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Original Research Article

Modulatory Effect of Phytoestrogens and Curcumin on Induction of Annexin 1 in Human Peripheral Blood Mononuclear Cells and their Inhibitory Effect on Secretory Phospholipase A₂

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Abstract

Purpose: To investigate the modulatory effects of phytoestrogens (coumestrol, daidzein and genistein) and curcumin on the induction and secretion of annexin-1 (ANXA-1) in human peripheral blood mononuclear cells (PBMCs) under inflammatory and non-inflammatory conditions, as well as their effect on the activity of phospholipase A₂-V (sPLA₂-V).

Methods: The modulatory effects of phytoestrogens and curcumin on the induction of ANXA1 were investigated via sandwich ELISA method, while their effects on the activity of sPLA₂-V were determined by photometric assays. Besides, the cell viability of these compounds was determined by standard trypan blue exclusion method using PBMCs.

Results: The results indicate a significant increase ($p < 0.05$) in the total content of ANXA1, particularly by coumestrol ($p < 0.01$), in both inflammatory and non-inflammatory cells. Besides, the compounds also exhibited a dose-dependent inhibition of sPLA₂-V activity; however, among these compounds, curcumin and genistein were the strongest inhibitors with an IC₅₀ value of $11.1 \pm 0.3 \mu\text{M}$ and $13.6 \pm 0.6 \mu\text{M}$ respectively.

Conclusion: The investigated compounds have a potential to induce synthesis and secretion of ANXA1 as well as inhibitory activity of sPLA₂-V, suggesting their inhibitory role in phospholipid metabolism and inflammation.

Keywords: Inflammation, Annexin-1, Phospholipase A₂, Phytoestrogens, Curcumin.

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INTRODUCTION

Inflammation, a widely addressed issue with clinical implications, has attracted a lot of researchers in multiple disciplines. Mechanistically, inflammation is a complex set of interactions among soluble and cellular components, recruited at and by destruction of connective tissue. The major soluble

components include certain intercellular messengers such as cytokines [1]. Among cellular components, blood-borne monocytes/macrophages are an integral part of inflammation.

Among all the anti-inflammatory mediators, Annexin 1 (ANXA 1) has been proven to be the key anti-inflammatory mediator to control the inflammatory cascades. ANXA1 is a 37-kd protein

made up of 346 amino acids and the first member of the annexin super family of calcium and phospholipid binding proteins [2]. ANXA1 and its N-terminal portion can influence many inflammatory mechanisms and regulate the synthesis of eicosanoids, leukocyte migration and apoptosis of inflammatory cells [3]. The ability of ANXA1 to regulate the synthesis of inflammatory mediators (such as eicosanoids) is due to its ability to restrain the activity of phospholipase A₂ (PLA₂). PLA₂ is the only super family of esterases that slices the acyl ester linkage at the *sn*-2 site of membrane phospholipids, liberating free fatty acids and lysophospholipids [4]. These hydrolases are pro-inflammatory cytokines responsible for the release of arachidonic acid and eicosanoids synthesis. PLA₂ can be segregated into three major classes, Ca⁺² dependent cytosolic PLA₂ (cPLA₂), Ca⁺² independent cytosolic PLA₂ (iPLA₂) and secretory PLA₂ (sPLA₂). Amongst these, sPLA₂s are the main contributors for the extensive production of arachidonic acid in inflammatory pathways [5].

To counteract the mediators of inflammatory cascades, a number of anti-inflammatory agents have been used. Among these, glucocorticoids (GCs) are the mainstay for the management of various inflammatory and immune disorders. GCs inhibit inflammation through a series of mechanisms, of which the induction of ANXA1 is a key mechanism. However, adverse effects associated with their long-term usage and development of resistance limit the use of GCs in chronic maintenance therapies. Among these deleterious effects, adrenal atrophy, cushing syndrome, hypertension, gastrointestinal bleeding, hypo-gonadism and fetal growth retardation [6] are associated with the long-term use of GCs. Considering the above mentioned clinical complications, global focus is to discover safer and more efficacious agents.

