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# Gossypetin 3′ Glycoside exerts neuroprotective activity by altering the expression of NR3C1 and Pro-Inflammatory Cytokines targeting the HPA axis

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**ABSTRACT**

**OBJECTIVES:** The hypothalamic-pituitary-adrenal (HPA) axis plays an important role in maintaining homeostasis and its dysregulation is characterized by elevated levels of glucocorticoids leading to depression. Therefore, therapies targeting the stress mechanisms at molecular level are of research interests. The objective of this study is to elucidate the neuroprotective and anti-inflammatory activity of Gossypetin-3′-O-glycoside (G-3′gly) isolated from the endophyte *Daldinia eschscholtzii*.

**MATERIALS AND METHODS:** The purified compounds were tested for its viability against AtT20 cell lines and $IC_{50}$ was calculated. Intracellular ROS scavenging activity, nitric oxide activity and lipid peroxidation assays were done to analyze the anti-inflammatory activity. Further the compound with most promising activity was used to study the levels of corticosterone, mRNA and protein expression of stress system genes and pro-inflammatory cytokines.

**RESULTS:** The neuroprotective effects of the isolated compounds were mediated by down regulating intracellular ROS as well as inhibiting LPS induced lipid peroxidation. Further the molecular mechanism of G-3′gly in Bisphenol A (BPA) induced AtT 20 cells evaluated using RT-PCR and ELISA showed that G-3′gly restored the altered levels of the stress system genes CRHR1, POMC and NR3C1 indicating the compound actively attenuates BPA induced activation of HPA axis genes. Further the activation of HPA axis was correlated with the release of pro-inflammatory cytokines TNF-α, IL-1β and IL-6.

**CONCLUSION:** Our study attributes to the possible anti-depressant and neuroprotective effects of the compound G-3′gly and its anti-stress mechanism thereby restoring the homeostasis.

**Keywords**: Gossypetin-3′-O-glycoside, Antioxidants, Lipid peroxidation, Anti-depressant, Neuroprotective.

## 1. Introduction

Depression is a well-known life threatening disorder and is strongly correlated with acute and chronic stress which is triggered by many environmental and genetic factors.[1, 2] Upon Chronic exposure, stress induces neuronal inflammation, neuronal degeneration and brain micro damage.[3] The main system regulating the stress response is the hypothalamus-pituitary-adrenal (HPA) axis, a complex molecular pathway which includes the feedback regulatory interactions between the hypothalamus, the pituitary and adrenal glands. The HPA axis is a key response element against stressors and its abnormal activation by chronic stressful conditions is regarded as an important risk factor for depression.[4]

Chronic stress often simultaneously increases the generation of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6 representing a chronic inflammatory state and also decreases the sensitivity of the glucocorticoid receptors.[5, 6] During chronic stress, the activated immune cells from the peripheral blood produce various cytokines, many of which crosses the blood brain barrier and activates the resident immune cells, the microglia which in turn provokes neuro inflammation.[7,8,9]

Studies show that anti-depressants, such as selective serotonin re-uptake inhibitors (SSRIs) are found to exert better therapeutic efficacy.[10,3] However, the side effects of these drugs have not yet been entirely eradicated and their molecular and therapeutic mechanisms are not fully understood.[11] On the other hand, extensive research on medicinal plant extracts and natural compounds from other microorganisms like endophytes which have been traditionally used to treat stress and other neurological disorders are emerging as they could help in identifying novel drugs to treat chronic stress.[12]

In our previous study, we have reported the isolation of Gossypetin 3′-glycoside (G-3′gly) along with four other compounds (α-NF, SA, GATE, 2-FA) from the endophytic fungus *Daldinia eschscholtzii* which showed strong anti-microbial activity[13]. Literature shows that G-3′ gly has also been reported from the medicinal plant *Talipariti elatum S* with potent anti-oxidant activities.[14] But only preliminary investigation has been carried out and only limited information is known in terms of this natural compound. It would be of interest to study the molecular pathways involving free radical scavenging activity and anti-inflammatory activity of G-

3′gly. Hence the current study attempts to identify the therapeutic influence of G-3′gly on neuro inflammation targeting the HPA axis stress associated genes.

## 2. Materials and Methods

### 2.2 *Invitro* assays

#### 2.2.1 Cell lines and culture medium

AtT20 Mouse pituitary cell lines (P32) were procured from National Centre for Cell Sciences (NCCS), Pune, India. Cells were cultured in DMEM supplemented with 10% inactivated FBS, penicillin (100 IU/ml), streptomycin (100 mg/ml) and amphotericin B (5 mg/ml) in a humidified atmosphere of 5% CO2 at 37˚C until confluent.

#### 2.2.2 Determination of cell viability and $IC_{50}$ values of isolated compounds by MTT assay

Isolated compounds at concentrations 3 – 100 µg/ml (dissolved in sterile water) were tested on AtT20 cell lines and cell viability was assessed using MTT assay as previously described with minor modifications.[15] Cell viability was determined as the relative percentage of treated cells to the untreated cells and the $IC_{50}$ values are calculated.

### 2.3 Intracellular ROS scavenging activity of the isolated compounds

Intracellular Reactive Oxygen Species (ROS) was measured using DCFH-DA fluorescent dye in AtT 20 mouse pituitary cells using a standardized method with minor modifications.[16] The analysis was done using BD Cell Quest Pro software.

### 2.4 In vitro nitric oxide inhibitory activity

Intracellular nitric oxide inhibition activity was measured in AtT20 cells according to the method described.[17]

### 2.5 In vitro Lipid peroxidation assay

Lipid peroxidation assay is based on the production of malondialdehyde (MDA) in AtT20 cells as previously described [18] with some modifications. For evaluation of MDA production rate, thiobarbituric acid (TBARS) assay was used.

