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Evaluation of *Chelidonium Majus* L. Alkaloid Effect on *VEGF* Gene Expression and Cell Apoptosis in the Burkitt Lymphoma Cell Line

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Abstract

Background: Burkitt lymphoma (BL) is a type of mature B-cell non-Hodgkin's lymphoma that commonly develops in children and young adults. Vascular endothelial growth factor *(VEGF*) is acknowledged as a vital regulator of angiogenesis in both normal and disease states. In light of the adverse effects linked to chemical treatments, this study aimed to explore the anticancer effects of *Chelidonium majus* L. alkaloid on the Daudi BL cell line and to evaluate the expression of the *VEGF* gene.

Materials and Methods: This project was an experimental study. The cytotoxic effects of the alkaloid derived from *Chelidonium majus* L. on the Daudi and normal cells were evaluated using MTT assays (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). The induction of apoptosis in cells was measured utilizing Annexin V/propidium iodide (PI) flow cytometry. Additionally, the expression levels of the VEGF gene were determined via a Real-time PCR assay. Data were entered into SPSS, version 21. Student's t-test and ANOVA test were used for comparisons of groups.

Results: The 50% cytotoxic concentration (CC_{50}) of the alkaloid from *Chelidonium majus* L. was found to be 56.35µg/mL in the Daudi cell. Findings from flow cytometry aligned with those from MTT assays. Real-time PCR assay results showed a significant decrease in the *VEGF* gene expression ($P<0.009$). Effects observed after 48 hours of treatment across various concentrations of *Chelidonium majus* L. alkaloid demonstrated dose-dependency. However, in peripheral blood mononuclear cells (PBMCs) as a control, the alkaloid did not significantly affect the expression of the *VEGF* gene ($P>0.05$).

Conclusion: The alkaloid is derived from *Chelidonium majus*L. Appears to influence angiogenesis by down-regulating the *VEGF* gene expression, suggesting its potential as a complementary agent in chemotherapy for Burkitt lymphoma. However**;** further research is required to evaluate the effectiveness of the *Chelidonium majus*L. Extract as a definitive treatment for Burkitt lymphoma.

Keywords: Burkitt lymphoma**,** Chelidonium majus, Vascular endothelial growth factor

Introduction

Burkitt lymphoma (BL) is classified as a B-cell non-Hodgkin's lymphoma characterized by a high mitotic rate (1). The disease is associated with several factors, including the Epstein-Barr virus (EBV), human immunodeficiency virus (HIV), and chromosomal translocations that result in the overexpression of the c-Myc oncogene (2, 3).

The WHO (World Health Organization) categorizes BL into three clinical types: endemic (linked to malaria and EBV), sporadic, and immunodeficiency-associated. Generally, BL can affect the lymph nodes, brain, spinal cord, intestines, kidneys, and other organs and cause tumors in the jaw and the mouth. The significance of angiogenesis in the growth and survival of lymphoma, as well as its

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impact on patients with leukemia and other hematologic malignancies, has been recognized since 1994 (4). Recent studies have demonstrated that the treatment of malignant lymphoma with anti-vascular endothelial growth factor (VEGF) agents can enhance cancer cell apoptosis and a reduction in blood vessels (5-9). Without timely treatment, BL can be fatal in children and elderly patients. Common treatments, which have many side effects, include radiotherapy, chemotherapy, and bone marrow transplant (10). While chemotherapy is widely regarded as an adjuvant treatment method for human cancer following surgery, because surgery alone cannot fully replace it, the response rates to these treatments are often low, and the degree of clinical improvement can be minimal. Moreover, severe toxicity and drug resistance are frequent challenges. Given these hurdles, the development of herbal drugs is crucial for advancing the treatment of BL (11-13). In many developed and developing countries, it is common to use medicinal plants to enhance the effectiveness of compounds used in treating diseases. These natural remedies play a significant role in the treatment of cancers and other malignancies (14). *Chelidonium majus* L. (*C. majus*), a member of the poppy family (Papaveraceae), is indigenous to Asia and Europe. This plant contains large amounts of isoquinoline and alkaloid secondary metabolites, including sanguinarine, chelidonine, chelerythrine, berberine, and coptisine, with antibacterial, antiviral, and anti-inflammatory properties. Generally, *Chelidonium majusL.* Is a rich source of compounds with antimicrobial, antitumor, and anti-inflammatory properties (15, 16). Additionally, the alkaloid-rich extract of *C. majus* eliminates chemotherapy-induced drug resistance and prevents cancer cell proliferation (17). In this study, the aim was to investigate the anti-angiogenesis effects of *C. majus* alkaloid and the gene expression of the *VEGF* in the Daudi BL cell line.

