ZITA MEDICAL MANAGEMENT

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PHARMAKEFTIKI, 37, 2S, 2025 | 127-130 RESEARCH

https://doi.org/10.60988/p.v37i2S.158

Apoptosis-mediated antitumor activity of the ethanolic extract of *Calotropis procera* flowers: *in vitro* testing on the MG63 human osteosarcoma cell line

Israa Abd Alryahi[1,*], Fadia Hameed Mohammed[2], Hala M. N. Al-Saily[2]

[1]*Babylon Education Directorate, Ministry of Education, Hillah, Iraq*
[2]*Department of Biology, College of Science, University of Babylon, Hillah, Iraq*

KEY WORDS:
apoptosis; cancer; osteosarcoma; MG63; Calotropis procera

ARTICLE INFO:
Received: January 24, 2025
Revised: February 08, 2025
Accepted: February 17, 2025
Available online: October 10, 2025

ABSTRACT

Developing cancer therapeutics that selectively target malignant cells without harming healthy tissue or encountering resistance remains a central challenge in oncology. This study aimed at evaluating the effects of an ethanol (70%) extract of *Calotropis procera* L. flowers on the osteosarcoma MG63 cell line. Following serial dilution, the extract was assessed by using a multi-parameter cytotoxicity assay in order to measure cell viability (valid cell count), total nuclear intensity, mitochondrial membrane potential, cell membrane permeability, and cytochrome c release. These parameters were analysed *via* high-content screening (HCS) in order to determine the extract's therapeutic potential. The HCS results demonstrated that treatment with 200 μg/mL of the *Calotropis procera* flower extract can produce a highly significant effect across all five of the aforementioned parameters in MG63 cells.

*** CORRESPONDING AUTHOR:**
Israa Abd Alryahi, Babylon Education Directorate, Ministry of Education, Hillah, Iraq; e-mail: sci956.asra.abad@student.uobabylon.edu.iq

1. Introduction

Cancer is characterized by the uncontrolled proliferation of cells that invade surrounding tissues and spread to distant sites through the lymphatic system and the bloodstream; a process known as "metastasis"[1]. Effective anticancer therapies must minimize harm to normal cells while targeting multiple oncogenic pathways[2]. Conventional treatments such as chemotherapy and radiotherapy often impose significant physiological stress and may further compromise patient

PHARMAKEFTIKI, 37, 2S, 2025 | 127-130

Table 1. High-content screening (HCS) assay of MG63 cells treated with various concentrations of an ethanolic Calotropis procera flower extract. Results are presented as mean ± standard deviation, in fluorescence intensity units. Identical superscript letters indicate no statistically significant difference ($p>0.05$) between the groups, while different superscript letters denote significant differences ($p<0.05$).

***Calotropis procera* flower extract** (μg/mL)	**Valid cell count**	**Total nuclear intensity**	**Cell membrane permeability**	**Mitochondrial membrane potential**	**Cytochrome c release**
untreated (control)	3,539.5 ± 159.09 ^{a}	404 ± 24.04 ^{c}	204.5 ± 27.57 ^{b}	827 ± 8.48 ^{a}	463 ± 15.55 ^{b}
200	2,070.5 ± 119.50 ^{c}	599.5 ± 16.26 ^{a}	294.5 ± 24.74 ^{a}	658.5 ± 20.50 ^{c}	600.5 ± 30.40 ^{a}
100	2,222 ± 60.81 ^{c}	463.5 ± 17.67 ^{b}	183.5 ± 6.36 ^{b}	740.5 ± 2.12 ^{b}	577.5 ± 6.36 ^{a}
50	3,024 ± 212.13 ^{b}	418.5 ± 3.53 ^{c}	188.5 ± 9.19 ^{b}	826.5 ± 7.77 ^{a}	454.5 ± 10.60 ^{b}
25	3,593 ± 63.64 ^{a}	406.5 ± 30.40 ^{c}	192 ± 22.62 ^{b}	811 ± 11.31 ^{a}	444 ± 18.38 ^{b}

health. Consequently, there is growing interest in alternative and complementary therapies for cancer management.

Complementary medicine frequently incorporates crude plant materials in the form of extracts, powders, or tablets[3]. *Calotropis procera* L. belongs to the family Apocynaceae and is widely distributed across central and southern Iraq. Phytochemical analyses of its flower extract have revealed a rich profile of pharmacologically active compounds, including sterols (stigmasterol, sitosterol, campesterol), proceragenin, α-amyrin, β-amyrin, rosmarinic acid, and cinnamic acid[4,5]. These bioactive compounds exhibit cytotoxic effects against various cancer cell types through diverse mechanisms, such as by inducing apoptosis, by inhibiting cell proliferation, by suppressing angiogenesis, and by modulating oxidative stress. As such, *Calotropis procera* represents a promising candidate for the development of novel anticancer agents with minimal toxicity to normal cells[2]. Given the lack of prior research on the anticancer potential of *Calotropis procera* flower extracts, this study investigates their potential antitumor activity against the osteosarcoma cell line MG63.