Curcumin, a natural compound found in *curcuma* species has been used successfully as an anti-inflammatory agent. The pharmacological perspective of curcumin is under investigation and includes demonstration of its targets such as transcription factors, cytokines, cell adhesion molecules, surface receptors, growth factors and various kinases [7] thereby, causing either direct cellular pathway inhibition or activation of secondary cellular responses.

Furthermore, coumestrol, genistein and daidzein sourced from soybeans are the phytoestrogens. Phytoestrogens are substances that promote estrogenic actions in mammals and structurally resemble mammalian estrogen 17 β -estradiol [8].

They mimic the biological activity of estrogens and have wide range of biological activities including estrogenic, antioxidant, anti inflammatory, antithrombotic, anti allergic, hypolipidemic and anti cancer properties [9]. Many biological and anti inflammatory effects of curcumin and phytoestrogens have been reported. However, to the best of our knowledge, their ability to modulate the induction of ANXA1 has not been reported to date. Therefore, the aim of the current study was to investigate the effect of phytoestrogens and curcumin on the induction of ANXA1 in human peripheral blood mononuclear cells as well as their inhibitory effects on the secretory phospholipase A₂-V (sPLA₂-V) enzyme.

EXPERIMENTAL

Materials

Curcumin, coumestrol, daidzein and genistein were obtained from Sigma, Steinheim Germany. Compounds were dissolved in 5 % dimethyl sulfoxide (DMSO) (Sigma, Steinheim, Germany) and diluted with PBS (phosphate-buffered saline) to obtain stock solutions of 1mg/mL. For the concentrations used in assay, calculated amount of stock solution were taken and diluted with complete medium. Complete medium consisted of RPMI-1640 supplemented with *L*-glutamin (Sigma, Steinheim, Germany), 10 % heat-inactivated newborn calf serum (Sigma, Steinheim, Germany), 10 mM HEPES (Sigma, Steinheim, Germany), and 100 U/ml penicillin and 100 μ g/ml streptomycin (PAA, Pasching, Austria). The final concentration of DMSO in the solution applied to the PBMC was < 0.01%.

Separation of human peripheral blood mononuclear cells (PBMCs)

PBMCs were obtained from blood donated by healthy volunteers. This study was approved by the human ethical committee of Universiti Kebangsaan Malaysia (UKM) (approval no. UKM 1.5.3.5/244/NF-050-2012) and conformed to the principles outlined in the Declaration of Helsinki [23]. Venous blood was collected in heparinized tubes and processed right away. Human PBMCs were isolated using gradient centrifugation in Lymphoprep (Axis-Shield PoC AS, Oslo, Norway). Blood was diluted at 1:2 with RPMI-1640 medium, carefully layered on Lymphoprep, and centrifuged at 600 g for 20 min at 20 °C. The PBMCs layer was removed and washed twice with RPMI-1640 and re-suspended in RPMI-1640 complete medium in a culture tube. The cells were adjusted to 5x10⁵ cells/ml by using haemocytometer.

Cell viability

Cell viability was determined by the standard trypan blue exclusion method. The PBMCs (5 x 10⁵ cells/ml) were incubated with various concentrations of the compounds ranging from 5 to 100 µg/ml, each in triplicate at room temperature for overnight. The blue dye uptake was a signal of cell death. The percentage viability was calculated from the total cell counts. The concentration of compounds at which viability was > 95% was used for further studies [10].

Incubation of compounds in inflammatory and non-inflammatory PBMCs

PBMCs were incubated overnight at 37 °C and 5 % CO₂ either in the presence or in the absence of the test compounds. PBMCs were stimulated by 1 µg/mL of lipopolysaccharide (LPS) from *Salmonella enteritica* (Sigma, Steinheim, Germany) for the inflammatory condition. Dexamethasone at 0.4 µg/mL (10⁻⁶ M) was used as a positive control and a mixture containing DMSO, PBS and RPMI-1640 was used as a negative control. Control groups were taken for stimulated as well as for non-stimulated PBMCs [11].

