IN VITRO STUDIES ON THE SYNERGISTIC EFFECT OF MORINDA CITRIFOLIA L. (NONI) AND METHOTREXATE ON CYTOTOXICITY OF HELA CELL LINES

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ABSTRACT

Objective: Cancer remains to be one of the leading causes of death around the world. Modern targeted therapies have undeniably improved cancer patients’ survival, but search still continues for safer and more effective drugs. This work is an attempt to assess the effectiveness of cotreatment of aqueous fruit extract of Morinda citrifolia L. (Noni) on methotrexate (MTX)-induced cytotoxicity on HeLa cancer cell line.

Methods: HeLa cells were treated with different concentrations of MTX and Noni alone and in combination for 24 and 48 h and studied for the cytotoxic effects by various approaches.

Results: There was a dose-dependent inhibition of growth when treated with MTX in a dose range of 0.045–45.4 µg/ml and Noni for a dose range of 0.3–30 mg/ml for 48 h. The IC₅₀ for MTX was 4.45 µg/ml (1 µM/ml), and for Noni, it was 8 mg/ml while the combination exhibited a more intensive growth inhibitory effect. Our results were confirmed with phase-contrast microscopy, whereby the number of viable cells decreased with an increase in the concentration of Noni. Cells were found to have rounded up and detached from the flask indicating apoptosis. Apoptosis in HeLa cell lines was further confirmed by DNA fragmentation and flow cytometry.

Conclusion: It can be concluded that Noni may enhance MTX cytotoxicity by inducing apoptosis and cell cycle arrest. Hence, cotreatment with Noni may reduce the dosage of MTX necessary for inhibiting proliferation of malignant cells and hence decrease the toxic effects of MTX on the system.

Keywords: Morinda citrifolia L., Methotrexate, HeLa cell lines, DNA fragmentation, Flow cytometry, Apoptosis.

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INTRODUCTION

Many nutraceuticals and plant active compounds are an important historical and ethnobotanical source for developing drugs with broad-spectrum pharmacological activities such as anticancer, antitumor, and antioxidant. An increasing incidence of cancer in developing and developed countries has promoted research toward dietary and medicinal plant extracts as supplements and prophylactics essentially due to the presence of phenolic acids, flavonoids, and phenolic diterpenes [1]. Chemotherapy and radiation on human cells continuously lead to the generation of reactive oxygen radicals, causing noxious effects due to potentially heavy loads of oxidants. Usage of alternative medicine is a necessity of the hour as they can be protective and can possess antioxidant and antitumor agents which can be more effective and reduce toxicity due to the presence of phytochemicals [2,3]. In the present study, we have used methotrexate (MTX) which is an antineoplastic drug widely used in the treatment of several diseases such as autoimmune diseases, lymphoma, and lymphoblastic leukemia but has been associated with mild-to-severe liver and intestinal toxicity. Aqueous fruit extract from Morinda citrifolia L. (Noni), which has a diverse array of active phytoconstituents with multitude of pharmacological activities, has been used for cotreatment in our study. Noni is used as an herbal remedy and has promising antioxidant properties [4].

Currently, there is a growing interest to understand and analyze the herb–drug interactions. Most of the oncologists do not recommend herbal medicines since it can interact with chemotherapeutic drug decreasing the therapeutic benefits. Rather, antioxidants are compounds which can act as radical scavengers if present in food products and avoid radical chain reaction, delay oxidation, and increase the life of cells by retarding lipid peroxidation [5]. Attractive approaches to prevent toxicity of different organs should involve the use of natural antioxidants. In this study, we introduce a combination of Noni with MTX to study anticancer effects against cervical cancer cell line, HeLa.

METHODS

HeLa cell lines P89 were obtained from the National Center for Cell Science, Pune. Fetal bovine serum was purchased from Pan Biotech, and Penicillin-Streptomycin antibiotic solution, sodium pyruvate solution, Dulbecco’s Modified Eagle Medium (DMEM), and Dulbecco’s phosphate-buffered saline (PBS ×10) were obtained from Mu Lifesciences, Mumbai, India. Folitrax 15 mg/ml of MTX injection was used, and MTX stock was filter sterilized and used in different concentrations ranging from 0.045 µg/ml to 45.4 µg/ml for the assays.