Materials and Methods

This project was an experimental study. It was determined that the study complied with the ethical principles as well as the national norms and standards for conducting medical research in Iran. IR.IAU.TNB.REC.1402.096

Cell culture:

Daudi cells, derived from the B lymphoblasts of a BL patient, were procured from the Pasteur Institute of Iran. These cells were cultured and maintained in RPMI-1640 culture medium (Gibco, USA), enriched with L-glutamine and 10% fetal bovine serum (FBS, Bio Idea Co., Iran). The culture was maintained under conditions of 5% CO2 and a temperature of 37°C. Peripheral blood mononuclear cells (PBMCs), used as a control, were isolated from a healthy individual using Ficoll-Lymphodex (Inno-Train, Germany). These cells were subsequently cultured in RPMI-1640 medium supplemented with 15% FBS under conditions of 37°C and 5% CO_2 (15, 16).

Preparation of *C. majus* L. alkaloid

The aerial parts of *C. majus L.* utilized in this study were gathered from the Haraz Amol Forest in Iran. The plant was identified at the Department of Pharmacognosy, Faculty of Pharmacy, Tehran Medical Sciences, Islamic Azad University (Tehran, Iran). Additionally, it was registered with the herbarium under the number 1630-AUPF. For the preparation of *C. majus*, the collected samples were dried under controlled temperature conditions and subsequently processed into a fine powder. This dry powder was then extracted three times with 80% methanol, which aided in the extraction of active secondary metabolites, such as alkaloids. Following this, 1N HCL and 1N NaCl were added to the methanolic extract and mixed at a temperature of

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25°C. Finally, the alkaline solution obtained from the previous step underwent a chloroform extraction(18, 19). The obtained product was an alkaloid extract.

Cellular cytotoxicity test

The cytotoxic effects of the alkaloid extracted from *C. majus* were evaluated in both the Daudi cells and PBMCs using the MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide) assay. The cytotoxic effects of *C. majus* alkaloid on Daudi and PBMC cells were analyzed through the MTT assay. In the MTT test, the cells were first examined morphologically and seeded at 30,000 cells per well in 96-well plates. Following a 24-hour incubation period, the cells were treated with various concentrations of *C. majus* alkaloid (10, 20, 40, 60, 80, 100, 120, and 140 µg/mL) in 200 µL of RPMI-1640, supplemented with 2% FBS serum in triplicate. After 48 hours of incubation, the cells were centrifuged, and the culture medium was removed. Each well was subsequently filled with 80 µL of RPMI-1640 and 20 µL of MTT solution (0.005 g/mL of PBS). After four hours of incubation that allowed for formazan crystal formation, 100 µL of dimethyl sulfoxide (DMSO) was added to each well. Three wells were designated as blanks, containing only 100 µL of DMSO, while three additional wells containing 100 µL of RPMI without the *C. majus* alkaloid were used as controls. After a 20-minute incubation period, the absorbance of each well was measured at a wavelength range of 540 nm and 630 nm. The cytotoxicity of all three compounds on Daudi cancer cells, as well as PBMCs, was determined using the following formula:

$$\text{Cytotoxicity } \% = 1 - \frac{Mean\ OD\ Test - Mean\ OD\ Blank}{Mean\ OD\ Control - Mean\ OD\ Blank} \times 100$$

Flow cytometry

Cellular apoptosis was evaluated using flow cytometry with Annexin V/PI staining. Both Daudi cells and normal cells were treated separately for 48 hours at a concentration of 56.35 µg/mL CC_{50} (50% cytotoxic concentration) of *C. majus* L. alkaloid for Daudi cells. Following the incubation period, the cells were analyzed by flow cytometry (BD FACS Calibur; Becton-Dickinson, USA) using an apoptosis detection kit. The evaluation was conducted following the instructions provided with the kit.