Extraction of extracellular, intracellular and cell surface-associated Annexin 1

Following incubation period, cells were gently centrifuged (300 g) for 5 min at 4 °C and the supernatant was separated for extracellular ANXA1. This centrifugation did not cause any lysis. The membrane bound ANXA1 was removed into the medium by washing the cells with Ca⁺² free salt solution containing EDTA (Sigma, Steinheim, Germany). The pelleted cells were re-suspended in PBS containing 2 mM EDTA and were further incubated for 2-3 min to get rid of ANXA1 attached to cell membranes. After incubation, cells were centrifuged again at the specifications mentioned before. The supernatant was isolated for measurement of membrane bound ANXA1. Subsequent to the removal of membrane associated ANXA1, cells were lysed and the lysate was taken for the calculation of entire intracellular ANXA1 [10].

Quantification of Annexin 1 by ELISA

ANXA1 was deliberated by sandwich Enzyme linked-immunosorbent Assay (ELISA) (USCN Life Science, China). The whole assay was performed as described by the manufacturer. ELISA was performed in duplicate, and data was obtained from three different donors.

Inhibition of secretory Phospholipase A₂ –V (sPLA₂-V) Assay

Human recombinant sPLA₂-V (Cayman) was employed as enzyme source. The activity of sPLA₂ enzyme was calculated by a photometric assay based on the Ellman method [12]. Briefly, hydrolysis of sn-2 ester bond of the substrate 1,2-bis(heptanoylthio)-glycerophosphocholine by PLA₂-V was followed by the exposure of free thiols. These thiols triggered the alteration of DTNB (5,5-dithio-bis-(2-nitrobenzoic acid) to 2-nitro-5-thiobenzoic acid which was detected photometrically at 405 nm. Afterwards, the assay was progressed in an aqueous buffer solution (pH 7.5) containing KCl (94 mM), CaCl₂ (9 mM), Tris (24 mM) and Triton-X 100 (280 µM). Prior to the assay, substrate and PLA₂-V were re-suspended in assay buffer, and DTNB was dissolved in an aqueous solution of Tris-HCl (pH 8). Enzyme and DTNB yielded final concentrations of 100 ng/mL and 87 µM respectively. Assays were performed in 96-well microliter plates at room temperature containing DTNB, substrate solution and the respective test substance. 100% activity of enzyme was calculated by adding substrate and enzyme only. DMSO served as a negative control and was inactive at the concentration used in the assay (1.7 %v/v).

Statistical analysis

The results are expressed as mean ± standard deviation. All statistical analyses were performed using Graph Pad Prism 5. One-way ANOVA, followed by a post-test (Tukey's multiple comparison) was carried out when major divergence at $p < 0.05$ and $p < 0.01$ was present.

RESULTS

Cell viability

The cell viability was assessed prior to every experiment. The viability of PBMCs incubated in the presence of test compounds at concentrations 10 µg/mL was always greater than 95%. The concentrations of compounds higher than 10µg/mL decreased the cell viability below 95%.

Effect on annexin-1 level and secretion in normal PBMC

The modulation of ANXA1 from PBMCs was observed by incubating the PBMC either in the presence or in the absence of curcumin, coumestrol, ginseng and diadmine at 10 µg/mL for all compounds. After incubation, intracellular,

