). Their structural similarity to steroid hormones allows these compounds to regulate cell proliferation, apoptosis, and differentiation processes efficaciously (Manikyam et al. 2018).

Although steroidal glycoside-rich fractions from *M. philippensis* have shown promise in previous studies, their anticancer potential has not been thoroughly investigated. In this study, we evaluated the cytotoxic and pro-apoptotic effects of Iroid glycoside fractions on MCF-7 human breast cancer cells, a commonly used model for estrogen receptor-positive (ER+) breast cancer.

The research utilizes the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and DAPI (4′,6-diamidino-2-phenylindole) assays for determining cell viability and apoptosis, respectively (Mosmann et al. 1983; Kumar et al. 2014; Balakrishna and Kumar 2015). The two-pronged approach facilitates the determination of the breast cancer lead molecule potential of *M. philippensis*-derived compounds. The research further seeks to complement ancient traditional herbalism with modern-day cancer pharmacology and thereby open up avenues for sustainable plant-derived therapeutics.

MATERIALS AND METHODS

Study area and sample collection

Aerial parts of *M. philippensis* were collected from the forests of Rajaji National Park, located in Haridwar, Uttarakhand, India (29.9373° N, 78.0891° E). Samples were shade-dried, powdered, and stored in airtight containers until extraction.

Preparation of herbal extract

Aerial parts of *M. philippensis* were used to extract the actives. The samples were air-dried in the shade, powdered, and treated with ethanol extraction (95%) under agitation at room temperature for 72 hours. The extract was filtered, concentrated by rotary evaporation, and precipitated with cold acetone (1:3 v/v) to eliminate polysaccharide and protein contaminants (Manikyam et al. 2017a,b). The pellet obtained was dried, weighed, and stored at −20°C prior to further analysis.

Preliminary phytochemical test for steroidal glycosides

Keller-Kiliani test (for cardiac glycosides)

To identify the occurrence of steroidal nucleus associated with sugar groups, typically present in cardiac/steroidal glycosides (Pal et al. 2022; Tripathi et al. 2024). Procedure: mix 1 mL of plant extract with 2 mL of glacial acetic acid containing a minute quantity of ferric chloride. Gently pour 1 mL of concentrated sulfuric acid down the sides of the test tube to create a distinct lower layer. Note the color change at the interface.

Liebermann–Burchard test (for phytosterols/steroids)

To identify unsaturated sterols and triterpenoids, particularly glycosylated ones like β-sitosterol-D-glucoside. (Pal et al. 2022; Tripathi et al. 2024). Procedure: Combine 2 mL of the ethanolic extract with 2 mL of chloroform in a dry test tube. Gradually add 2 mL of acetic anhydride and a few drops of concentrated sulfuric acid. Leave the mixture undisturbed for a few minutes.

Salkowski test (for steroids):

Add 2 mL of concentrated sulfuric acid to 2 mL of chloroformic plant extract slowly along the side of the test tube. Note the color at the interface (Pal et al. 2022; Tripathi et al. 2024).

MTT cell proliferation assay

The MCF-7 cell line was procured from the National Center for Cell Sciences, Pune, with a seeding density of 2.0 × 104 cells/well and was kept in liquid nitrogen for future testing. MTT (5 mg/mL) was dissolved in PBS and sterile filtered using a 0.22 μM filter, and it was utilized for the study as a stock solution (Manikyam et al. 2021).

Prior to assay, the test system MCF-7 cells were cultured at 37 ± 1°C in a gaseous atmosphere with 5% ± 1% carbon dioxide in a humid environment in tissue culture flasks. The flasks contained 10% fetal bovine serum and Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, USA) and penicillin (100 units) and streptomycin (100 μg) antibiotics (Invitrogen, USA) to have the subconfluence of cells (70 to 90% confluent); cell seeding for cytotoxicity testing.