RNA extraction and cDNA synthesis:

The Daudi cells were separately treated with various concentrations of *C. majus* alkaloid (10, 30, 56.35, 80, and 100 µg/mL). Following a 48-hour incubation period, RNA was extracted from the Daudi cells using the ROJE Technologies kit, (Iran), by the kit's protocol. The concentration of the extracted RNA was determined using UV spectrophotometry. The RNA was then reverse transcribed using a cDNA synthesis kit (Yekta Tajhiz Azma, Iran), as per the kit instructions.

Quantitative real-time PCR assay

The expression levels of the *VEGF* gene and GAPDH, which served as an internal control, were quantitatively measured using real-time RT-PCR. This process was conducted on a Light Cycler® 96 instrument (Roche Molecular Systems, Germany), using the SYBR Green PCR Master Mix (Yekta Tajhiz Azma, Iran), following the manufacturer's instructions. The calculation of *VEGF* gene expression was performed using the ΔΔCt method. The specific primers for the *VEGF* gene and GAPDH are listed in Table I.

Statistical analysis

Statistical analyses were conducted to determine the significance level. Student's t-test was employed for comparisons between the groups, while an ANOVA test was used for comparisons involving more

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than two groups. Differences in gene expression were analyzed using GenEx 6 software, and statistical analyses were performed using SPSS Version 21. Graphs were also created with GraphPad Prism 8. A P-value of less than 0.05 was considered to indicate statistical significance.

Results

Cytotoxic effects of *C. majus* L. alkaloid on Daudi and PBMC cells

The CC_{50} of *C. majus* alkaloid on Daudi cells and PBMCs was determined using the MTT assay after 48 hours. The results of the MTT assay for various concentrations of *C. majus* alkaloid on Daudi cells and PBMCs are depicted in Figure 1. The CC_{50} value of *C. majus* alkaloid for the Daudi cell line was found to be 56.35µg/mL after 48 hours. Interestingly, the MTT assay results indicated that this concentration of *C. majus* alkaloid did not exhibit the same cytotoxic effect on PBMCs (normal cells).

Apoptosis assay

The cell apoptosis effects of *C. majus* alkaloid on the Daudi cell line and PBMCs were examined by flow cytometry. The Daudi and PBMC cells were treated with a CC_{50} concentration of *C. majus* alkaloid. As shown in Figure 2, the alkaloid derived from *C. majus* was found to induce apoptosis in cancer cells. However, it did not have the same adverse effects on normal cells at the same concentration. The results of the Annexin V/PI cell apoptosis assay revealed that the CC_{50} concentration of *C. majus* alkaloid on the Daudi cell line led to 1.46% early apoptosis and 37.94% late apoptosis. In contrast, the same concentration caused only 4.8% early apoptosis and 2.3% late apoptosis in normal cells.

Gene expression analysis by real-time quantitative PCR

The *VEGF* gene expression was evaluated by the real-time quantitative PCR assay. The analysis of *VEGF* gene expression after treatment with different concentrations of *C. majus* alkaloid showed that *VEGF* genes were dose-dependently inhibited by *C. majus* alkaloid (Figure 3). The results showed that in the Daudi cells, the *VEGF* gene expression decreased after 48 hours of *C. majus* alkaloid treatment (Table II). In the PBMCs treated with *C. majus* alkaloid (at the abovementioned concentrations), the *VEGF* gene expression did not alter significantly.

Table I: Sequences of primers and corresponding data for each gene used for real-time PCR

Gene	Primer sequence (5′→3′)	PCR Product length (bp)	PCR Protocol (Denaturation, Cycles, Extension)	Reference
VEGF*	F: ATCTGCATGGTGATG R: GGGCAGAATCATCACGAAGT	218	95°C/15 min (95°C/20s-58-65°C /20s-72°C/30s)× 5 (95°C/15s-60°C /20s-72°C/20s)× 40 72°C/5 min	(7)
GAPDH	*F: CTCTTGCTACTCTGCTCTG* *R: GCCTGCCTGGTGATAATC*	*179*	*94°C/5 min* *(94°C/40s-56°C/40s, 72°C/45s) × 30* *72°C/5 min*	*(8)*

*Vascular Endothelial Growth Factor

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Table II: Statistical analysis of VEGF gene expression in the Daudi cells at different concentrations of C. majus alkaloid after 48 hours.

