Comparative Analysis of Apigenin-3 Acetate versus Apigenin and Methyl-Prednisolone in Inhibiting Proliferation and Gene Expression of Th1 Cells in Multiple Sclerosis

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Abstract

Objective: In spite of the advances in therapeutic modalities, morbidity, due to multiple sclerosis (MS), still remains high. Therefore, a large body of research is endeavouring to discover or develop novel therapies with improved efficacy for treating MS patients. In the present study, we examined the immunomodulatory effects of apigenin (Api) on peripheral blood mononuclear cells (PBMCs) isolated from MS patients. We also developed an acetylated form of Api (apigenin-3-acetate) to improve In its blood-brain barrier (BBB) permeability. Additionally, we compared its anti-inflammatory properties to original Api and methyl-prednisolone-acetate (a standard therapy), as a potential option in treating MS patients.

Materials and Methods: The current study was an experimental-interventional research. The half maximal inhibitory concentration (IC_{50}) values for apigenin-3-acetate, apigenin, and methyl-prednisolone-acetate were determined in healthy volunteers' PBMCs (n=3). Gene expressions of T-box transcription factor (*TBX21* or *T-bet*) and *IFN-γ*, as well as proliferation of T cells isolated from MS patients' PBMCs (n=5), were examined in co-cultures of apigenin-3-acetate, Api and methyl-prednisolone-acetate after 48 hours of treatment, using quantitative reverse transcription polymerase chain reaction (qRT-PCR).

Results: Our findings showed that apigenin-3-acetate, apigenin, and methyl-prednisolone-acetate at concentrations of 80, 80, and 2.5 M could inhibit Th1 cell proliferation after 48 hours (P=0.001, P=0.036, and P=0.047, respectively); they also inhibited T-bet (P=0.015, P=0.019, and P=0.022) and interferon-γ (*IFN-γ*) gene expressions (P=0.0001).

Conclusion: Our findings suggested that Api may have anti-inflammatory properties, possibly by inhibiting proliferation of IFN-producing Th1 cells. Moreover, comparative immunomodulatory effects were found for the acetylated version of apigenin-3-acetate versus Api and methyl-prednisolone-acetate.

Keywords: Apigenin, Apoptosis, Multiple Sclerosis, Proliferation, Th1

Introduction

Multiple sclerosis (MS), as the most common source of neurological disability among adults, is an autoimmune-mediated inflammatory disease that affects central nervous system (CNS) and results in serious physical or cognitive disabilities (1). The disease can develop at any age and even may affect children or elderly people, but it most often occurs in adults in their 20 and 30 seconds, where women are twice as likely to suffer from MS compared to men (2-4). Neurologists have suggested four classes or subtypes for MS patients, comprising relapsing-remitting MS (RRMS), primary progressive MS (PPMS), secondary progressive MS (SPMS), and progressive relapsing MS (PRMS) (5). The first subtype accounts for almost 85% of MS cases. It is characterized by episodes of reversible acute attacks, followed by progressive neurological remission periods (6).

MS is mediated mainly by pathogenic T cells that target myelin antigens (self-antigens), thereby inducing a broad spectrum of neurodegenerative processes that subsequently result in development of the disease. A dysregulated activity of the several subtypes of T cells, including T-helper 1, T-helper 17, and regulatory T cells [interferon-γ (IFN-γ), interleukin-17 (IL-17), and transforming growth factor-beta (TGF-β)-producing cells, respectively], has been revealed to show a role in MS pathogenesis. Notably, IFN-γ-producing CD4$^+$ T cells (primarily Th1 cells) have been identified in the brain tissue of individuals suffering from the disease

early stages. Hence, they are assumed to play crucial roles in orchestrating inflammatory responses, leading to the recruitment and stimulation of immune cells, which negatively impact physiological activities of the oligodendrocytes (7, 8).

With advances in our knowledge of the pathobiology of MS, several therapies have been introduced for the management of affected patients. However, considering the relapsing nature of MS and less responsiveness to or serious side-effects of the currently available anti-inflammatory and immunosuppressive drugs, especially in long-term use (such as heart rate change, flu-like symptoms, rare brain infections, chest pain, hair loss, bladder infection, leukemia occurrence, etc.), unsolved challenges related to the treatment of MS patients still exist and need to be solved (9, 10). Therefore, this is an active research area to devise novel therapies or change existing options to improve their safety and therapeutic potential (11, 12).

During the last decades, herbal compounds or plant extracts with anti-inflammatory effects have widely been studied for potential use in clinical conditions, like MS disease (13-15). Among these compounds, apigenin (4′, 5, 7-trihydroxyflavone), is an easily extractable plant-derived flavonoid with strong anti-inflammatory characteristics, demonstrated *in vitro* and *in vivo* studies (13, 16). Previous studies have reported multiple biological functions for apigenin, such as anti-inflammatory, anti-oxidative, free radical-scavenging, and anti-carcinogenic effects (13, 17). The hydroxyl groups in flavonoids can form complexes with oxidizing species, allowing these compounds to scavenge and stabilize free radicals and reducing oxidative damage, as a hallmark of many chronic diseases (18, 19). Strikingly, apigenin (Api) has also been proven to counteract the neurodegenerative effects of nitric oxide (NO), inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) (20). Moreover, Api was shown to reduce production of inflammatory cytokines, especially tumor necrosis factor (TNF), IL-6, and IL-1, by inhibiting expression of NF-κB and AP-1 transcription factors (21, 22). The therapeutic potential has also been investigated in several inflammatory conditions comprising alzheimer's disease (23-25), parkinson's disease (26), Arthritis (27, 28), lupus (29), etc.