Noni extract was diluted in DMEM and filter sterilized. Concentrations ranging from 0.3 mg/ml to 30 mg/ml were used for the assays.

MTT-based cytotoxicity assay

Cytotoxicity assay was performed as described by Mosmann (1983) [6]. Cells were trypsinized and 2500 cells/200 µl were seeded per well of 96-well plate. Experiments were prepared in triplicates, and the cells were incubated at 37°C in CO₂ incubator. After 16 h, the medium from the well was replaced with medium containing different concentrations of M. citrifolia L. fruit extract (0.3–30 mg/ml). Separate wells were treated with varying concentration of MTX (0.045–45.4 µg/ml) and with IC₅₀ of MTX in combination with varying concentrations of M. citrifolia L. fruit extract. Control and negative control were also maintained. After 48 h of incubation with drug, 20 µl of MTT stock solution was added per well and incubated for 4 h at 37°C for formazan crystal formation.
The supernatant was carefully discarded taking care of not losing the formazan crystals. The crystals were dissolved by adding 100 µl of DMSO, and absorbance was read on a Bio Rad microplate reader (version 680) at 570 nm with a reference wavelength of 655 nm. The experiment was repeated 3 independent times in triplicates and percentage of inhibition was calculated.

**Phase-contrast microscopy**

Approximately 1×10⁴ cells were seeded in a 24 multi-well plate for phase-contrast microscopy. After 24 h, the medium was discarded and cells were supplemented with fresh medium. Cells were treated with MTX at a final concentration of 0.045–45.4 µg/ml alone, 0.3–30 mg/ml of *M. citrifolia* L fruit extract, and in combination along with IC₅₀ of MTX of varying concentrations of *M. citrifolia* L. fruit extract for 48 h. A separate well of control was also maintained. Phase-contrast microscopy study of treated and non-treated cells was performed using Inverted Microscope Nikon Eclipse TS100.

**DNA laddering assay**

DNA was extracted by the method of Gong et al. (1994) [7].

HeLa cells (2×10⁵) were seeded for DNA laddering assay in culture plates. After 24 h, the medium was discarded and cells were washed with ×1 PBS (pH 7.4) and fresh DMEM medium was added. Cells were treated with final concentration IC₅₀ (4.54 µg/ml) of MTX of alone, IC₅₀ (8 mg/ml) of Noni alone, and in combination with IC₅₀ of Noni and MTX. A separate control was also maintained. After 48 h incubation, the supernatant was collected and adhered cells were harvested by trypsin treatment.

Electrophoresis was carried out at 60 V until the loading dye migrated about three-fourth of the gel. DNA molecular marker of 500–1000 bp was run alongside the samples. The DNA was visualized by observing the gel using gel documentation system (Syngene).

**Clonogenic assay**

Clonogenic assay was performed as per the method of Buch et al. (2012) [8].

Clonogenic assay was performed on exponentially growing cells. A total of 500 cells were seeded in 6 multi-well plates in 1 ml of complete DMEM medium and allowed to adhere overnight. After 16 h, the medium was removed and replaced with medium containing IC₅₀ (4.54 µg/ml) of MTX alone, IC₅₀ (8 mg/ml) of Noni alone, and in combination with IC₅₀ of Noni and MTX. A separate control was also maintained. After 48 h, cells were fed with fresh DMEM medium. After 9 days of treatment, media were carefully removed and then washed with PBS. Cells were then fixed with methanol: acetic acid (3:1) and stained using 0.5% crystal violet in methanol for 15 min. Cells were washed again with PBS and colonies were observed. Treated and non-treated cells were photographed and each experiment was repeated in triplicate.

**Flow cytometry-based apoptosis detection**

Flow cytometry analysis of apoptotic cells was done as per the method of Riccardi and Nicoletti (2006), Balakrishnan and Bhat (2014), and Dai et al. (2017) [9-11]. Approximately 1×10⁵ cells were seeded in multiple 6-well plate for flow cytometry and apoptosis detection. After 16 h, the medium was discarded and cells were supplemented with fresh medium containing IC₅₀ (4.54 µg/ml) of MTX alone, IC₅₀ (8 mg/ml) of Noni alone, and in combination with IC₅₀ of Noni and MTX. A separate control was also maintained. After 24 h of treatment, the supernatant was collected and adhered cells were harvested by trypsin in treatment in a tube for each concentration-treated cells. The tubes were centrifuged at 2500 rpm for 10 min to obtain a cell pellet. The supernatant was discarded and cell pellet was washed with 1 ml of PBS (pH 7.4).