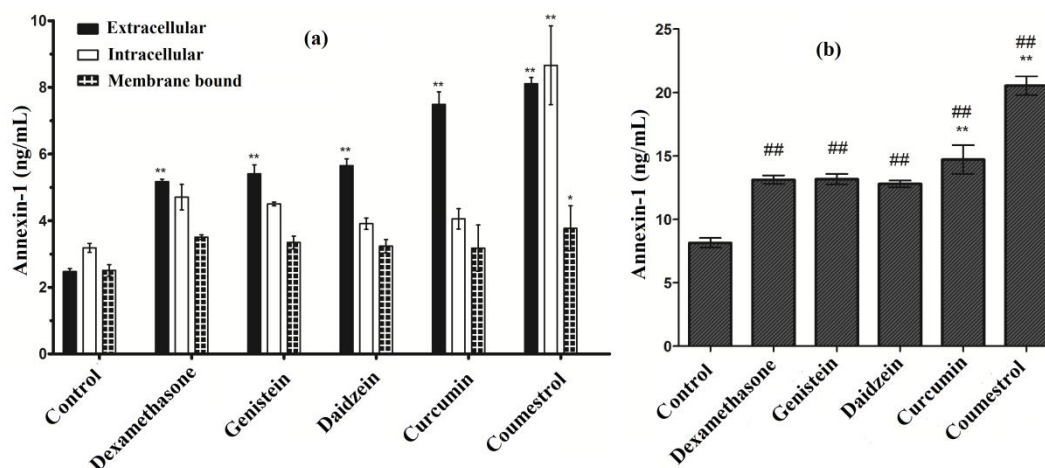


Figure 1: (a) Concentration of extracellular, intracellular and plasma membrane bound levels of ANXA1 in PBMC in the absence of compounds (control), Dexamethasone (0.4 $\mu\text{g/mL}$) and in the presences of compounds (10 $\mu\text{g/mL}$). (b) Total content of ANXA1 level in PBMC.

extracellular and membrane bound ANXA1 levels were calculated as described in methods. Considerable increase was observed in the basal levels of intracellular ANXA1 with all compounds; however, coumestrol exhibited maximum increase in the intracellular level of ANXA1. A significant increase in the level of extracellular ANXA1 was found for all the compounds as compared to control (Figure 1a). Results presented in Figure 1a illustrate an increase in plasma membrane bound ANXA1 and statistically significant increase by coumestrol. These results designate the induction of synthesis and stimulation of secretion of ANXA1 in PBMCs.

Effect on Annexin-1 level and secretion in inflammatory PBMCs

The levels of ANXA1 were also measured in inflammatory conditions. For this purpose, PBMCs taken from the same blood donors were subjected to inflammatory condition. Intracellular, extracellular and membrane bound ANXA1 level was measured as described above. Figure 2a illustrates an increase in the extracellular level of ANXA1 in stimulated PBMCs, relative to control. An increase in intracellular and plasma membrane-bound ANXA1 was also observed. In inflammatory PBMC, a net increase was seen in the total content (extracellular, intracellular and membrane bound) of ANXA1. The level of ANXA1 increased to 15 ± 0.7 and 11.5 ± 0.9 ng/mL for coumestrol and curcumin, respectively. Likewise, an increasing trend was seen for genistein (8.9 ± 0.3 ng/mL) and daidzine (9.8 ± 0.3 ng/mL), correspondingly. For all the compounds, an increasing trend in the levels of ANXA1 was seen in both normal and inflammatory cells. Similar to non-inflammatory

Collectively, a manifold increase in total level of ANXA1 was observed after combining the extracellular, intracellular and membrane bound contents of ANXA1 (Figure 1b). As compared to the untreated cells (8.1 ± 0.4 ng/mL), the total content of ANXA1 was substantially increased (21.5 ± 0.8 ng/mL) with coumestrol. With curcumin, genistein and daidzein, the level of ANXA1 increased to 14.7 ± 1.1 , 13.2 ± 0.4 and 12.8 ± 0.3 ng/mL respectively. Amongst all, coumestrol exhibited the strongest stimulation of ANXA1. On the other hand, curcumin also showed worth mentioning results. Genistein and daidzine also moderately up regulated ANXA1.

condition, the potency of coumestrol was also highest in inflammatory condition.