Based on the mentioned notes, in this study, we tested immunomodulatory effects of Api on peripheral blood mononuclear cells (PBMCs) isolated from MS patients. We also developed an acetylated form of Api (apigenin-3-acetate) to improve its blood-brain barrier (BBB) permeability and compared its anti-inflammatory properties to Api and methyl-prednisolone-acetate (as a standard therapy), as a potential option in treating MS patients.

Materials and Methods

Patients and peripheral blood mononuclear cells isolation

The current study was an experimental-interventional research and it was conducted in the Department of Immunology at the Isfahan University of Medical Sciences, Isfahan, Iran. Patients recruited into this study were those diagnosed according to the revised McDonald criteria (30) and enrolled in the MS center at Kashani Hospital, Isfahan, Iran. The blood samples were only taken from patients with RRMS before their first corticosteroid dose. Patients who had received anti-inflammatory and immunosuppressive drugs before blood sampling, were diagnosed with other inflammatory diseases, or pregnant were excluded.

Ten millilitres of peripheral blood samples were collected from the all subjects (five patients and three healthy donors) in heparinized tubes to isolate PBMCs. According to the manufacturer's guideline (inno-train Diagnostik GmbH, Germany), PBMCs were isolated using Ficoll-Paque centrifugation and washed with phosphate-buffered saline (PBS, pH=7.3) several times. Afterwards, PBMC cells were centrifuged at 2800 rpm for 25 minutes. In order to perform cell count, a hemocytometer was used and trypan blue dye exclusion (0.4% trypan blue in PBS) was used to assess cell viability. Cells with 98% viability were used for further experiments.

Carboxyfluorescein succinimidyl ester dye labeling

To determine proliferation capacity of PBMCs, carboxyfluorescein diacetate succinimidyl ester (CFSE) staining was performed. For this purpose, cells were suspended in 1 ml of Roswell Park Memorial Institute (RPMI1640, Sigma-Aldrich Co., Ireland) at a concentration of $5 \times$-$1 \times$ cells/ml. Then, CFSE staining solution at a concentration of 5 mM was added to the cell mixture to make a solution in 5 µM concentration for cell suspension. The cells were incubated in aluminium foil at 37°C, 5% CO_2 for 15 minutes, and after that, 10 ml of the whole RPMI medium was added to quench the staining. Then, after twice washing the stained cells with RPMI medium, the cells were used in the following experiments.

Preparation of the pharmaceutical compounds

To prepare a 10000 µM stock solution, powders of apigenin-3-acetate (Api-3A, 5 mg powder, Isfahan Pharmaceutical Sciences Research Center, Iran), Api (5 mg powder, Aktin Chemicals, China), and methyl-prednisolone-acetate (M-Pre-A, 40 mg ampoule, Kaspean Taemin, Iran) were dissolved in 1.851, 1.262 and 9.615 ml of dimethyl sulphoxide (DMSO), respectively. All subsequent dilutions were prepared in RPMI medium to run Api-3A and Api concentrations of 100, 10, and 1 µM, as well as M-Pre-A concentrations of 5, 0.5, and 0.05 µM.

Dose response and time coursing

Flow cytometry was used to determine amount of inhibition in CD4^{+} lymphocyte proliferation (IC_{50}) with Api-3A concentrations of 1, 10, and 100 µM over 24 hours, 48 hours, and 72 hours. For this purpose, carboxyfluorescein

succinimidyl ester dye **(**CFSE) labelled PBMCs were cultured (/well) in complete RPMI 1640 medium (BIO-IDEA, Iran), containing 10% heat-inactivated fetal bovine serum (FBS, Gibco, USA) and 1% penicillin/ streptomycin (Sigma-Aldrich, USA), while it was activated with soluble anti-CD3 and anti-CD28 monoclonal antibodies (mAb) OKT-3 (0.1 µg/ml, Mabtech, Sweden). After 24 hours, the cells were re-stimulated by soluble IL-2 (100 U/ml, Pepro Tech, UK) and simultaneously treated with various concentrations of Api-3A (100, 10, and 1 µM) for different incubation times (24, 48, and 72 hours) under normal culture conditions (37°C, 95% humidified atmosphere with 5% CO_2). Negative control cells were treated with DMSO and RPMI, instead of a drug. Then, DMSO was added to the negative control with equivalent volumes of the maximum doses of Api-3A.

In the following, after collecting cultured cells from the wells, they were stained with anti-human CD4-Percp. Cy5.5 (Biolegend, USA) at 4°C for 20 minutes. Using flow cytometry, a determinate dose capable of inhibiting 50% of proliferation in the best time course was calculated and nominated by Excel and graphs were provided by GraphPad Prism 6.0 (GraphPad Software, San Diego, CA). IC_{50} determination for M-Pre-A has also been accomplished using 0.05, 0.5, and 5 µM concentrations in the elected hour mediated by Api-3A with Excel. The time course for real-time polymerase chain reaction (PCR) experiments was also determined by three times pilot testing of the chosen dose at 24, 48, and 72 hours intervals.

FITC Annexin V-Propidium Iodide staining for apoptosis assay and cytotoxicity effects

The cytotoxicity of Api-3A, Api, and M-Pre-A at the optimum dose was evaluated using FITC annexin V-PI (Abcam, USA) double staining. Briefly, PBMCs (cells/ ml) were stimulated with soluble CD3, CD28, and IL-2, treated with their IC_{50} concentrations, and incubated in 24-well culture plates for the elected time (37°C, 5% CO_2 humidified atmosphere). The cells were then harvested, centrifuged, re-suspended in 200 µl of binding buffer, and stained with FITC Annexin V (5 µl/195 µl). The cells were incubated (10 minutes, in darkroom) and were washed with binding buffer. Once more, the cells were re-suspended in 200 µl of binding buffer and stained with PI (10 µl/190 µl). Cell apoptosis and cytotoxicity effects of Api-3A, Api, and M-Pre-A in comparison with DMSO were then analyzed by a FACS Calibur flow cytometry (Becton Dickenson, Bioscience, USA); using FITC signal detector; Ex=488 nm; Em=530 nm for Annexin V (usually FL1) and PI signal detector for PI (FL3), and analyzed by Cell Quest software (Becton Dickenson, Bioscience, USA).