The layers of the cells were washed with Phosphate Buffer Solution (PBS) and with 0.25% trypsin in 0.2 g/L EDTA in the culture flask at 37 ± 1°C until cells detached and floated, and DMEM with 10% FBS was then added to the flask to flush out the cells. Centrifugation was performed at 900 rpm for 5 minutes. DMEM was employed to suspend the cells, and cell counting was conducted to calculate the viability cell number/mL, and the cell number was adjusted to 2 × 105 cells/mL. Adjusted cells were taken at 0.1 mL and seeded in every well of 96-well plates. Mixing was done repetitively while seeding was performed to obtain a uniform cell suspension for plating the cells in every well. The well identified plates were incubated at 37 ± 1°C for 24 ± 1 h in gaseous atmosphere of 5% ± 1% carbon dioxide and incubated for 24 ± 1 h. And cells were exposed to different concentrations of the test item. Spent medium was replaced with varying concentrations of test item solutions, and the incubation time to 48 ± 1 h at 37 ± 1°C, in an atmosphere of 5 ± 1% carbon dioxide, with

positive, negative, and blank in assigned wells. Following incubation for 48 ± 1 h, the item and positive control medium were removed, and cells were incubated for 4 h with 20 µL of MTT 5 mg/mL solution. Formazan crystals were developed in incubation by reduction of MTT and 150 µL of DMSO was added to dissolve. The absorbance was measured at 570 nm after 10 min of incubation.

Reduction in the count of viable cells means a reduction in metabolic activity, resulting in a reduction in formazan formation. This direct relationship can be tracked by optical density at 570 nm. The formula for percentage viability is given below:

% viability = 100 (O.D test item/O. D of control)

% activity = 100 - %viability

DAPI nuclear staining assay

Cell seeding and treatment

MCF-7 cells were seeded onto sterile glass coverslips in 6-well plates at a concentration of 1×10^5 cells per well and incubated for 24 hours for cell adhesion. Cells were subsequently exposed to the IC_{50} value of *M. philippensis* extract for 24 hours. Control cells were treated with DMSO alone (Manikyam et al. 2021).

Fixation and staining: Following treatment, cells were washed in Phosphate-Buffered Saline (PBS) twice and fixed with 4% paraformaldehyde for 15 minutes at room temperature. Cells were permeabilized with 0.1% Triton X-100 in PBS for 10 minutes and stained with 1 µg/mL DAPI (4′,6-diamidino-2-phenylindole) solution for 10 minutes in the dark.

% of apoptotic cells = (apoptotic cells + late apoptotic cells) / (total number of cells) × 100

Microscopic analysis

The stained coverslips were mounted on glass slides using fluorescence mounting medium. Nuclear morphology was observed in a fluorescence microscope with an excitation wavelength of 358 nm and an emission wavelength of 461 nm. The apoptotic features of chromatin condensation and nuclear fragmentation were noted and compared with control cells (Manikyam et al. 2021).

RESULTS AND DISCUSSION

Keller-Kiliani test (for cardiac glycosides): A blue or green ring at the junction of the two layers, not confirmed, indicates the absence of a deoxy sugar moiety, indicating the presence of cardiac or steroidal glycosides. Liebermann–Burchard test (for phytosterols/steroids): The development of a blue-green color is a positive indication of the presence of steroidal or triterpenoid compounds, including their glycosidic forms. Salkowski test (for steroids): A reddish-brown ring at the interface is the indication of the presence of steroidal compounds.

MTT assay

Steroidal glycoside content was extracted from aerial parts of *M. philippensis* and screened by qualitative analysis, which was found to be positive for steroid and glycoside tests and negative for the rest of the tests as discussed. Pure steroidal glycoside was also characterized for its anticancer activity against MCF-7 cancer cell lines by DAPI and MTT assays. Steroidal glycoside exhibited >67% apoptosis activity against negative controls presented in Table 3. The result of the MTT assay indicated that the compound possesses the highest % average anticancer activity, 50%, at a 200 µg/mL concentration in correlation with the positive test sample (SLS), as illustrated in Tables 1 and 2. *M. philippensis* extract-treated cells showed an IC50 at a concentration (17.32 µg/mL) when compared to SLS (107.02 µg/mL) (Figure 2).

DAPI

The cells were seeded in a 24-well flat-bottom microplate with cover slips and kept at 37°C in a CO_2 incubator overnight. The 200 µg/mL of compounds were treated at 48 hrs. Post incubation, cells were washed with PBS and fixed with 4% paraformaldehyde for 30 min. 20 µL of DAPI was incubated for 5 min at room temperature in the dark, then observed under a fluorescent microscope. Randomly selected the fields in the microscope and counted the number of cells that underwent apoptosis, and calculated the percentage of apoptotic cells.