Inhibition of sPLA₂-V

Secretory PLA₂ inhibitory activity was determined using the Ellman method. The activity was assessed by detecting free thiols using Ellman's reagent DTNB (5, 5'-Dithio-2 nitrobenzoic acid). The inhibition of sPLA₂ activity from human source was determined by different concentrations of the inhibitors. The IC₅₀ values for sPLA₂ were calculated by linear XY scattered plot (Table 1). The inhibition of sPLA₂ activity varied for different inhibitors at different concentrations. ANXA1 inhibited sPLA₂ in a dose-dependent manner. As the concentration of ANXA1 was increased, a corresponding decrease in the enzyme activity was observed. ANXA1 preferentially inhibited sPLA₂ enzyme activity with an IC₅₀ value of 4.9×10^{-7} and showed > 80% at 400 ng/mL. Dexamethasone also exhibited sPLA₂ inhibition in a dose dependent manner with an IC₅₀ value of 0.61 ± 0.1 μM (Figure 3 and Table 1). Similarly, curcumin, genistein and daidzein

inhibited the sPLA₂ activity in a concentration dependent manner (Figure 4 and Table 1). Of all the compounds, curcumin exhibited the strongest inhibition on sPLA₂ enzyme activity with an IC₅₀ value of 11.1 ± 0.3 μM. Among the

phytoestrogens, genistein showed the strongest activity with an IC₅₀ value of 13.6 ± 0.6 μM. Coumestrol also moderately inhibited sPLA₂ activity in a concentration dependent manner

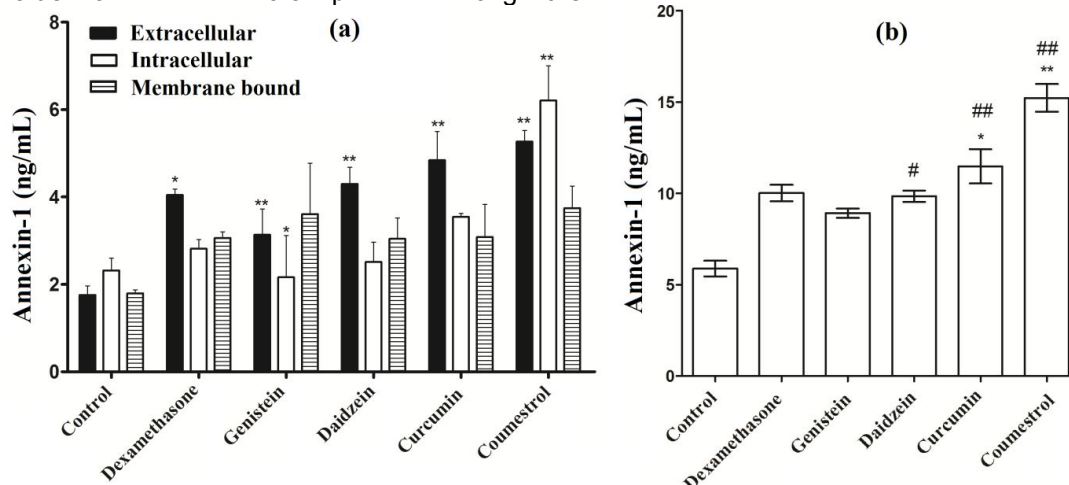


Figure 2: (a) Concentration of extracellular, intracellular and plasma membrane bound levels of ANXA1 in inflammatory PBMC in the absence of compounds (control), Dexamethasone (0.4 μg/mL) and in the presence of compounds (10 μg/mL). (b) Total content of ANXA1 level in inflammatory PBMCs.

