

## The Flavonoid, Quercetin, Inhibits HIV-1 Infection in Normal Peripheral Blood Mononuclear Cells

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**Abstract: Problem statement:** Flavonoids and related polyphenols, have been known to possess cardioprotective, anti-tumor, anti-inflammatory, anti-allergic and anti-viral activities. Previous studies have shown that flavonoid, quercetin significantly downregulates expression of pro-inflammatory cytokines in cultured cells via modulation of NF $\kappa$ B and p38MAPK signaling pathways. In the current study, we hypothesize that quercetin exerts anti-HIV activity by differential modulation of pro-and anti-inflammatory cytokine expression in normal PBMCs. **Approach:** Cultures of PBMC received either different concentrations of quercetin (1-50  $\mu$ M) or media alone. The anti-HIV effects of quercetin was evaluated in an *in vitro* infection model by quantitaing the HIV-1 LTR gene suppression by quantitative Real Time PCR, HIV-1 p24 antigen production by ELISA and viral infectivity by MAGI cell assay. **Results:** Our results showed that quercetin significantly downregulates p24 antigen production, LTR gene expression and viral infectivity in a dose dependent manner (5-50  $\mu$ M) as compared to HIV infected untreated control PBMCs. Further, we reported that quercetin significantly downregulated the expression of the pro-inflammatory cytokine, TNF- $\alpha$  with concomitant upregulation of anti-inflammatory cytokine IL-13 as determined by measurement of gene expression and protein production. A higher level of IL-13 is known to inhibit TNF- $\alpha$  production and also HIV-1 infection. Thus, differential modulation of pro-and anti-inflammatory cytokines could be one of the possible mechanisms for the anti-HIV effects of quercetin. **Conclusion:** Better understanding of the mechanisms underlying the anti-HIV effects of quercetin may help to develop a new neutraceutical agent useful in the treatment of HIV-1 infected subjects in conjunction with conventional therapeutic regimens.

**Key words:** Quercetin, anti-HIV, inflammatory cytokines

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### INTRODUCTION

Discovery of effective and less toxic antiviral agents is a high priority in the search for more effective therapies against HIV. Flavonoids comprise a large class of low molecular weight polyphenolic compounds ubiquitously distributed in food plants. Flavonoids are enriched in certain vegetables, fruits, seeds and beverages (e.g., tea and wine) and are regarded as a class of semi essential nutrients for humans. Dietary intake of flavonoids is highly variable, with estimation ranging from 23<sup>[1]</sup> to more than 500 mg day<sup>-1</sup><sup>[2]</sup>. Flavonoids display a diverse array of biological effects including antioxidant, anti-inflammatory and anti-tumor activities with no apparent toxic side effects on cells<sup>[3-6]</sup>.

These beneficial effects of flavonoids have been attributed to their antioxidant and anti-inflammatory properties.

Quercetin, one of the polyphenolic flavonoids, is known to have effects on a variety of inflammatory processes and immune functions<sup>[7-14]</sup>. Tumor Necrosis Factor (TNF)- $\alpha$  is a major pro-inflammatory cytokine that regulates the growth, proliferation, differentiation and viability of activated leukocytes. Previous studies have shown that quercetin suppresses TNF- $\alpha$  induced IL-8 and MCP-1 expression in cultured human synovial cells<sup>[15]</sup>. Likewise, several studies have demonstrated the inhibitory effects of quercetin on the expression of inflammatory cytokines by cultured cells<sup>[16,17]</sup>. Recent study reported in HMC-1 human mast cell line has

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indicated that quercetin effects on inflammatory cytokines are mediated through the modulation of NF- $\kappa$ B and p38 MAPK signaling pathways<sup>[18]</sup>. Previously, we have also demonstrated the inhibitory effects of quercetin on TNF- $\alpha$  production in normal Peripheral Blood Mononuclear Cells (PBMCs) and this effect is mediated by downregulation of NF $\kappa$ B and I $\kappa$ B<sup>[19]</sup>. Moreover, our laboratory has also shown that quercetin inhibits HIV-1 entry co-receptor CCR5 in normal PBMC in a dose dependant manner<sup>[20]</sup>.