The cells were then fixed by adding pre-chilled 2 ml of 70% ethanol dropwise to the pellet. The cells at this stage were stored for a week at −4°C and then processed for analysis. During analysis, cells were first centrifuged at 2500 rpm for 10 min. Ethanol was discarded and cells were washed with PBS (pH 7.4) to remove ethanol completely. 500 µl of PBS was again added to the cell pellet, and the cells were passed gently through 26-gauge needle 3–4 times. 50 µl of RNase A and 5 µl of Triton X-100 were then added to the microfuge tube and incubated at 37°C for 30 min. 12.5 µl of propidium iodide was added per tube at a concentration of 1 mg/ml and incubated in the dark at 37°C for 20 min. The DNA content of the cells was analyzed on BD Accuri Flow Cytometer. C6 analysis software program was used for acquisition and percentage of cells undergoing apoptosis.

**Statistical analysis of data**

Data are presented as mean ± standard deviation. The statistical significance was assessed using one-way analysis of variance followed by Student’s t-test. In figures and tables, symbols represent statistical significance as indicated: *p<0.05, **p<0.01, ***p<0.001 and NS: Non-significant.

**RESULTS**

**Cytotoxicity of Noni and MTX**

Cytotoxic effect of MTX and Noni alone and in combination on HeLa cell lines was checked first with an exposure time of 24 h, but since it did not show any significant effect on the HeLa cells, then it was increased to 48 h.

**Flow cytometry graph**

**Fig. 1**: Cell viability of HeLa cells with different concentrations of methotrexate by MTT assay. Data represent cell viability with different concentrations of MTX after 48 h with mean ± standard deviation value from three independent determinations in triplicates.

**Flow cytometry graph**

**Fig. 2**: Cell viability on HeLa cells with different concentrations of Noni by MTT assay. Data represent cell viability with different concentrations of Noni after 48 h with mean ± standard deviation value from three independent determinations in triplicates.
Fig. 3: Cell viability of HeLa cells with different concentrations of Noni and $IC_{50}$ value of methotrexate (MTX) in combination with MTT assay. Data represent cell viability with different concentrations of Noni in the presence of $IC_{50}$ value of MTX after 48 h with mean±standard deviation value from three independent determinations in triplicates.

Plate 1: Representative photomicrographs of methotrexate (MTX)-treated HeLa cell’s morphology. Cells exposed to increasing concentrations of MTX. (a) None. (b) 0.0454 µg/ml. (c) 0.227 µg/ml. (d) 0.44 µg/ml. (e) 2.27 µg/ml. (f) 4.54 µg/ml. (g) 6.810 µg/ml. (h) 9.080 µg/ml. (i) 22.7 µg/ml. (j) 45.4 µg/ml.

Study of the morphology of HeLa cells after MTX and Noni treatment

Morphological alterations in HeLa cell lines treated with MTX alone, Noni alone, and combination of Noni and MTX along with control were observed under an inverted microscope. Control plates with 75–80% confluency appeared to be healthy. Considerable cytotoxicity was observed in HeLa cells treated with varying concentration of MTX in range of 0.0454–45.4 µg/ml for 48 h. Distinctive apoptotic features such as cellular shrinkage, reduction in cell volume, cytoplasmic change, and nuclear condensation were noted. The combination of Noni and MTX showed a synergistic effect with an increase in concentration, leading to more pronounced morphological changes.

Fig. 2 shows the cell viability results of Noni in a dose range of 0.3–30 mg/ml, used for standardization. $IC_{50}$ value for Noni was found to be 8 mg/ml.

Fig. 3 shows the results of the combination of Noni in varying concentrations with MTX $IC_{50}$ value of 4.45 µg/ml. Noni was found to inhibit the viability of cells with an increase in concentration along with $IC_{50}$ value of MTX.
constriction and membrane blebbing was also observed (Plate 1e-h).
With an increase in the concentration of MTX (Plate 1h-k), the number
of attached viable cells decreased and maximum cells were found to be
floating.