Flow cytometry

After culturing, stimulating, and treating the CFSE-labeled PBMCs from MS patients with the elected dose of each drug, they were stained with anti-CD4 and anti-CXCR3 surface markers and matched isotype controls

as negative controls according to the Biolegend (USA) recommended method, and finally, proliferation of CD4^{+}, CXCR3^{+} (Th1) cells were evaluated by flow cytometry using a FACS Calibur (BD, USA) and analyzed by Cell Quest software (BD, USA) in the following.

RNA extraction and cDNA synthesis

Total RNA was extracted from the PBMCs using the Yekta tajhiz azma kit (Yekta tajhiz azma Co., Iran) in accordance with the manufacturer's instructions. Concentration and purity of the total extracted RNAs were measured by Nano Drop (Biochrom WPA, UK).

Subsequent to the RNA extractions, 9 µl of total RNA was directly reverse transcribed (RT) in a 20 µl final volume, using BioFact MicroRNA reverse transcription kit (Biofact Co., South Korea) in accordance with the manufacturer's instructions. The 20 µl reactions were incubated in an Applied Biosystems 2720 Thermal Cycler (in a 96-well plate) for 5 minutes at RT , 60 minutes at 50°C, 5 minutes at 95°C and, then held at 4°C. The products were kept at -20°C, for quantitative reverse transcription PCR (qRT-PCR) amplification.

Quantification of *TBX21* and *IFN-γ* gene expressions by quantitative reverse transcription polymerase chain reaction

qRT-PCR analysis of *TBX21 and IFN-γ* was done using the SYBR Green Master Mix protocol (Biofact Co., South Korea) to detect gene expressions under the following cycling conditions: primary denaturation at 95°C for 15 minutes, 40 cycles of amplification consisting of denaturation at 95°C for 20 seconds and extension at 60°C for 60 seconds, followed by melting curve analysis to verify the qRT-PCR product identity. The 10 µl reaction system, contained 1 µl cDNA, 0.25 µl of each pair of oligonucleotide primers, 3.5 µl deoxyribonuclease (DNase)-free and ribonuclease (RNase)-free water, and 5 µl SYBR Green Master Mix. Each run included a non-template control consisting of 1 µl nuclease-free water instead of cDNA template. The primers were designed by Primer Express 2.0 Software (Perkin-Elmer, USA) and used to quantify *T-bet* and *IFN-γ* mRNA:

T-bet:
F: 5´-CAGATGATTGTGCTCCAGTCC-3´
R: 3´-CTGAGTAATCTCGGCATTCTGGTA-5´

IFN-γ:
F: 5´-TGTATTGCTTTGCGTTGGAC-3´
R: 3´-TGACCAGAGCATCCAAAAGA-5´

β-act:
F: 5´-ATAGCACAGCCTGGATAGCAACGTAC-3´
R: 5´-CACCTTCTACAATGAGCTGCGTGTG-3´

Normalization of the gene levels was performed using those of *β-act*, as an internal control, and relative

expression levels were assessed using the method.

Statistics

All results were as mean ± standard error of the mean (SEM). They were analyzed by one-way analysis of variance (ANOVA) and unpaired t tests, followed by Kolmogorov-Smirnov test to determine normal distribution of data. To compare the groups with non-normal distributions, Mann-Whitney and Kruskal-Wallis tests were used. All statistical calculations were achieved using GraphPad Prism version 6.0 (GraphPad Software Inc., USA) and SPSS (Version. 22, IBM, USA). The standard level of significance was $P<0.05$.

Ethics approval

The study was approved by the Ethical Committees of Isfahan University Medical of Science, Isfahan, Iran (IR. MUI.MED.REC.1398.407). Informed consent was obtained from all persons before participating in this study.

Results

Calculation of IC_{50} for apigenin, apigenin-3-acetate, and methyl-prednisolone-acetate

We determined the half maximal inhibitory concentration (IC_{50}) of newly synthesized apigenin-3-acetate with the PBMCs obtained from healthy donors, who were then followed by testing its anti-inflammatory effects on PBMCs isolated from MS samples (Table 1).

Table 1: Characteristics of the study participants

Characteristics	MS patients	Healthy volunteers
Number	5	3
Age (mean) Y	22-46 (34.6)	26-39 (33)
Gender (female/male)	3/2	2/1
EDSS	<3	--

MS; Multiple sclerosis and EDSS; The Expanded Disability Status Scale.