Concentration	Mean (mRNA Fold Change)	SD*	p-value
10 µg/mL	0.585213	0.12531	0.0291
30 µg/mL	0.524491	0.036519	0.0020
56.35 µg/mL	0.330672	0.109706	0.0088
80 µg/mL	0.291379	0.085738	0.0048
100 µg/mL	0.199212	0.03269	0.0006

* Standard deviation

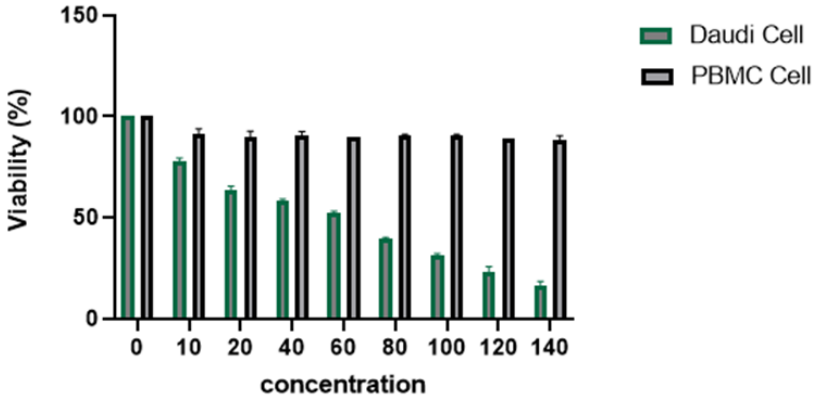

Figure 1. Comparison of CC_{50} obtained for C. majus alkaloid at 48 hours on the MTT assay. The CC_{50} value of C. majus alkaloid on the Daudi cell line was determined to be 56.35 µg/mL. However, this concentration did not have a significant effect on normal cells. The error bars in the graph represent the standard deviation.

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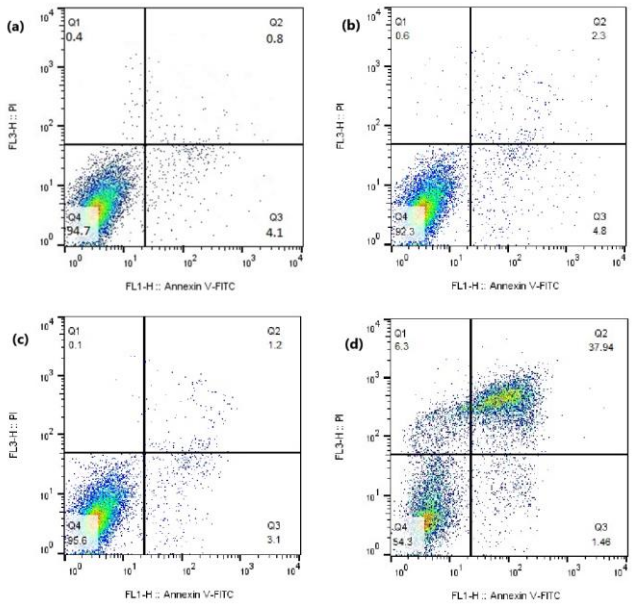

Figure 2. The flow cytometric diagram of Annexin/PI testing. (a) The PBMC line untreated as the control, (b) the PBMC line treated with 56.35 µg/mL of *C. majus* alkaloid, (c) the untreated Daudi cell line as the control, and (d) the Daudi cell line treated with 56.35 µg/mL of C. *majus* alkaloid.