HeLa cells treated with varying concentrations of Noni from 0.3 mg/ml to
30 mg/ml also showed concentration-dependent cytotoxicity (Plate 2).

Plate 3 shows HeLa cells treated with a combination of MTX
IC\textsubscript{50} (4.45 µg/ml), and varying concentrations of Noni also show cellular
shrinkage and reduction in volume (Plate 3 - e, f and g); similarly,
floating cells are observed in Plate 3g-j also.

Plate 4 shows morphology of HeLa cells treated with control (a),
8 mg/ml of Noni (b), 4.54 µg/ml MTX treated cells (c), and 8 mg/ml
Noni + 4.54 µg/ml MTX treated cells (d) showing reduction in cell
number as compared to that of control.

**Effect of MTX and Noni on HeLa cell clonogenic assay**

Clonogenic assay measures the ability of the cell to retain its normal
morphological characteristics, proliferation, and the effectiveness
of a cytotoxic agent is added to cancer cells [12]. With increase in
concentration of cytotoxic agent there is loss of reproductive integrity
and inability to proliferate leading to cell death [13].

**Plate 2: Representative photomicrographs of Morinda citrifolia L. fruit extract-treated HeLa cells, morphology.** Cells exposed to increasing concentrations of *M. citrifolia* L. fruit extract. (a) None. (b) 0.3 mg/ml. (c) 1.5 mg/ml. (d) 3 mg/ml. (e) 5 mg/ml. (f) 8 mg/ml. (g) 10 mg/ml.
(h) 15 mg/ml. (i) 20 mg/ml. (j) 25 mg/ml. (k) 30 mg/ml

**Plate 3: Shows HeLa cells treated with**

**Plate 4: Shows morphology of HeLa cells treated with**

**Fig. 4: Effect of Noni on methotrexate (MTX)-induced DNA fragmentation.** Alphabets indicate wells loaded with DNA isolated from cells after different treatments stained with (a) DNA Ladder 500-1000 bp, (b) untreated cells, (c) 4.54 µg/ml MTX treated, (d) Noni 8 mg/ml + MTX 4.54 µg/ml treated, (e) Noni 8 mg/ml treated. Arrow indicates characteristic ladder pattern observed in apoptotic cells seen in lane C.
Colony formation ability of HeLa cells when treated with MTX and Noni fruit extract was assessed. Our study shows that HeLa cells are sensitive to MTX alone, Noni alone, and combination of Noni and MTX in comparison to control (Plate 5). Since cells number was less than 50, plating efficiency could not be calculated.

As can be observed in Plate 5, cells treated with MTX IC<sub>50</sub> value (4.54 µg/ml), Noni IC<sub>50</sub> value (8 mg/ml), and combination of 8 mg/ml Noni and 4.54 µg/ml MTX showed distinct fragments that ultimately lead to collapse of the cells, nuclear disintegration, destabilization of nuclear envelope, and lamin proteolysis (Plate 4 - b, c, d). These photographs establish the synergistic effect of Noni fruit juice on MTX-induced cytotoxicity.

**Effect of Noni and MTX on DNA fragmentation**

Apoptosis is a distinctive feature of cell death due to the release of nuclear DNA from apoptotic cells. HeLa cell lines treated with MTX and Noni were analysed for DNA fragmentation. DNA ladder 500-100bp was used for comparison. (Fig. 4a) DNA of cells treated with MTX IC<sub>50</sub> (Fig. 4c) showed ladder with some smearing. There was no DNA fragmentation or smearing of DNA in control (Fig. 4b), but the combination of Noni + MTX and Noni alone showed smearing which might be due to necrosis of cells (Fig. 4d and e). This also serves as a vital biochemical marker for measurement of drug-mediated cytotoxicity.
Cells treated with MTX IC$_{50}$ 4.54 µg/ml, however, showed 25.86%±7.78 of apoptotic cells and 74.14% viable cells. Fig. 7a and b shows a number of cells captured with no gate and gate R$^2$ later. Fig. 7c and d shows the sub G1 phases which determine the number of apoptotic cells in the IC$_{50}$ 4.54 µg/ml.