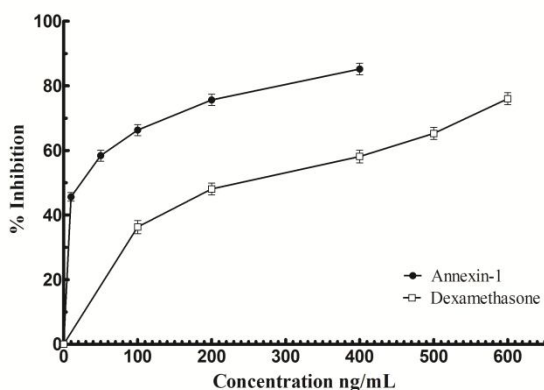


Figure 3: Concentration-dependent inhibition of sPLA₂ enzyme by annexin-1(circle) and dexamethasone (square). Inhibition is expressed as percent of control. The data represents mean ± SD (n = 3).

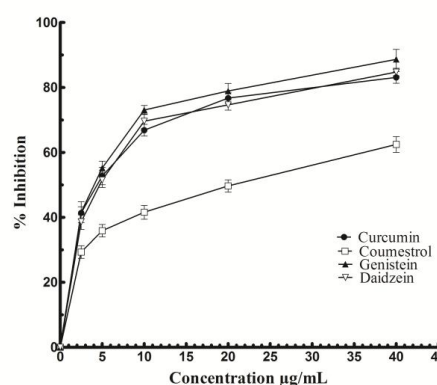


Figure 4: Concentration-dependent inhibition of sPLA₂-V enzyme by curcumin, coumestrol, genistein and daidzein. Inhibition is expressed as percent of control. The data represents mean ± SD (n = 3).

Table 1: IC₅₀ (μM) values of phytoestrogens, curcumin and annexin 1 against sPLA₂-V activity

Substance	IC ₅₀ (μM)
Curcumin	11.1±0.3
Coumestrol	84.2±1.1
Genistein	13.6±0.6
Daidzein	18.0±0.6
Annexin-1	4.9 × 10 ⁻⁷ ± 0.3 × 10 ⁻⁷
Dexamethasone	0.6±0.01

DISCUSSION

ANXA1 role as an anti-inflammatory protein has been brought to limelight in recent years. ANXA1 was initially thought to be just a cytosolic protein

to exert its anti-inflammatory effects through inhibition of PLA₂. However, recent studies on ANXA1 have uncovered new effects and actions of ANXA1. In resting conditions, human neutrophils, monocytes and macrophages constitutively contain ANXA1 [13]. ANXA1 expression and function with GCs treatment are already known but the intracellular signalling pathways involved in the expression of ANXA1 in response to steroidal hormones are still unclear. Although some reports have illustrated that GCs up-regulate ANXA1 synthesis by genomic mechanisms [14], different intracellular studies state that this effect is independent of the activation of nuclear GCs receptors. In agreement with these reports, a recent study reported that in CCRFCEM cells, ANXA1

synthesis was independent of the nuclear GCs receptors [15]. Dexamethasone significantly increases the cellular content of ANXA1 when incubated overnight. The overnight incubation of PBMCs with 10 $\mu\text{g}/\text{mL}$ of investigated compounds result in the up-regulation of ANXA1. Moreover, a significant increase in the amount of ANXA1 excreted out of plasma membrane was also observed. It has been reported that the increase in the cellular turnover of ANXA1 may cause a rapid export of the proteins from intracellular stores to extracellular sites and as a consequence the de novo synthesis of proteins may take place to replenish the depleted intracellular levels [16]. Based on these findings, our results indicate that the investigated compounds may have a potential to efficiently stimulate the ANXA1 secretion.