IC_{50} is a measurement method indicating the required amount of a particular drug or other substance to inhibit a given biological process by half. For this purpose, we investigated the IC_{50} of Api-3A and M-Pre-A by evaluating the effects on the proliferation inhibition of CD4$^+$ lymphocytes isolated from PBMCs of healthy donors. In this regard, the cells were treated with logarithmic escalating concentrations of Api-3A (1, 10, and 100 µM) for 24, 48, and 72 hours, respectively. A drastic reduction in the CD4 cell proliferation was observed after 48 hours of treatment, and the IC_{50} was calculated at a concentration of 80 µM Api-3A (Fig.1A). The elected dose was adapted for Api investigation, in order to further comparing Api-3A with Api, as its basic compound. Consequently, IC_{50} determination for M-Pre-A was made after 48 hours of culturing cells in doses of 0.05, 0.5, and 5 µM and it was calculated at a 2.5 µM concentration (Fig.1B). IC_{50} was calculated using Graphpad prism 6. (GraphPad Software Inc., USA).
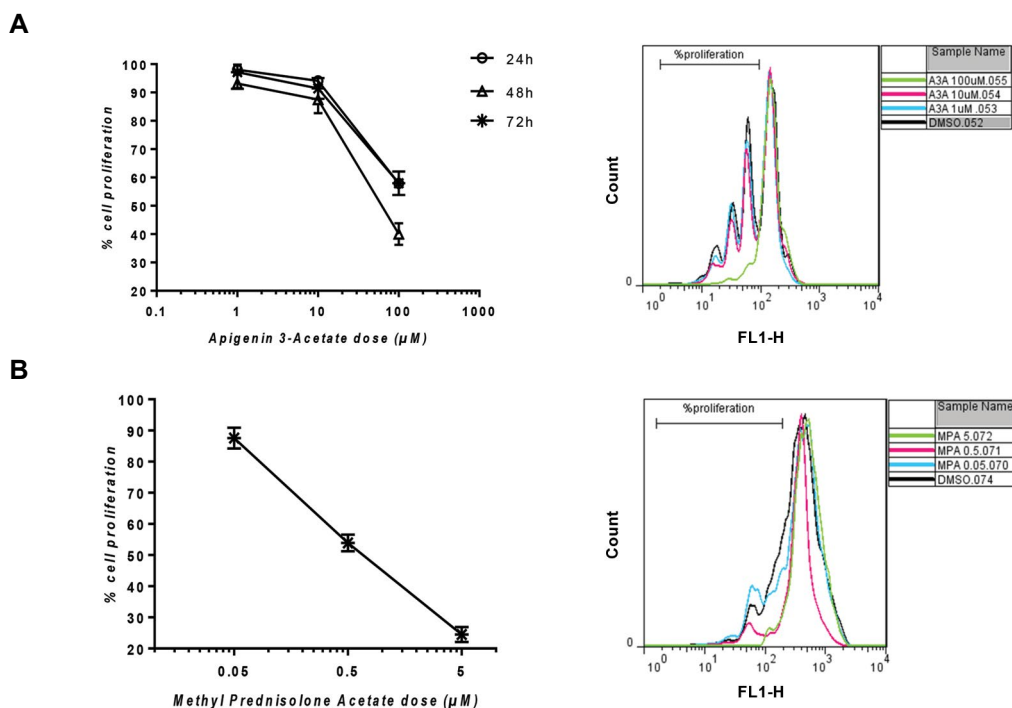

Fig.1: IC_{50} calculation. CFSE-stained PBMCs of the heathy donors were co-cultured with the escalating logarithmic doses of **A.** Api-3A (1, 10, and 100) for 24, 48, and 72 hours and **B.** MPA (0.05, 0.5, and 5) for 48 hours in order to determine the capable dose of inhibiting the CD4 cells proliferation by half using flow cytometry. Data were analyzed by excel to calculate IC_{50}. Graphs were depicted by Prism software. Overlay of graphs were painted by FlowJo 7.6.1 software. 80 µM and 2.5 µM concentrations were calculated IC_{50} doses of Api-3A and MPA following 48 hours of culture, respectively. Data were pooled from three independent healthy people and expressed as means ± SEM. IC_{50}; Half maximal inhibitory concentration, CFSE; Carboxyfluorescein succinimidyl ester, PBMCs; Peripheral blood mononuclear cells, Api-3A; Apigenin-3 Acetate, and MPA; Methyle Prednisolone acetate.

Apigenin 3-acetate showed apoptotic effects on PBMCs-isolated from MS patients compared to apigenin, and methyl-prednisolone-acetate

FITC annexin V-PI double staining was accomplished in order to identify cytotoxicity of Api-3A, Api, and M-Pre-A on PBMCs using flow cytometry. The cells were incubated for 48 hours after treating with their IC_{50} concentrations. We did not observe any significant difference in the early and late apoptosis as well as necrosis of PBMCs, when treating with Api-3A (18.79%), Api (17.72%), and M-Pre-A (16.25%) in comparison with DMSO (17.15%, Fig.2).