Cells treated with Noni and MTX, however, showed 21.13%±2.68 of apoptotic cells and 78.87% viable cells. Fig. 8a and b shows a number of cells captured with no gate and gate R$^2$ later. Fig. 8c and d shows the sub G1 phases which determine the number of apoptotic cells in the Noni and MTX.

Our results indicate that cytotoxicity of Noni or MTX by themselves and in combination as detected by MTT assay and clonogenic assay showed a decrease in survival of cancer cells. As shown in Fig. 9a, combination of Noni and MTX can enhance apoptosis as compared to either Noni alone (p<0.001) or MTX alone (p<0.001). Fig. 9b is a pictorial representation of multiple samples run in gated (P5 in R$^2$). Black peak represents control with 89.41% viable cells and 10% apoptotic cells. Red peak represents Noni extract-treated cells with 75.20% viable cells and 24.8% apoptotic cells. Blue peak represents cells treated with Noni +MTX showing 71.83% viable cells and 28.17% apoptotic cells; similarly, green peak represents cells treated with MTX showing 61.36% viable cells and 38.64% apoptotic cells.

Even though the number of apoptotic cells appears to be more in pictorial graph (Fig. 9b), Fig. 9a shows a greater number of apoptotic cells in Noni and MTX in comparison to that of MTX alone in histogram. This could be due to the scatter of cells obtained during acquisition of data.

**DISCUSSION**

Natural plant compounds have attracted attention as alternative therapeutics in the fight against diseases, primarily due to their low toxicity and high therapeutic index. Plant-derived drugs such as vinblastine, vincristine, taxol, and camptothecin are already accepted as potent chemotherapeutic agents. Here, we explore the antiproliferative potential of fruit extracts of *M. citrifolia* alone and in combination with a well-accepted drug, MTX, by *in vitro* methods.

Our results for viability assay performed on MTX alone, Noni alone, and combination of Noni with MTX-treated cells were found to be highly significant. Singh et al. (2013) have also reported that addition of varying concentration of Noni alone, cisplatin alone, and combination of both reduced the cell survivals, respectively, by 22%, 29%, and 50% in HeLa cell lines [15]. The concentration of cisplatin used in their study was 10 µg/ml and Noni extract used was 10%, v/v for 24 h.

In our study, we have observed that about 50% of HeLa cells showed cytotoxicity even with a concentration of 8 mg/ml of Noni alone and MTX 4.54 µg/ml alone, whereas a minimum dose of MTX 4.54 µg/ml in combination with Noni (8 mg/ml) is sufficient to be 52% cytotoxic after 48 h, showing an additive effect and hence proving that small amount of chemotherapeutic drug can be used along with Noni as a chemoadjunct to boost the cytotoxic potential.

Noni by itself has a cytotoxic effect on HeLa cell lines. Cells rounded up and detached from the plate and their neighbouring cells with subsequent condensation of cytoplasm. Dissolution of nuclear envelope along with separation of the nucleus into distinct fragments leading to collapse of cells into several small intact vesicles were also observed. A similar result was seen when we combined the IC$_{50}$ value of MTX with increasing concentration of Noni. Plasma membrane blebbing, collapse of cells, number of viable cells decreased with an increase in concentration, and retraction were observed in treatment with IC$_{50}$ value of MTX and increasing concentration of Noni.

Our studies indicate that Noni co-treatment with lower dose of MTX can induce morphological changes such as, necrosis and cell death.
Fig. 5: (a-d) Representative histogram showing quantitative detection of apoptotic population in control HeLa cells by flow cytometry.

Fig. 6: (a-d) A representative histogram showing quantitative detection of apoptotic population in 8 mg/ml of Noni-treated HeLa cells by flow cytometry.
proving to be very good cytotoxic agent, equivalent to higher doses of MTX alone.

Similar cytotoxicity with extracted chlorophyllin from fresh leaves of *M. citrifolia L.* has been also reported on Hep G2 and Vero cell lines [16], which suggest higher safety ratio, indicating potential use in cancer treatment as the extract inhibits only the growth of cancer cells but not normal cells.

Clonogenic assay is a conventional and more frequently used *in vitro* assay to visually witness cloning efficiency. Chemotherapeutic drug leads to remarkable phenotypic changes, [17] and damage to chromosomes leading to apoptosis [18]. This assay detects the ability of all cells that have retained the capacity for producing a large number of progenies after treatment.