Although, earlier reports showed the antiviral activity of quercetin, the pharmacological action of quercetin on inflammatory cytokines and the underlying mechanisms are not sufficiently investigated. Therefore, we hypothesize that quercetin exerts anti-HIV activity by differential modulation of pro-and anti-inflammatory cytokine expression in normal PBMCs. In the current study, we report the inhibition of HIV-1 replication and virus infectivity by quercetin in normal PBMC as evaluated by HIV-1 p24 antigen production, HIV-1 LTR gene suppression and MAGI cell assay and the mechanism may involve suppression of pro-inflammatory cytokine (TNF- $\alpha$ ), with a reciprocal upregulation of anti-inflammatory cytokine, IL-13.

## MATERIALS AND METHODS

**Cell culture:** Normal Peripheral Blood Mononuclear Cells (PBMC) were isolated by density gradient centrifugation process from HIV-1, HIV-2 and hepatitis B seronegative donor leukopacks as described earlier by us<sup>[19]</sup>. PBMC ( $3 \times 10^6$  cells mL<sup>-1</sup>) were cultured in RPMI 1640 medium (Invitrogen, Grand Island, NY) containing 10% fetal bovine serum, penicillin 100 U mL<sup>-1</sup>, streptomycin 100 mg mL<sup>-1</sup> and 2 mM L-Glutamine (Gibco-BRL, Gaithersburg, MD) at 37°C in 5% CO<sub>2</sub> incubator.

**HIV-1 IIIB infection:** HIV-1 IIIB, CXCR4-tropic, X4 virus strain (Cat #398) was procured through NIH AIDS Research and Reference Reagent Program. PBMCs ( $3 \times 10^6$  cells mL<sup>-1</sup>) were infected with HIV-1 IIIB virus at a concentration of  $10^{3.0}$  TCID<sub>50</sub> mL<sup>-1</sup> cells for 3h as described by the supplier. Cells were washed and cultured in the presence of quercetin (Sigma-Aldrich, St. Louis, MO) at concentrations ranging between 0-50  $\mu$ M for 7 days at 37°C in 5% CO<sub>2</sub> incubator. These concentrations selected for the *in vitro* studies are similar to levels found in plasma of human subjects that have ingested 150 or 300 mg of quercetin<sup>[21]</sup>. The culture supernatants were harvested and stored at -70°C until further analysis for HIV-1 p24 antigen protein production by commercially available

ELISA kit as per manufacturer's instructions (Zeptomatrix, Buffalo, NY) and cell pellets were used for RNA extraction and analyzed by qRT-PCR for LTR gene expression.

**Quantitative Real Time PCR (qRT-PCR):** RNA from cell pellets was extracted using RNeasy mini kit (Qiagen, GmbH, Germany) followed by cDNA synthesis using high capacity reverse transcriptase cDNA kit (Applied Bio systems, Foster City, CA, USA) to perform qRT-PCR. Relative abundance of each mRNA species was assessed using SYBR green master mix from Stratagene using Mx3000P instrument which detects and plots the increase in fluorescence versus PCR cycle number to produce a continuous measure of PCR amplification. To provide precise quantification of initial target in each PCR reaction, the amplification plot is examined at a point during the early log phase of product accumulation. This is accomplished by assigning a fluorescence threshold above background and determining the time point at which each sample's amplification plot reaches threshold (defined as the threshold cycle number or C<sub>T</sub>). Differences in threshold cycle number are used to identify the relative amount of PCR target contained within each tube<sup>[22]</sup>. Relative mRNA species expression was quantitated and expressed as transcript accumulation index (TAI =  $2^{-\Delta\Delta CT}$ ), calculated using the comparative C<sub>T</sub> method<sup>[23]</sup>.

**MAGI Assay to measure HIV-1 infectivity:** To observe the effects of quercetin on HIV-1 infectivity in PBMC, we used the MAGI cell assay. This assay was intended to quantitatively measure virus infectivity<sup>[24]</sup>. The MAGI (multinuclear activation of a galactosidase indicator) cells are HeLa derived cells stably transfected with CD4 and a reporter construct consisting of the  $\beta$ -galactosidase gene (which is modified to localize to the nucleus) driven by a truncated HIV-1 LTR. Expression of the  $\beta$ -galactosidase gene is Tat-dependent such that an incoming virus must produce active Tat protein to drive expression of the reporter. MAGI cells ( $4 \times 10^4$  cells well<sup>-1</sup>) were plated in a 24 well plate and were treated in duplicate with 100  $\mu$ L cell suspension of PBMC's that were infected with HIV-1 IIIB virus and subsequently treated with quercetin (5-50  $\mu$ M). A total of 200  $\mu$ L of DMEM supplemented with 10% FCS, 100 U mL<sup>-1</sup> penicillin, 100  $\mu$ g mL<sup>-1</sup> streptomycin, 0.25  $\mu$ g mL<sup>-1</sup> fungizone and 300  $\mu$ g mL<sup>-1</sup> glutamine containing DEAE-Dextran at a concentration of 15mg mL<sup>-1</sup> was added to the MAGI cells and infected cells were incubated for 3 days at 37°C, 5% CO<sub>2</sub>. Cells were fixed and stained with 5-

bromo-4-chloro-3-indolyl-D-galactopyranoside (X-Gal) and blue cells were counted as infected cells.