2. Methodology

2.1. Preparation of a raw powder and ethanolic extract of *Calotropis procera* flowers

Fresh, fully bloomed, and healthy flowers of *Calotropis procera* were collected in March 2024 from Hillah, Iraq. The plant material was identified and authenticated by Dr Shaimaa Mohi (Department of Biology, College of Science, University of Babylon). The dry powder of the *Calotropis procera* flowers was prepared according to Al Ghezil *et al.*[6], while the ethanolic extraction was done according to Ekpenyong *et al.*[7], with modifications.

2.2. Cell line used

The osteosarcoma MG63 cell line was stored in the vapor phase of liquid nitrogen at temperatures below -130°C, in cryogenic vials. These vials were maintained at the Tissue Culture Laboratory of the Centre for Natural Product Research and Drug Discovery of the Department of Pharmacology of the Faculty of Medicine of the University of Malaya (Kuala Lumpur).

2.3. High-content screening (HCS)

HCS was performed by using the Cellomics Multiparameter Cytotoxicity Kit (Thermo Fisher Scientific, USA) in order to simultaneously evaluate multiple cellular parameters in the MG63 cell line. These parameters included valid cell count, total nuclear intensity, cell membrane permeability, mitochondrial membrane potential, and cytochrome c levels. The fluorescence distribution and intensity within the MG63 cells were captured and analysed using the ArrayScan HCS Reader, thereby enabling a quantita-

PHARMAKEFTIKI, 37, 2S, 2025 | 127-130

tive assessment of the cytotoxic effects induced by the *Calotropis procera* flower extract.

2.4. Statical analysis

Data were analysed using SPSS version 23. One-way analysis of variance (ANOVA), followed by Duncan's multiple range test, was used in order to determine significant differences between groups. A *p*-value lower than 0.05 was considered as statistically significant.

3. Results and Discussion

The HCS technology enables precise characterization of complex cellular behaviours. By combining automated high-throughput microscopy with advanced image analysis, HCS facilitates the exploration of intricate phenotypes and yields rich cellular data[8]. In this study, HCS was employed in order to assess apoptosis-related changes in the MG63 osteosarcoma cell line following treatment with four concentrations of an ethanolic *Calotropis procera* flower extract (200, 100, 50, and 25 μg/mL). The assay evaluated five key cellular parameters: valid cell count, total nuclear intensity, cell membrane permeability, mitochondrial membrane potential, and cytochrome c release.

As shown in Table 1, the 200 μg/mL concentration produced a highly significant effect across all parameters compared to untreated control cells. The 100 μg/mL concentration exhibited a statistically significant effect ($p<0.05$) on valid cell count and cytochrome c release, with a less pronounced effect on nuclear intensity and mitochondrial membrane potential. Lower concentrations (50 and 25 μg/mL) yielded results comparable to those of the control group ($p>0.05$).

The *Calotropis procera* flower extract has reduced valid cell counts and has suppressed cancer cell viability and proliferation, thereby suggesting cell cycle arrest at the G0/G1 phase. Morphological changes (such as cell rounding and size reduction) were indicative of apoptosis. The observed increase in nuclear intensity may reflect chromatin condensation; a hallmark of apoptotic progression. Additionally, the extract has enhanced membrane permeability in MG63 cells, which typically exhibit rigid membranes and multidrug resistance due to elevated cholesterol content[9]. At higher concentrations, the extract has significantly decreased mitochondrial membrane potential and increased cytochrome c release. These effects suggest mitochondrial involvement in the apoptotic response. The phytochemicals in *Calotropis procera* flowers may act through multiple mitochondrial mechanisms, including oxidative stress induction, inhibition of the electron transport chain, modulation of apoptotic proteins, and disruption of energy metabolism[10]. Such mechanisms are desirable in the context of anticancer therapy. Future work should focus on evaluating gene expression profiles in cancer cells treated with the *Calotropis procera* flower extract in order to further elucidate its molecular mechanisms of action.

4. Conclusion

Based on our findings, the ethanolic extract of *Calotropis procera* flowers demonstrates the ability to induce apoptosis in cancer cells, highlighting its potential as a promising candidate for anticancer therapy.

Acknowledgements

The authors express their sincere gratitude to the staff of the Biotechnology Centre of the Al-Nahrain University, Baghdad, Iraq, for their assistance in conducting the assays, and to the reviewers for their valuable feedback and constructive suggestions.

Conflicts of interest

None exist.

ORCIDs

0009-0007-3381-5483 (I.A. Alryahi); 0000-0002-9292-9348 (F.H. Mohammed); 0000-0003-4757-2914 (H.M.N. Al-Saily)

PHARMAKEFTIKI, 37, 2S, 2025 | 127-130

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HOW TO CITE:
Alryahi I.A., Mohammed F.H., Al-Saily H.M.N. Apoptosis-mediated antitumor activity of the ethanolic extract of *Calotropis procera* flowers: *in vitro* testing on the MG63 human osteosarcoma cell line. *Pharmakeftiki* 37(2s), 127-130, 2025. https://doi.org/ 10.60988/p.v37i2S.158