Morphological assessment of the nucleus by fluorescence microscopy with the help of cell-permeable nucleic acid stain, like DAPI, is normally employed for analysis of apoptosis. Steroidal glycoside extract of *M. philippensis* treated cells showed condensed chromatin with clear apoptosis when compared to the negative control using the DAPI assay method, as illustrated in Figure 1.

Table 1. % of apoptosis standard compound SLS

Concentration (µg/mL)	% of Inhibition
25	22
75	45
100	58
200	88
400	100

Table 2. % of apoptosis test compound extract (*Mallotus philippensis*)

Concentration (µg/mL)	% of Inhibition
25	66
50	88
100	95
200	100

Table 3. % of apoptosis test compound

Control	4.25±2.90
Extract	67.87±6.13

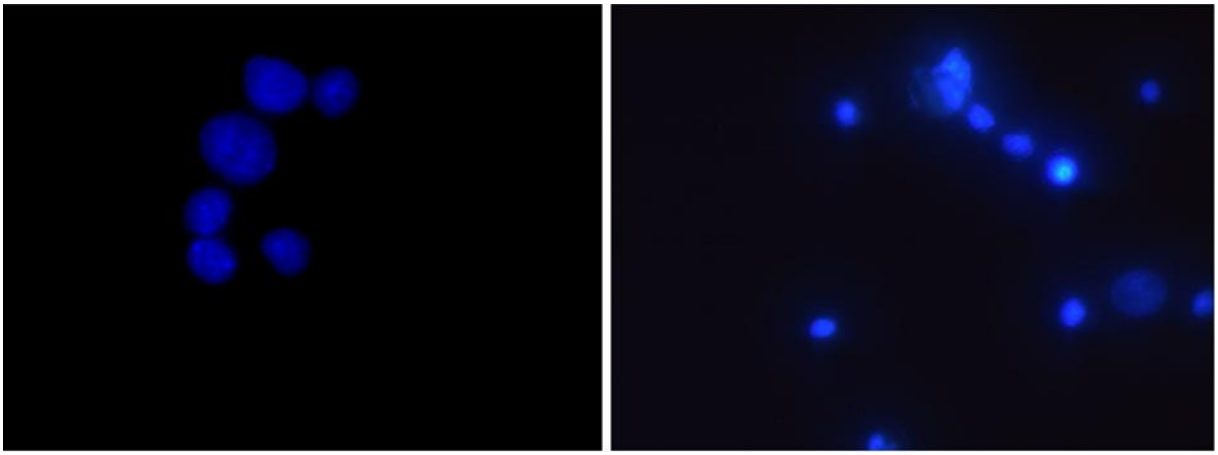

Figure 1. *Mallotus philippensis* extract-treated cells showed condensed chromatin, right image, with clear apoptosis when compared to the negative control, left image, using DAPI assay method

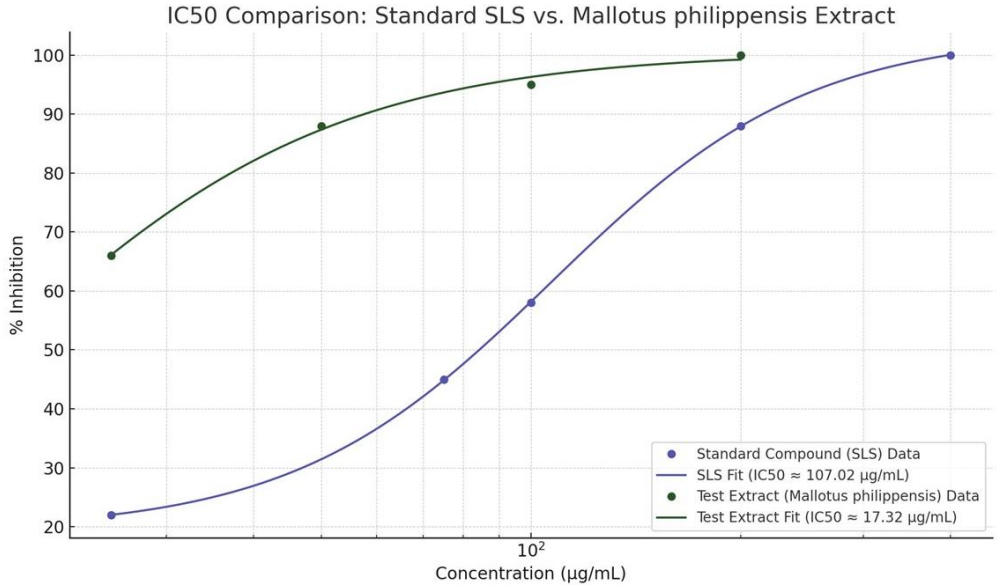

Figure 2. *Mallotus philippensis* extract-treated cells showed shown IC50 at a lower concentration (17.32 µg/mL) when compared to SLS (107.02 µg/mL)