A

B

Annexin V
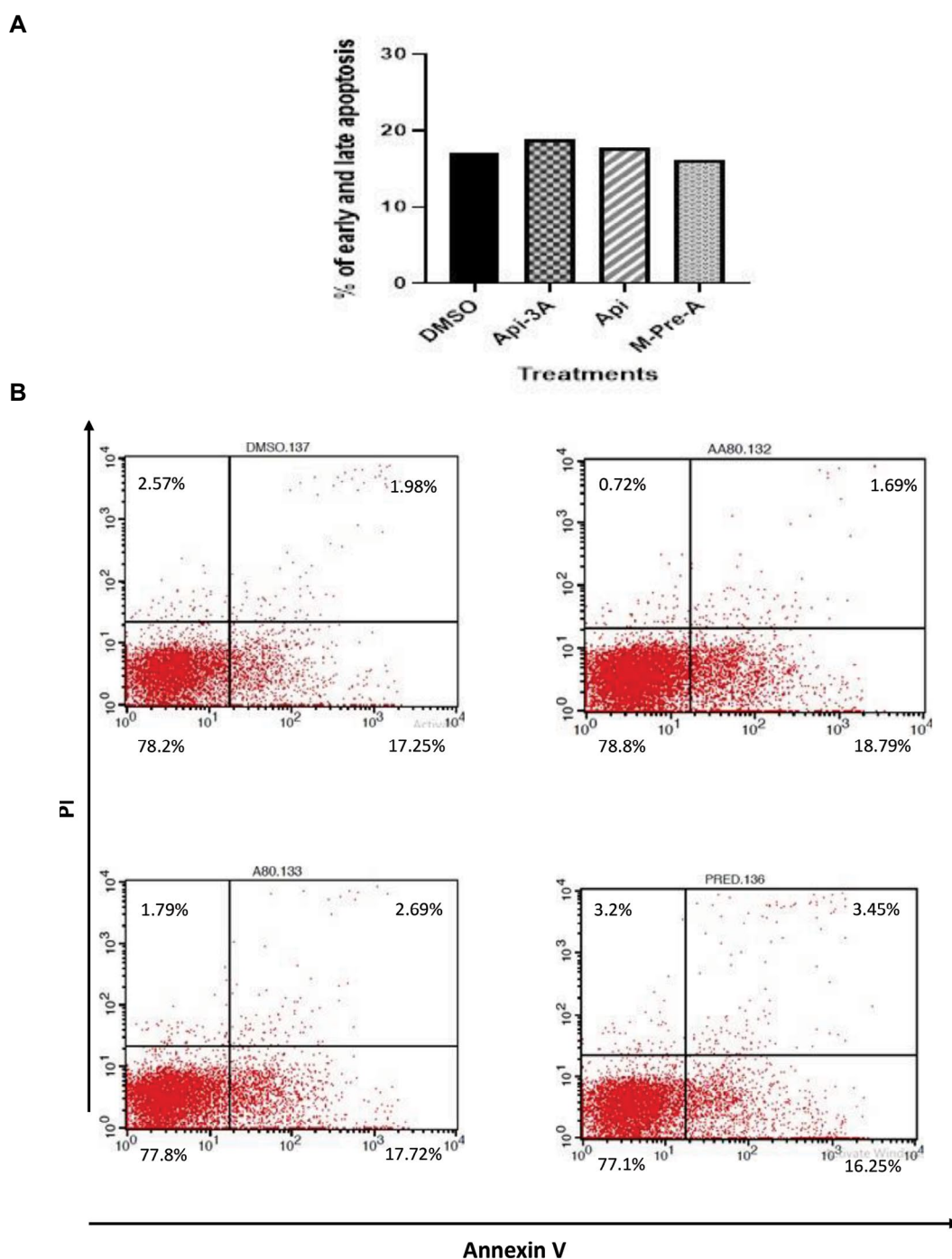
Fig.2: Cytotoxicity effects of Apigenin-3 Acetate (Api-3A), Apigenin (Api), and Methyl-Prednisolone-Acetate (M-Pre-A) on PBMCs. **A.** After 48 hours of treating PBMCs with Api-3A (80 μM), Api (80 μM), and M-Pre-A (2.5 μM), no significant cytotoxicity effects were observed from these three compounds in their IC_{50} doses on viability of PBMCs, comparing to DMSO (18.79, 17.72, 16.25, and 17.25% of late apoptosis and necrosis for Api-3A, Api, M-Pre-A and DMSO, respectively). **B.** The upper right, upper left, lower right and lower left quadrants respectively represent late apoptosis, necrosis, early apoptosis and live cells. PBMCs; Peripheral blood mononuclear cells, DMSO; Dimethyl Sulfoxide, and IC_{50}; Half maximal inhibitory concentration.

Effects of apigenin-3-acetate, apigenin and methyl-prednisolone-acetate on the proliferation of Th1 cells

CFSE-stained PBMCs of newly diagnosed MS patients were treated with Api-3A (80 μM), Api (80 μM) and M-Pre-A (2.5 μM) for 48 hours, followed by staining with anti-CD4 and anti-CXCR3 antibodies (for indicating Th1 cells) and assessement by CFSE flow cytometric analysis. Accordingly, Api-3A diminished Th1 proliferation to approximately 41% (P=0.001), while the proliferation reductions by Api and M-Pre-A were approximately 26% and 33% (P=0.036, and P=0.047) respectively, compared to the proliferation inhibition by DMSO as the control. In this experiment inhibition rate of DMSO was normalized to zero, Fig.3).

Quantitative reverse trascription polymerase chai reaction time coursing

qRT-PCR time coursing of the elected dose in 24, 48 and 72 hours showed that effectiveness and reduction of *T-bet* (Fig.4) and *IFN-γ* (Fig.5) genes were much more significant 48 hours post-treatment.

Apigenin-3 acetate exhibited similar effects on down-regulating T-bet in Th1 cells-isolated from MS patients versus apigenin and methyl-prednisolone-acetate

After co-cultureing Th1 cells isolated from MS patients with 80 μM of Api-3A for 48 hours, we examined expression levels of *T-bet* by qRT-PCR, in order to investigate whether or not Api-3A would affect gene expression in Th1 cells (Fig.4). The same experiments were done with the same doses of Api and M-Pre-A. As it is obvious (Fig.4), Api-3A was able to significantly down-regulate expression of *T-bet* in Th1 cells isolated from MS patients compared to DMSO (P≤0.001). Down-regulation of *T-bet* was also significant for 2.5 μM of M-pre-A and 80 μM of Api for 48 hours of treatment, respectively (P=0.015, and P=0.022). However, no significant difference was observed for the expression of *T-bet* in treating Th1 cells with Api-3A, Api or M-Pre-A. This showed similar potential for Api-3A in reducing *T-bet* expression compared to its Api counterpart and M-Pre-A as standard therapy.