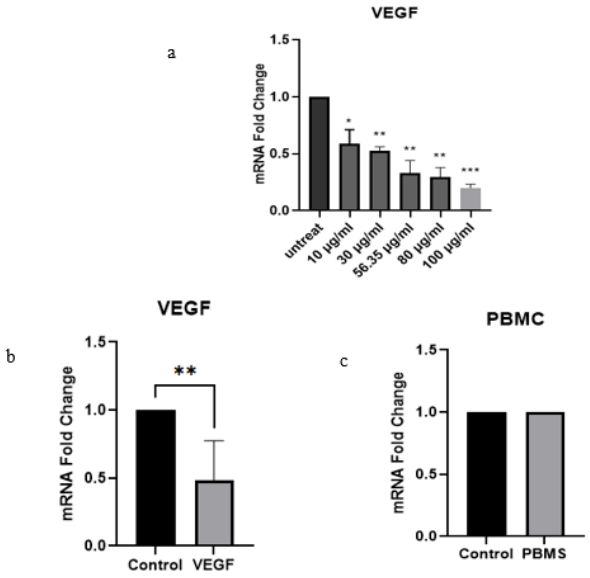

Figure 3. Expression of *VEGF* gene in the Daudi cells and PBMCs after 48 hours of treatment. (a) The *VEGF* gene expression is dose-dependently inhibited by different concentrations of *C. majus* alkaloid (*P=0.0291, **P<0.009, ***P=0.0006). (b) There is a significant decrease in the downregulation of the *VEGF* gene compared to the untreated control. The results are expressed as standard deviation (**P<0.009). (c) The expression of the *VEGF* gene compared to the untreated control is not significant in normal cells (P>0.05). (Using Student's t-test and ANOVA test).

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Discussion

Tumor growth and metastasis are dependent on angiogenesis, which is triggered by chemical signals from tumor cells during the rapid growth phase (20). Considering the importance of angiogenesis in the treatment of lymphoma cancer, the *VEGF* gene has been validated as a therapeutic target in various human cancers (21). Therefore, the development of new therapeutic strategies based on herbal medicines can improve patient survival. This study investigated the cytotoxicity, apoptosis, and gene expression related to the C. *majus* alkaloid through MTT assays, flow cytometry, and real-time quantitative PCR. The results indicated that *C. majus*L. Alkaloid decreased the *VEGF* gene expression in the Daudi BL cell line compared to PBMCs which were used as normal cell controls. In this context, the concentrations of *C. majus* alkaloid used to inhibit the proliferation of Daudi cancer cells had no harmful effects on normal lymphocytes. Treatment of the Daudi cell line with different concentrations of *C. majus* alkaloid revealed that *C. majus* alkaloid inhibited lymphoma cell proliferation in a dose-dependent manner by acting on cellular apoptosis. In 2023, Tuzimski et al. investigated the cytotoxic effects of *C. majus* on various cancer cells, especially melanoma cells, such as FaDu, SCC-25, MCF-7, and MDA-MB-231. The root extract exhibited the highest cytotoxic activity against melanoma A375 cells, with an IC_{50} value of 12.65µg/mL. It also showed significant cytotoxicity against K-MEL-3 cells, with an IC_{50} value of 1.93 µg/mL (22). Another study by Aljuraisy et al. showed that the aqueous extract of *C. majus* possessed cytotoxic effects on cancerous cell lines (pelvic RD and cervical HeLa cell lines). The most significant inhibitory efficacy of the extract was observed after 72 hours of exposure to the highest concentration of *C. majus* (1000 µg/mL) (23). In this study, it was shown that the alkaloids derived from the aerial parts of C. *majus* exhibit significant cytotoxicity against the Daudi cell line, after 48h, with a CC50 value of 56.35µg/mL. Additionally, no toxic effects were observed on normal cells. The differences in the results between this study and previous research could be attributed to several factors, including the primary source of the plant used for extraction, the type of extract used, and variations in cell types. Additionally, in 2018, Herrmann et al. demonstrated that chelidonine, an alkaloid found in *C. majus*, possesses pro-apoptotic and anti-metastatic properties in head and neck squamous cell carcinoma (HNSCC) cell lines. While mucosal keratinocytes were significantly affected by chelidonine, fibroblasts exhibited much greater resistance (24). In the present study, the whole alkaloid of C. *majus*, which exhibited dose-dependent effects, has been used. Interestingly, normal cells (PBMCs) demonstrated resistance to the alkaloid. Moreover, a study by Abu-Bakr Hassan et al. in 2016 showed that the ethanolic extract of *C. majus* had an anti-angiogenic potential against Ehrlich Ascites Carcinoma (EAC) in mice. In their study, the EAC cells were introduced into mice, following which mice with solid tumors were treated with *C. majus*. The activity of pro-angiogenic mediators was then assessed in the serum of various mouse groups (21). In 2007, a study conducted by Basini et al. demonstrated that sanguinarine, an alkaloid derived from *C. majus*, can inhibit vessel growth. To investigate this, they examined the effect of 300 nM sanguinarine on the production of VEGF in swine granulosa cells. They found that the VEGF signaling pathway was the primary effector (25). In the present study, using the real-time quantitative PCR, it