**Stimulated cytokine gene expression and protein secretion:** PBMCs ( $3 \times 10^6$  cells  $\text{mL}^{-1}$ ) were stimulated with Phorbol Myristate Acetate (PMA) ( $5 \text{ ng mL}^{-1}$ ) and  $\text{Ca}^{2+}$  ionophore ( $50 \text{ ng mL}^{-1}$ ) for 24-96 h and simultaneously treated with quercetin (1-50  $\mu\text{M}$ ). Pro-inflammatory cytokine, TNF- $\alpha$  and anti-inflammatory cytokine, IL-13 gene expression was studied by qRT-PCR after 24 h of stimulation, whereas protein secretion was determined in cell culture supernatants by ELISA after 96 h of stimulation. TNF- $\alpha$  and IL-13 protein secretion was quantitated using a commercially available ELISA kit obtained from Bio Source International (Camarillo, CA) as per manufacturer's instructions.

**Statistics:** Experiments were performed at least three times and the values obtained were averaged. Data are represented as Mean  $\pm$  SE. Comparisons between two groups were conducted using Student's paired t-test. Differences were considered significant at  $p \leq 0.05$ , with two-tailed test. Data analysis was performed with the Statistical Program, Graphpad prism software (La Jolla, CA).

## RESULTS

**Quercetin inhibits HIV-1 LTR gene expression in PBMCs:** In the present study, the anti-HIV activity of flavonoid, quercetin was evaluated using an *in vitro* infection model in PBMCs. Data showed in Fig. 1 shows the effect of quercetin on HIV-1 LTR gene suppression in PBMC infected with HIV-1. PBMC ( $3 \times 10^6$  cells  $\text{mL}^{-1}$ ) were cultured with and without quercetin at different concentrations for 7 days after the HIV-1 IIIB infection. Cells were harvested, RNA was extracted and reverse transcribed to obtain cDNA which was used further to quantitate LTR gene expression by qRT-PCR. Results obtained were transformed to obtain Transcript Accumulation Index (TAI) and plotted as % LTR expression with quercetin treatment. Our data shows that quercetin significantly downregulated LTR gene expression in a dose dependent manner at concentrations of 5  $\mu\text{M}$  (17%,  $p = 0.069$ ), 10  $\mu\text{M}$  (40%,  $p = 0.014$ ), 25  $\mu\text{M}$  (72%,  $p = 0.0005$ ) and 50  $\mu\text{M}$  (88%,  $p = 0.000006$ ) compared to HIV infected untreated control cultures (100%) (Fig. 1a).

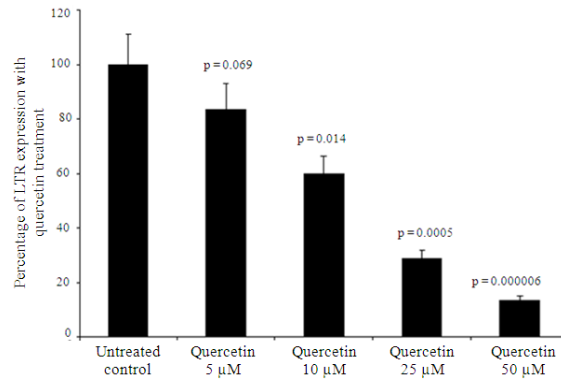


Fig. 1a: Effect of quercetin on LTR gene suppression in PBMC infected with HIV-1 IIIB

**Note:** Effect of quercetin on LTR gene suppression in PBMC infected with HIV-1. PBMC ( $3 \times 10^6$  cells  $\text{mL}^{-1}$ ) infected with HIV-1 IIIB were treated with quercetin 0-50  $\mu\text{M}$  for 7 days. Cells were harvested, RNA was extracted and reverse transcribed followed by quantitative real time PCR for LTR gene. Relative expression of mRNA species was calculated using the comparative  $C_T$  method. The data represents the means  $\pm$  SE of 3 independent experiments. Statistical significance was calculated by students "t" test