The studies on the effect of curcumin-induced apoptosis in human mammary carcinoma cells (MDA-MB-23) reported by Latifah *et al.* (2006) are similar to our results, which again emphasize Noni fruit juice's synergistic effect on cytotoxicity induced by MTX [19].

Consistent slow growth rate, inability to form clones, no surviving colonies, and only few cells were observed in all the three plates that were treated with Noni and MTX. We observed that the cell number in MTX alone, Noni alone, and combination of Noni and MTX was less than 50 as compared to that of control.

Induction of apoptosis in HeLa cell lines was confirmed by DNA fragmentation. These degraded DNA produces a ladder pattern which is a hallmark of cytotoxicity and drug-induced apoptosis in cancer cells as observed on an agarose gel [20]. Xie *et al.* (2016) have stated that MTX can be lethal to cancer cells, as well as normal cells, and hence, the use of a combination of Noni and MTX can be more beneficial in the treatment of cancer [21]. Breakdown of cellular DNA caused due to activation of endogenous nuclear endonucleases and proteases, cleave double-stranded DNA at sites situated on linker DNA leading to DNA laddering. The release of these enzymes from cytoplasmic membrane is a sign of inhibition of DNA replication.

Treated or damaged cells can be evaluated by cell cycle analysis. Certain checkpoints are interrupted due to damage that occurs in the cell during cell cycle, hence preventing normal cells from being transformed into cancer cells [9].

G1 phase nuclei of apoptotic cells contain less DNA than healthy cells. Apoptosis is evaluated by quantification of percentage cells in the sub-G1 phase. A sub G0/G1 peak was seen before G1 in a flow cytometry histogram. Cycle cell analysis carried out to check the synergistic effect of Noni and MTX on HeLa cell lines showed an increment in sub G0/G1 population indicating the occurrence of apoptosis which was in concurrence to decrement in the percentage of viable cells in MTT and clonogenic assays.

Sharma *et al.* (2016) reported that cell cycle distribution in MCF-7 and MDA-MB-231 cell when exposed to Noni ethyl acetate extract at IC\textsubscript{50} for 24, 48, and 72 h showed a noticeable increase in the G1/S phase in a time-dependent manner [20]. They have reported 81% apoptotic cells which are comparable to our results for the same time duration [22]. Our results are also in accordance with Gupta *et al.* (2013) and Alitheen *et al.* (2016) [2,23]. The latter have used the combination of doxorubicin and damnacanthal, and the population of MCF-7 cells decreased more when treated in a combination of two rather than individual agents alone.

Combination therapy (Noni and MTX) remarkably increased apoptotic index, thereby promising herbal-based anticancer agent. An important homeostatic mechanism can be established by induction of apoptosis

![Fig. 7: (a-d) A representative histogram showing quantitative detection of apoptotic population in 4.54 µg/ml of methotrexate-treated HeLa cells by flow cytometry](image-url)
which regulates cell division and cell death leading to cytotoxicity that can maintain suitable cell number. Noni has been identified for its antiproliferative potential against tumor cells. In accordance, if plant extracts and their constituents have an ability to inhibit cancer cells without disturbing normal cells, they can have an immense potential in the treatment of cancer [24,25]. The synergistic combinations of Noni with existing anticancer drugs can enhance efficacy, reduce side effects, boost immune modulation, as well as abrogate drug resistance.

CONCLUSION
The results of this study show that Noni extract was able to induce growth inhibition, apoptosis, and cell cycle arrest alone and in
combination with MTX. The combined use of Noni and lower MTX dose could potentially reduce the side effects/toxicity of the drug while not compromising on its therapeutic potential.

Noni as a chemoadjuvant can be one of the effective methods in reducing MTX dose administered to cancer patients during chemotherapy, thereby reducing toxicity to normal cells as well as side effects caused by chemotherapeutic drugs during the course of treatment.

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AUTHORS’ CONTRIBUTION

Corresponding author, Dr. Thakamani Marar designed the experimental work, reviewed the manuscript, and also put a lot of efforts to improve the quality of the manuscript. Dr Bhakti A Mhatre carried out all laboratory work, data collections, and writing the manuscript.

CONFLICTS OF INTEREST

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no financial support for this work that could have influenced its outcome.

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