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was demonstrated that *C. majus* has an anti-angiogenic effect on BL cells, but does not affect human cells. Other studies have indicated that alkaloids play a crucial role in tumor growth and invasion. For instance, berberine, one of the alkaloids found in *C. majus*, has been shown to inhibit angiogenesis in breast cancer and hepatocellular carcinoma (26-28). In another study, M Deljanin et al. found that Chelidonium *majus* crude extract has time- and dose-dependent activity against migration and cytotoxic activity against human tumor cell lines A549, H460, HCT 116, SW480, MDA-MB 231, and MCF-7. However, does not affect peripheral blood mononuclear cells from healthy individuals. Cell cycle analysis was performed using flow cytometry and cell morphology assessment, revealing that the extract induces apoptosis. The inhibitory effect of cancer cells was evaluated using the wound healing assay (29). The results of this research are consistent with those of previous studies, with the only difference being the type of tumor cells. In various studies, researchers have demonstrated that chelidonine, a major alkaloid, exhibits potent cytotoxic effects against several types of cancer cells. These include leukemia, PANC-1 (a type of pancreatic cancer, with an IC_{50} value of 20.7 µg/mL), and HT-29 cells (a type of colon cancer, with an IC_{50} value of 20.6 µg/mL). Furthermore, chelidonine, which was isolated from the ethanolic extract of *C. majus*, has been reported to inhibit proliferation and induce apoptosis in the HeLa cells (30-32). In 2024, Zhu M et al. indicated that the extract of Celastrus orbiculatus inhibits the proliferation of human Burkitt lymphoma Raji and Ramos cells and promotes apoptosis by regulating apoptosis-related proteins. To evaluate cell viability, they used the CCK-8 assay. Western blotting was employed to detect alterations in apoptosis-related proteins, while annexin V-PI and JC-1 staining experiments were conducted to assess apoptosis (33). The findings of the aforementioned studies align with the results of this research. By flow cytometry method, we observed that the alkaloid from *C. majus* L. induced apoptosis in the Daudi cell line, but had no significant effect on PBMCs. Furthermore, it significantly decreased the level of the *VEGF* mRNA in the Daudi cell line compared to both PBMCs (a normal cell type) and the untreated Daudi cell line, which served as a control.

Conclusion

Based on the findings, the alkaloid from *C. majus* L. has the potential to suppress VEGF expression in the Daudi cell line. This suggests that this compound could significantly inhibit angiogenesis. Our results offer a stronger theoretical and experimental basis for upcoming in vivo studies. The data discussed here also bolsters the case for *C. majus* L. a promising new anti-tumor agent. Therefore, it may be considered a candidate drug for use as an adjuvant in the treatment of BL.

Data Availability

The authors declare that all data obtained during this study are available within the manuscript.

Ethical Considerations

This project was found to be in accordance with the ethical principles and the national norms and standards for conducting medical research in Iran. IR.IAU.TNB.REC.1402.096.

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Authors' Contributions

MP and FA conceived and designed the research. MS Carried out experiments and acquired the data. MP and MS interpreted the data and carried out data analysis and statistical analysis. MS wrote the original draft of the manuscript. MP, FA, MHP, and MS modified the paper. All authors read and approved the final manuscript.

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Conflict of Interest

Authors have no conflict of interest to declare.

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