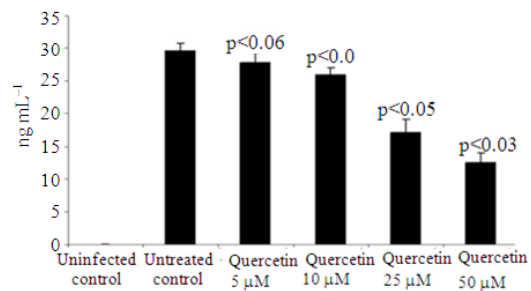


Fig. 1b: Effect of quercetin on p24 production in PBMC infected with HIV-1 IIIB

**Note:** Effect of quercetin on p24 production in PBMC infected with HIV-1. PBMC ( $3 \times 10^6$  cells  $\text{mL}^{-1}$ ) infected with HIV-1 IIIB were treated with quercetin 0-50  $\mu\text{M}$  for 7 days. After incubation, supernatants were collected and analyzed for HIV-1 p24 antigen levels by ELISA. The data represents the means  $\pm$  SE of 3 independent experiments. Statistical significance was calculated by students "t" test

**Quercetin inhibits HIV-1 p24 antigen production in PBMC:** Further we performed measurement of HIV-1 p24 protein levels from cell culture supernatants by ELISA. Data showed in Fig. 1b shows HIV-1 p24 antigen levels in culture supernatants of PBMC infected with HIV-1 and cultured with or without quercetin at 5-50  $\mu\text{M}$  concentrations. A dose dependent decrease in HIV-1 p24 protein production was observed upon treatment with quercetin at varying concentration 5  $\mu\text{M}$  (28 ng  $\text{mL}^{-1}$ ), 10  $\mu\text{M}$  (25.7 ng  $\text{mL}^{-1}$ ;  $p < 0.004$ ), 25  $\mu\text{M}$  (16.9 ng  $\text{mL}^{-1}$ ;  $p < 0.05$ ) and 50  $\mu\text{M}$  (12.1 ng  $\text{mL}^{-1}$ ;  $p < 0.03$ ) compared to HIV infected untreated control cultures (29.8 ng  $\text{mL}^{-1}$ ).

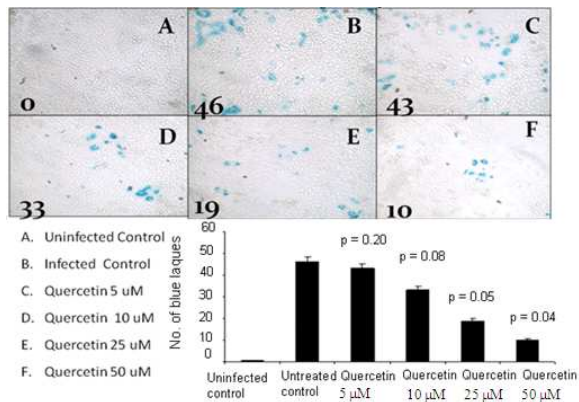


Fig. 2: Effect of quercetin on HIV-1 IIIB virus infectivity in PBMC by MAGI cell assay

**Note:** Effect of quercetin on MAGI cell assay. MAGI cells ( $4 \times 10^4$  cells well<sup>-1</sup>) were treated with 100 µL cell suspension of PBMCs that were infected with HIV-1 IIIB virus and subsequently treated with quercetin (5-50 µM) and incubated for 3 days at 37°C, 5% CO<sub>2</sub>. Cells were fixed and stained with 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-Gal) and blue cells were counted as infected cells. The data represents the means ± SE of 3 independent experiments. Statistical significance was calculated by students "t" test

**Quercetin inhibits HIV-1 virus infectivity in PBMC by MAGI cell assay:** We also measured HIV-1 infectivity using the MAGI assay that allows detection of HIV-1 after a single viral replication cycle in supernatant of quercetin treated infected PBMC cultures. Our results showed a decrease in number of HIV-1 infected cells as represented by the decreased number of blue cells with increasing concentrations of quercetin. Percentage of HIV-1 infected cells at 5, 10, 25 and 50 µM concentrations of quercetin were 43% (p = 0.2, NS), 33% (p = 0.08, NS), 19% (p = 0.05) and 10% (p = 0.04) respectively compared to the 46% in the HIV-1 infected untreated control (Fig. 2).