Apigenin-3 acetate significantly down-regulated expression of *IFN-γ* in Th1 cells-isolated from MS Patients

We also assessed effects of Api-3A on the expression of *IFN-γ* as the main pro-inflammatory cytokine produced by Th1 cells using qRT-PCR. As shown in Figure 5, treating Th1 cells-isolated from MS patients with Api-3A for 48 hours resulted in a remarkable reduction of *IFN-γ* expression. Api and M-pre-A were also capable to reduce *IFN-γ* expression into a significant level (P≤0.0001). However, these findings were more outstanding for Api-3A compared to Api and M-Pre-A.
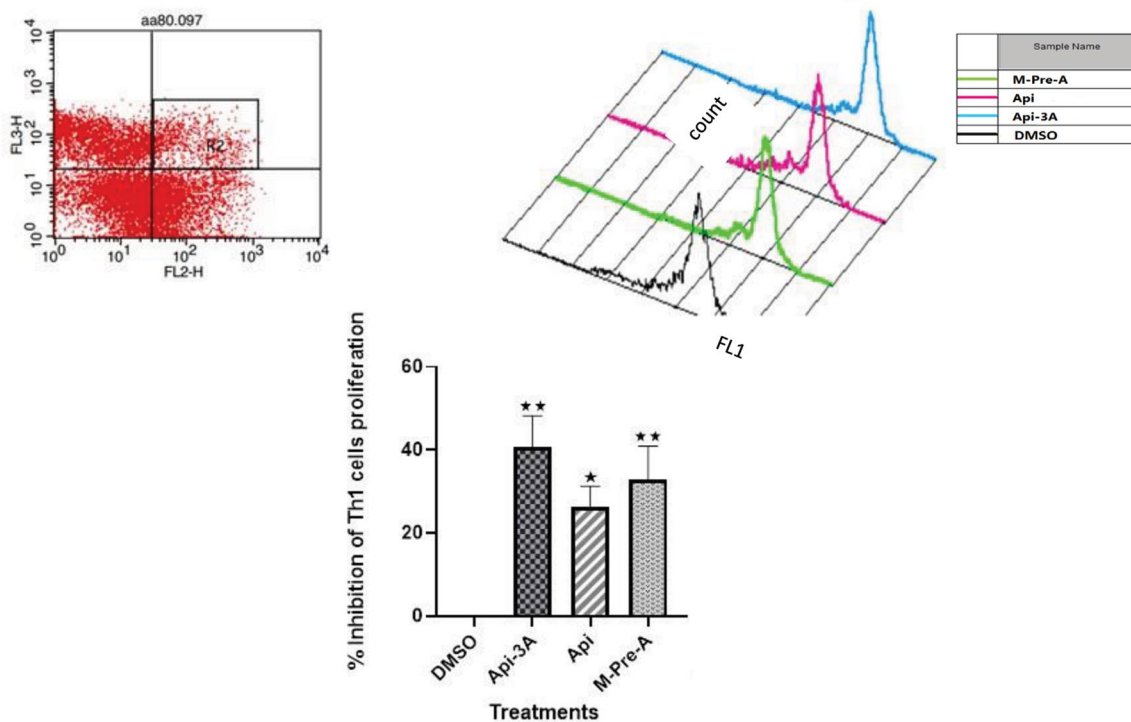
Fig.3: Effects of Apigenin-3 Acetate (Api-3A), Apigenin (Api) and Methyl-Prednisolone-Acetate (M-Pre-A) on the proliferation of Th1 cells in MS patients. After 48 hours of incubating the CFSE-stained PBMCs with or without the selected doses of Api-3A, Api, and M-Pre-A (80 μM, 80 μM and 2.5 μM, respectively), the cells were collected, stained with anti-CD4 and anti-CXCR3 antibodies to detect Th1 cells. They were then analyzed by flow cytometer. In this regard, double positive cells were gated and then proliferation percentage of these gated cells were assessed using Cell Quest software comparing to DMSO. Data, represented from five independent experiments for MS patients and expressed as means ± SEM respectively, indicate statistically significant differences respectively between Api-3, Api and M-Pre-A with DMSO (40.61, 26.21, and 32.82% of Th1 cells proliferation inhibition by Api-3A, Api and M-Pre-A, respectively; with the highest rate for Api-3A; P=0.001, P=0.036, and P=0.047). The FL2-H and FL3-H channels respectively represent the cells labeled with anti-CD4 and anti-CXCR3 markers and the FL1-H channel represents CFSE stained cells. MS; Multiple sclerosis, CFSE; Carboxyfluorescein succinimidyl ester, PBMCs; Peripheral blood mononuclear cells, and DMSO; Dimethyl sulfoxide.
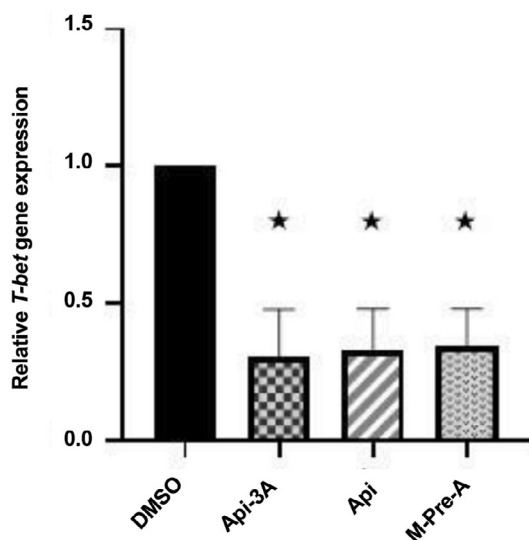
Fig.4: Effects of Apigenin-3 Acetate (Api-3A), Apigenin (Api) and Methyl-Prednisolone-Acetate (M-Pre-A) on *T-bet* gene expressions in PBMCs of MS patients. After 48 hours of incubating PBMCs of MS patients with or without the selected doses of Api-3A, Api, and M-Pre-A, relative gene expression of *T-bet* was measured using qRT-PCR. All components were capable to significantly inhibit expression of *T-bet* gene expression (0.69, 0.67 and 0.65 reduction of *T-bet* gene expression by Api-3A, Api and M-Pre-A, respectively; P=0.015, P=0.019, and P=0.022). X-axis represents the relative *T-bet* gene expression and Y-axis represents the components used for treating cells including DMSO, Api-3A, Api and M-Pre-A. Depicted results are representative of five independent newly diagnosed MS patients and expressed as means ± SEM. PBMCs; Peripheral blood mononuclear cells, MS; Multiple sclerosis, and qRT-PCR; Quantitiative reverse transcriptase polymerase chain reaction.
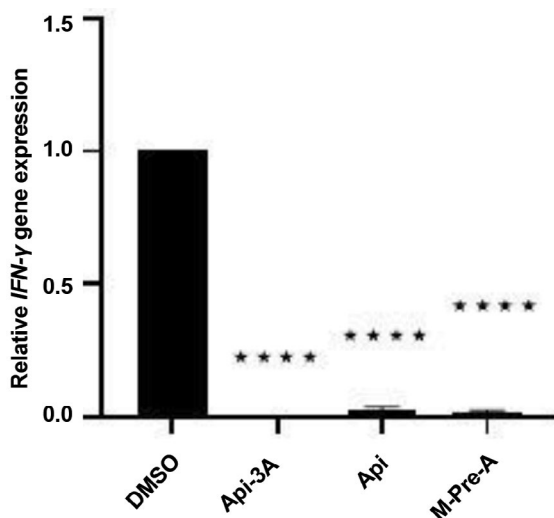
Fig.5: Effects of Apigenin-3 Acetate (Api-3A), Apigenin (Api) and Methyl-Prednisolone-Acetate (M-Pre-A) on *IFN-γ* gene expressions in PBMCs of MS patients. After 48 hours of incubating the PBMCs of MS patients with or without the selected doses of Api-3A, Api, and M-Pre-A, relative gene expression of *IFN-γ* gene was measured using qRT-PCR. All components were strongly capable to inhibit expression of *IFN-γ* gene expression (0.99, 0.97, and 0.98 reduction of *IFN-γ* gene expression by Api-3A, Api and M-Pre-A, respectively; P<0.0001). X-axis represents relative *IFN-γ* gene expression and Y-axis represents the components used for treating cells including DMSO, Api-3A, Api, and M-Pre-A. Depicted results are representative of five independent newly diagnosed MS patients and expressed as means ± SEM. *IFN-γ*; Interferon-γ, PBMCs; Peripheral blood mononuclear cells, MS; Multiple sclerosis, qRT-PCR; Quantitiative reverse transcriptase polymerase chain reaction, and DMSO; Dimethyl sulfoxide.