Recent studies on the induction of ANXA1 expression have shown that chemically similar estrogen hormones also have a role in the induction of the ANXA1. In studies carried out previously, researchers had demonstrated that in human lymphoblastic CCRF-CEM cell line, estrogen hormone and 17 β -estradiol ($\text{E}_2\beta$) induced the synthesis of ANXA1 [15]. It is well established that $\text{E}_2\beta$ exerts major effects on cell growth, differentiation and function by specifically interacting with intracellular estrogen receptors (ER) [17]. The complex $\text{E}_2\beta$ -ER migrates to the nucleus of the cells, where it binds to estrogen-responsive elements present in the genomic DNA. It was reported that the up-regulation of ANXA1 was due to the action of $\text{E}_2\beta$ on estrogen receptors.

Coumestrol, genistein and diadzein are phytoestrogens. Phytoestrogens promote estrogenic actions in mammals. Mechanistically, phytoestrogens have been shown to bind to two types of estrogen receptors: estrogen receptor α and receptor β , respectively. On the basis of above mentioned facts, it is hence proved that the investigated compounds resemble $\text{E}_2\beta$ in structure and functions, and also exhibit results similar to $\text{E}_2\beta$. Curcumin is renowned for its anti-inflammatory effects and treatment of various diseases. In this study, it has been observed that curcumin up-regulates ANXA1 in PBMC, but the mechanisms by which curcumin up-regulates ANXA1 are still unknown.

Among the PLA_2 enzymes, sPLA_2 enzymes play an important role in the pathogenesis of inflammatory diseases [18]. Elevated levels of sPLA_2 enzymes are detected in many inflammatory conditions. Inhibiting sPLA_2 s would probably be a preferable strategy since their induced levels are predominantly associated with

pathological conditions. Though, a selective inhibition of just one isoform of sPLA_2 may not be sufficient to exert the desired effect. The originally recognized activity of ANXA1 as an inhibitor of phospholipase A_2 (PLA_2) was at first proposed to be accountable for its anti-inflammatory actions [19]. The inhibition of PLA_2 activity, including the production of arachidonic acid, was thought to be a result of ANXA1 binding to the substrate, rather than directly to the enzyme, leading to the depletion of substrate sites and a subsequent reduction of PLA_2 activity. However, this idea was reassessed and it is now obvious that a secretory form (sPLA_2) as well as a cytosolic form of PLA_2 (cPLA_2) exist. ANXA1 has shown the inhibition of sPLA_2 in a concentration dependent manner. The same mechanism has also been observed for cPLA_2 inhibition [20].

Anti-inflammatory properties of GCs have been attributed to the liberation and enhanced synthesis of ANXA1, which inhibits PLA_2 , resulting in a decreased eicosanoid synthesis. However in the current study, dexamethasone has been found to have inhibited the sPLA_2 in a concentration dependent manner. In the previous studies, dexamethasone had been reported to inhibit the PLA_2 in U937 cells but, the expression of ANXA1 was not inducible by GCs in these cells [21].

Flavonoids are antioxidants known to act as anti-inflammatory compounds by scavenging the free radicals. Therefore, a single molecule having both potencies of PLA_2 inhibition as well as antioxidant activity can serve as a better anti-inflammatory molecule. In the present study, the flavonoids, genistein and diadzein inhibited $\text{sPLA}_2\text{-V}$ in a concentration dependent manner. Many inhibitors inhibit the sPLA_2 activity either by binding to the substrate, or by chelating with calcium. But the inhibition of other sPLA_2 isomers by genistein was observed to be independent of substrate and chelation of calcium, though sPLA_2 isomers exhibited more than 70% homology [22]. Diadzein and genistein belong to the same class and have structural and functional similarities. So, it is possible that diadzein has the same mechanism as followed by the genistein.

CONCLUSION

The results illustrate that the phytoestrogen and curcumin have potentials to induce synthesis and secretion of ANXA1 in peripheral blood mononuclear cells (PBMCs). Our findings on $\text{sPLA}_2\text{-V}$ inhibition provide further evidence to rationalize the anti-inflammatory activities of the tested compounds. The present findings on

sPLA₂-V inhibitory functionality should encourage further investigations that would address other PLA₂ isoforms in inflammatory cascades.

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