**Differential modulation of pro-and anti-inflammatory cytokine by quercetin in PBMC:** Previously, we have reported that quercetin inhibits expression of pro-inflammatory cytokine TNF-α in normal PBMC via modulation of the NFκB system<sup>[19]</sup>. In the current study, we investigated whether quercetin exerts differential effect on expression of pro- and anti-inflammatory cytokines in normal PBMC. PBMCs were stimulated with PMA and calcium ionophore and simultaneously treated with quercetin (1-50 µM) for 24-96 h. After 24 h incubation, cells were harvested to determine pro-inflammatory cytokine, TNF-α and anti-inflammatory cytokine, IL-13 gene expression by qRT-PCR. Result indicates that quercetin downregulated TNF-α gene expression by PBMCs in a dose dependent manner in the concentration range of 10-50 µM (Fig. 3a).

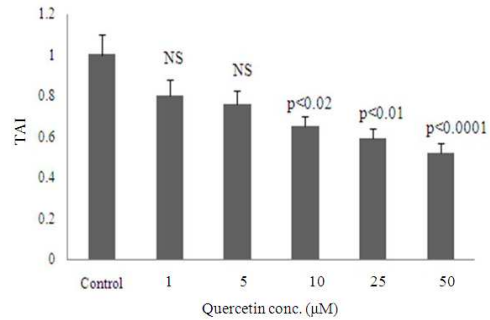


Fig. 3a: Effect of quercetin on pro-inflammatory cytokine, TNF-α gene expression

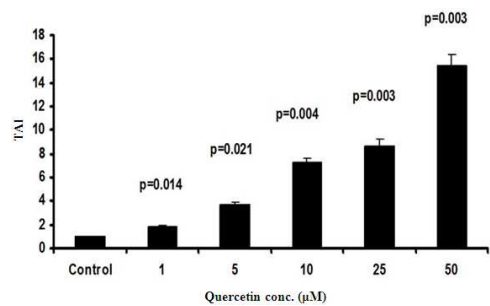


Fig. 3b: Effect of quercetin on anti-inflammatory cytokine IL-13 gene expression

**Note:** Effect of quercetin on stimulated TNF-α and IL-13 gene expression in PBMC. PBMCs ( $3 \times 10^6$  cells mL<sup>-1</sup>) were stimulated with PMA (5 ng mL<sup>-1</sup>) and Ca<sup>2+</sup> ionophore (50 ng mL<sup>-1</sup>) for 24 h and simultaneously treated with quercetin (1-50 µM). Cells were harvested, RNA was extracted and reverse transcribed followed by qRT-PCR for TNF-α (Fig. 3a) and IL-13 (Fig. 3b) gene expression. Relative expression of mRNA species (TAI) was calculated using the comparative C<sub>T</sub> method. The data represents the means ± SE of 3 independent experiments. Statistical significance was calculated by students "t" test

On the contrary, IL-13 gene expression was significantly upregulated in the quercetin (1-50 µM) treated cultures compared to untreated cultures (Fig. 3b).

To substantiate gene expression results, we measured cytokine protein secretion from culture supernatants stimulated with PMA and treated with quercetin (1-50 µM) for 96h. Data showed in Fig. 4a shows that quercetin at 5 µM (200 pg mL<sup>-1</sup>, p = 0.026) and 10 µM (189pg mL<sup>-1</sup>, p = 0.016) significantly inhibited secretion of TNF-α cytokine, while 25 µM and 50 µM quercetin treatment resulted in complete inhibition of TNF-α secretion compared to untreated cultures. Interestingly, IL-13 protein secretion was significantly upregulated (p < 0.001) in a dose dependent manner in the concentration range of 1-50 µM of quercetin (Fig. 4b).