Discussion

The present study demonstrates that steroidal glycoside-enriched fractions derived from the aerial parts of *M. philippensis* exhibit significant anticancer activity against MCF-7 human breast cancer cells. The findings from both the MTT and DAPI assays confirm that these plant-derived compounds exert cytotoxic and pro-apoptotic effects at a level comparable to standard chemotherapeutic agents (Manikyam et al. 2021). Importantly, these results build upon and validate the ethnomedicinal use of *M. philippensis* in traditional systems for treating various ailments, including tumors and skin disorders. The MTT assay showed a concentration-dependent cytotoxic effect, with maximum inhibition of 100% observed at 200 µg/mL. This aligns with earlier reports where other phytosterol-rich or glycoside-containing plant extracts, such as those from *Psidium guajava* L. or *Paris polyphylla* Sm., have similarly shown high cytotoxicity against hormone-sensitive cell lines like MCF-7. The ability of the extract to induce cell death at a relatively low concentration underlines its potential therapeutic potency and warrants further exploration of its constituent compounds.

Further validation was provided through DAPI nuclear staining, which revealed hallmark features of apoptosis such as nuclear condensation and fragmentation. The apoptosis index reached nearly 68% in treated cells compared to less than 5% in controls, indicating that the extract likely activates apoptotic pathways rather than causing nonspecific necrosis. This supports previous findings about the steroidal compounds like β-sitosterol-D-glucoside and rottlerin, which have been shown to induce apoptosis via mitochondrial pathways, caspase activation, and suppression of anti-apoptotic proteins like Bcl-2 (Manhas et al. 2021).

Interestingly, *M. philippensis* extract comparable activity with Sodium Lauryl Sulfate (SLS)—used as a positive control—demonstrates not just activity but efficacy. While SLS is a standard detergent compound known for causing cytotoxicity, its use in this study serves as a benchmark. The ability of a natural extract to perform on par with SLS emphasizes the potential of *M. philippensis* as a source of lead compounds for cancer alternative therapy.

It is also noteworthy that the plant is a rich source of multiple compound classes—rottlerin, iso-rottlerin, mallotophilippens, kamala chalcones, and various triterpenoids—which may work in synergy. Synergistic interactions between different bioactive components can enhance efficacy and reduce toxicity, a strategy that has long been recognized in traditional polyherbal formulations (Manhas et al. 2021).

Moreover, these findings are relevant given the limitations of conventional chemotherapy, such as systemic toxicity, resistance development, and high cost. Natural products offer a sustainable, biocompatible alternative that could be harnessed either as primary therapeutics or as adjuvants to existing treatments. Particularly in low- and middle-income countries, affordable and accessible plant-based therapeutics can significantly improve cancer management outcomes. In vivo studies using xenograft models and toxicity profiling will also be crucial to establish clinical safety and efficacy. The pharmacokinetic properties of the lead compounds, including Absorption, Distribution, Metabolism, and Excretion (ADME) (Bodas et al. 2022), must be thoroughly investigated before advancing to preclinical or clinical trials.

This study provides the anticancer potential of *M. philippensis* steroidal glycosides and reinforces its value in modern pharmacognosy and drug discovery. The data support traditional claims and justify deeper pharmacological investigations, paving the way for novel anticancer agents from this underutilized medicinal plant.

This provides steroidal glycoside-enriched fractions of *M. philippensis* that have strong anticancer activity against the MCF-7 breast cancer cell line, causing profound apoptosis and growth inhibition similar to standard chemotherapeutic controls. *Mallotus philippensis* extract, especially enriched with lipophilic steroids and glycosides, is indicative of its ethnopharmacological significance and potential as a source of new drug development. Isolation of individual active compounds, determination of their molecular modes of action, and assessment of their efficacy in in vivo cancer models are suggested areas of further research.

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