Discussion

Although several studies showed that Api had anti-

inflammatory and immunomodulatory effects (28, 29), its clinical application in autoimmune diseases such as MS received little attention, most likely due to its low permeability across the BBB. Thus, a strategy to improve Api permeation through the BBB would be of interest and it may introduce a novel candidate for treatment of MS patients. In this regard, in the present study, we developed an acetylated form of Api (Api-3A) and examined its modulatory effects on Th1 cells isolated from MS patients compared to parental Api and methyl prednisolone acetate (M-Pre-A, as a standard option).

The data presented here clearly demonstrated that Api-3A, Api, and M-Pre-A have the potential to inhibit proliferation of Th1 cells in PBMCs of MS patients in a dose-dependent manner. This inhibition in Th1 cell proliferation was much higher when treating them with Api-3A and M-Pre-A rather than Api (40.61, 32.82, and 26.20%, respectively), which points to the same-level or even much higher efficacy of Api-3A compared to the commonly used drug for MS patients (M-Pre-A).

The inhibitory effects of M-pre and Api on PBMC proliferation was proven in the previous study (20). For instance, Leussink et al. (31) concluded that both cell apoptosis and anergy after exposing cells to glucocorticoids (GC), such as Methyl-Prednisolone (M-Pre), could result in inhibition of peripheral blood lymphocyte (PBL) proliferation (especially CD4 T cells), and development of anti-inflammatory conditions. This document is contrary to our results obtained by flow cytometry assessment, which showed inhibition of Th1 proliferation without a significant difference in the apoptosis percentage of PBMCs after treatment with M-Pre-A, compared to the DMSO group. Furthermore, investigating anti-proliferative effect of M-Pre in another autoimmune disease (32, 33) showed that using different doses of M-Pre, at least 48% inhibition of PBMC proliferation was prohibited in Rheumatoid Arthritis (RA) patients. Additiotnally, by compring healthy people and RA patients, it was indicated that size of this prohibitory effect on PBMCs was not significant. This was consistent with our findings indicating that the chosen dose of M-Pre-A resulted in 50 and 33% inhibition of PBMC proliferation, respectively in healthy donors when calculating IC_{50} and in MS patients.

According to the results reported by Namgoong et al. (34) on 34 different structures, Api was one of the most active flavonoids that could greatly inhibit lymphocyte proliferation. According to their investigations, unsaturation at positions 2 and 3, along with the absence of the hydroxyl group at position 3 in these molecules and other flavonoids, could possibly be very important in their inhibitory effect (35). In our newly synthesized compound, these properties were still retained in proportion to the basic Api. According to these results, Lee et al. (16) concluded that methylation and addition of a lipophilic compound at position 4' of the Api compound, as well as the presence of this translocation at positions 7 and 4, increased Api inhibitory activity on cell proliferation.

As mentioned earlier, the Api-acetate compound contains lipophilic acetate at positions 4′, 5′, and 7, which may be a reason for the better inhibitory effect of Api-acetate on Th1 cell proliferation compared to the basic Api.

On the other hand, in relation to the anti-proliferative efficacy of Api, Xu et al. (36) previously proved that Api caused apoptosis of recurrently activated T cells through regulation of the NF-κB signaling pathway, which has been well known for its importance in survival, proliferation, and T-cell effector functions. Its activation is the common denominator in the anti-apoptotic pathways (37). Eventhough the elected dose in our study was much higher than the under-investigated dose in their study (80 μM versus 12.5 μM), we did not observe any significant difference in the apoptosis percentage of the cells treated with Api compared to the cells treated with DMSO. This represented that the elected dose of Api in our study had appropriate efficacy and yet the lowest apoptosis and cell death rate.