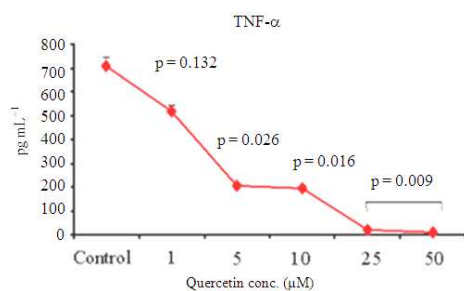


Fig. 4a: Quercetin downregulates TNF- $\alpha$  protein secretion in PBMC

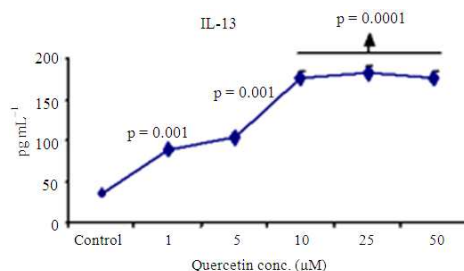


Fig. 4b: Quercetin upregulates IL-13 protein secretion in PBMC

**Note:** Effect of quercetin on TNF- $\alpha$  and IL-13 protein secretion in PBMC. PBMCs ( $3 \times 10^6$  cells mL<sup>-1</sup>) were stimulated with PMA ( $5$  ng mL<sup>-1</sup>) and Ca<sup>2+</sup> ionophore ( $50$  ng mL<sup>-1</sup>) for 96 h and simultaneously treated with quercetin ( $1$ - $50$   $\mu$ M). After incubation, culture supernatants were collected and analyzed by ELISA for TNF- $\alpha$  (Fig. 4a) and IL-13 (Fig. 4b) protein levels. The data represents the means  $\pm$  SE of 3 independent experiments. Statistical significance was calculated by students "t" test

## DISCUSSION

Flavonoids represent a group of phytochemicals that are known to offer significant health benefits to humans. Flavonoids have been recognized to exert antibacterial and antiviral activity, anti-inflammatory, antiangiogenic and antiallergic effects, analgesic, hepatoprotective, cytostatic, apoptotic, estrogenic and antiestrogenic properties<sup>[3-14]</sup>. Quercetin is the most ubiquitous flavonoid in nature that exhibits a wide range of biological activities arising mainly from its antioxidant property and ability to modulate several enzymes or cell receptors. The current study shows that quercetin exerts significant anti-HIV activity by inhibiting HIV replication and reducing virus infectivity in PBMCs as measured by HIV-LTR gene expression, p24 antigen production and MAGI cell assay. Further, we also report that quercetin downregulates the

expression of pro-inflammatory cytokine, TNF- $\alpha$  with a concomitant upregulation of anti-inflammatory cytokine, IL13. The differential modulations of pro-and anti-inflammatory cytokines play a major role in the infection and pathogenesis of HIV disease.

Previous studies from our laboratory demonstrate that quercetin differentially regulates gamma interferon (IFN- $\gamma$ ) and IL-4 expression in PBMCs<sup>[25]</sup>. Further, we have also shown that flavonoids from grape seed extracts downregulates the expression of HIV-1 entry co-receptors, CCR2b, CCR3 and CCR5 in normal PBMCs<sup>[26]</sup>, which suggests that flavonoids can interfere with the binding of the virus to the cell receptor and, thus can prevent HIV entry into the normal lymphocyte. In addition, the findings from this study support data demonstrating the ability of quercetin to modulate a variety of inflammatory cytokines and their role in HIV infection. HIV-1 infection and virus replication are regulated by a complex network of cytokines secreted by a variety of cells. TNF- $\alpha$  is one of the major pro-inflammatory cytokines that significantly upregulate HIV-1 production in Monocytes-Derived Macrophages (MDM) through the activation of NF- $\kappa$ B signaling<sup>[27]</sup>. On the other hand, IL-13 exerts anti-inflammatory activity by inhibiting production of pro-inflammatory cytokines such as TNF- $\alpha$  and inhibits HIV-1 production<sup>[28]</sup>. Previous studies have reported that IL-13 acts on macrophages before and after HIV-1 infection and blocks the completion of reverse transcription, inhibits virus production and reduces the infectivity of the progeny virions<sup>[29]</sup>. Further, IL-13 is also known to suppress TNF- $\alpha$  induced activation of NF- $\kappa$ B, Activation Protein-1 and apoptosis, which describes its immunosuppressive and anti-inflammatory effects<sup>[30]</sup>. Our results showing significant downregulation of TNF- $\alpha$  by quercetin could probably be attributed to increased levels of IL-13. These findings suggest that in addition to downregulation of HIV entry co-receptors by quercetin, differential modulation of pro-and anti-inflammatory cytokines expression could be the potential mechanisms for the anti-HIV activity of quercetin.

## CONCLUSION

Quercetin displays a diverse array of biological effects, which is mainly attributed to its antioxidant and anti-inflammatory properties. Elucidating the molecular mechanisms of quercetin-induced anti-HIV effects may help to develop novel flavonoid-based nutraceutical agents useful in the treatment of HIV infection. Furthermore, these studies may provide new

information and impetus for developing a potential clinical trial of quercetin as an adjunct nutritional supplement, along with the existing antiviral therapeutic regimen, in the treatment of HIV infection.

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