A study by Verbeek et al. (38) showed that two components (Api and luteolin) were more effective in inhibiting proliferation of human and mouse T cells activated against myelin antigens than other flavonoids in the flavanone and flavonol subtypes. This study investigated inhibition of all T lymphocytes and found a significant inhibition of these lymphocytes in the exposure of Api, whereas, in our study, the Th1 subgroup was studied solely indicating lower inhibition of proliferation rate. In the Verbeek study (38), Api could have the inhibitory effect on T lymphocyte proliferation possibly by other members of the lymphocyte family, such as TCD8+ and other T helper cells, including Th17 cells.

Furthermore, the regulatory effect of Api in IFN-γ production has been proven earlier (35, 36). Reduction of the Th1 cells transcription factor (T-bet) and their indicator cytokine (IFN-γ) by Api-3A, Api, and M-Pre-A were also observed in our study. Additionally, it has been proven in previous studies that GCs and M-Pre were responsible for shifting the Th1-dominant cellular responses in autoimmune disease toward Th2-dominant responses (specific efficacy on Th1 cells cytokines and not Th2 cells cytokines). It has been hypothesized that selective effects of M-Pre on Th1 cells might be due to different numbers of GC receptors or different GC receptor affinities in these Th1 and Th2 cells (36-38).

Moreover, more potent inhibitory efficacy of Api-3A, Api and M-Pre-A on *IFN-γ* gene expression rather a *T-bet* gene expression as well as T-cell proliferation can be vindicated by the wide-range production of IFN-γ via different kinds of inflammatory cells and pathways in the inflammatory conditions. It can be proposed that multilateral efficacy of these drugs on different immunological cells (including monocytes, macrophages, Th1 cells, T-reg cells and Th17 cells) would result in the significant reduction of IFN-γ. In an interesting study performed by Momcilović et al. (39), it was found that severe decrease of IFN-γ in the presence of methylprednisolone, toward zero by antibodies against

this cytokine, would increase IL-17 in the presence or absence of methyl Prednisolone. This means that IFN-γ acted as a negative regulator for IL-17, even in very small amounts, and complete exclusion of its inhibitory affected IL-17. Moreover, by noticing our investigations on *IFN-γ* gene expression in our study, no significant decrease in the expression of this gene was observed in any of the three-drug combinations; thus, none of them was able to bring it down to zero.

Besides the decreased expression of α4-chain observed in peripheral DCs and T cells (38, 39), and reduction of monocyte adherence to vascular endothelium due to the down-regulations of vascular cell adhesion molecule-1 (VCAM-1), intra-cellular adhesion molecule-1 (ICAM-1), and E-selectin upon Api treatment as well as reducing the ability of cells for crossing the BBB, as a result (38, 39). Adding three acetate groups in positions 4, 5, and 7 to the recently produced combination (Api-3A) was investigated in our study, whose increased lipophilicity would be able to increasingly elevate its potential for passing the BBB and more specified effects.

But before any actions, it was necessary to investigate whether or not these recently added acetate groups led to more anti-inflammatory and anti-proliferative effects compared to the basic compound. The results were acceptable. Additionally, significant difference in the inhibitory effects of Api-3A and its parent compound was only observed by evaluating proliferation inhibition; in other evaluating processes, we also faced (although slightly and not significantly) this difference.

Depite realising that the observed efficacy of Api-3A was not solely due to its parent combination and these three acetate groups also played a part in inserting the efficacy, we had to make sure about their conversion in the body (whether they converted to their basic compound or the other unique derivatives) and subsequently insert their efficacies. Perceiving this fact required more *in vivo* studies in line with our study, which was pioneer for the *in vitro* phase in the long journey of investigating Api-3A, as a newly synthesized compound.

The limitation of this study was few number of under-investigated MS patients; it will be better generalized and concluded, if it is more extensively investigated, in the future. On the other hand, in contrast to a 2004 published study demonstrating any beneficial effects on murine EAE by oral flavonoids, including Api and quercetin (20), an updated study by Ginwala indicated a prominent decrease in disease severity and some anti-inflammatory reactions in the treated animals by Api (40). This could be encouraged to continue studying this basic compound and its novel design, Api-3A, in animal models of EAE *in vivo* as well as the patients with MS, in the future, to ensure that its passage through the BBB is improved, compared to the basic compound without acetylation.

A notable point leading to the confirmation of our results was that we investigated the multilateral anti-

immunological effects of Api-3A and its parent compound, Api, in the same dose. So that we could greatly understand their different rates of efficacy. Api-3A had a significant efficacy on Th1 cell proliferation, *T-bet* and *IFN-* γ gene expressions, while there was neither significant increase in apoptosis (either early or late apoptosis) nor necrosis compared to the control test. That would increase our reliance on our promising compound. This result would be confirmation of the fact that Api-3A exerted no toxicity or deleterious side effects.

Conclusion

The importance of controlling Th1 cells, as the initiator cells responsible for progression of many treatment steps in MS patients, is well understood. In addition, controlling activity of cells affecting the main site of inflammation, especially brain tissue, would make us able to particularly prevent the general suppression of the immune system and its destructive effects, such as increased susceptibility to a variety of diseases. Therefore, considering the modulatory and anti-inflammatory properties of Api-3A on Th1 cells and the higher possibility of this compound crossing the BBB than the basal compound can make this compound a useful choice in treating MS patients.

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Authors' Contributions

N.K., S.M.Gh., M.R., L.A., N.E.; Carried out the study concept, design and writing of the manuscript. N.K., M.R., L.A.; Carried out the experimental works. N.K., M.R., F.A., A.P., N.E., L.A.; Carried out data collection. N.K., S.M.G., M.R., L.A., R.H., N.E.; Carried out analysis of data and interpretation. All authors read and approved the final manuscript.

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