### 2.6 RNA extraction and quantitative PCR analysis

Of all the five compounds, G-3′gly showed most potent anti-inflammatory and neuroprotective activity. Hence RT-PCR analysis was carried out to analyze the mRNA expressions of HPA axis stress genes CRHR1, POMC, NR3C1 and inflammatory cytokines TNF-α, IL-1β, IL-6 upon treatment with G-3′gly. Bisphenol A (BPA), at a concentration of 40 µg/ml[19] which is an endocrine disruptor was used to induce stress in AtT-20 cells.

Total RNA was extracted from control, BPA induced cells, BPA induced and treated with G-3′gly and standard control fluoxetine (Flx) at concentration – 2 µM[20] treated cells using QAmp RNA mini kit and cDNA synthesis using Thermo Scientific RevertAid First strand cDNA synthesis kit according to the manufacturer's protocol. The data obtained was analyzed by comparative cycle threshold method and expressed as relative expression normalized to GAPDH values. Data was obtained in triplicates and results are shown as Mean ± Standard deviation.

**2.7 Measurement of Corticosterone levels in AtT 20 cells using ELISA**

Corticosterone is quantitatively measured using ELISA as per the manufacturers protocol (Abcam, ab 108821). AtT 20 cells were treated with BPA, BPA+ G-3′ gly and standard control Flx for 24 h. The values were measured using a microplate reader (Multiskan GO, Thermoscientific, USA). Experiments were performed as triplicates. The concentrations were calculated based on the standard curve and then normalized to the control.

**2.8 Quantitative ELISA**

The pro-inflammatory cytokine levels (TNF-α, IL-1β, IL-6) were measured using quantitative ELISA method according to the manufacturers protocol (R&D Systems). AtT 20 cells were treated with BPA, BPA+ G-3′ gly and standard control Flx for 24 h. The values were measured using a microplate reader (Multiskan GO, Thermoscientific, USA). Experiments were performed as triplicates.

**2.9 Statistical analysis**

Results obtained were analyzed and were then evaluated by analysis of variance (One-way and Two-way ANOVA). P-values less than 0.05 ($p<0.05$) are considered to be significant.

**3. Results**

**3.1 Neuroprotective activity: Determination of cell viability and $IC_{50}$ concentrations of the isolated compounds**

Compounds α-NF, SA, GATE, 2-FA, G-3′gly were evaluated for their ability to influence cell viability of AtT 20 cells. 3 – 100 µg/ml of compound concentration was used to assess the cell viability via MTT assay. All the five compounds showed significant change in cell viability ranging from 46% - 85%, 61% - 92%, 42% -89%, 49% - 81% and 64% -95% respectively (Figure 1a-e).

**3.2 Intracellular free radical scavenging activity in AtT 20 mouse pituitary cell lines**

Intracellular free radical scavenging assay was assessed using FACS which revealed that the compounds at their $IC_{50}$ concentrations significantly reduced the extent of free radical generation. AtT 20 cells when treated with compounds α-NF, SA, GATE, 2-FA, G-3′gly along with peroxide (50 µm) showed a significant ($p< 0.001$) reduction in the fluorescence intensity when compared to cells treated with peroxide alone (94%). α-NF exhibited 35%, SA- 31%, GATE - 29%, 2-FA -38% and G-3′gly - 15% ROS generation (Figure 2a).

**3.3 In vitro nitric oxide inhibitory activity of isolated compounds in AtT 20 cells**

In the present study, the NO scavenging capacity of the compounds was determined by decrease in the absorbance at 550 nm, as a result of reduction of NO production. The inhibitory activity of NO was demonstrated in all the compounds at their $IC_{50}$ concentrations in AtT 20 mouse pituitary cells. The cells when treated with compounds along with LPS showed a significant ($p< 0.01$) decrease in the generation of NO when compared to the LPS induced cells (92% NO generation). The compounds α-NF, SA, GATE, 2-FA, G-3′gly showed 32%, 39%, 36%, 41% and 22% NO generation respectively (Figure 2b).

**3.4 In vitro lipid peroxidation activity of isolated compounds in AtT 20 mouse pituitary cells**

In this study, concentration of MDA as a marker of lipid peroxidation was determined using Thiobarbituric acid reactive substances (TBARS) assay in AtT 20 mouse pituitary cells. The compounds significantly ($p< 0.01$) inhibited lipid peroxidation at their $IC_{50}$ concentrations in AtT 20 cells. The pink-coloured MDA-TBA abduct formed significantly decreased upon treatment with compounds α-NF, SA, GATE, 2-FA, G-3′gly (5.9, 5.1, 4.1, 6, 3.5 nmol/g MDA) when compared to the LPS induced cells (12 nmol/g) (Figure 2c).

**3.5 Mechanism of action of G-3′gly on stress system genes**

To understand the possible mechanism of the regulation of HPA axis, the mRNA expression levels of CRHR1, POMC, NR3C1was analyzed by RT PCR. Our results showed that the expression of CRHR1 is significantly higher in BPA induced cells with four-fold increase ($p < 0.01$) when compared to control cells. When treated with G-3′gly, the cells showed 2-fold decrease ($p < 0.01$) in the expression of CRHR1 (Figure 3a). The Flx group showed the decreased expression of CRHR1 by 3 folds when compared to BPA induced group. Similarly, the expression of POMC in BPA induced AtT20 cells showed significant 6-fold increase ($p<0.01$) (Figure. 3b) when compared to control cells. Upon treatment with G 3′-gly, a 3-fold decrease in the expression of POMC was seen. The standard control, Flx showed 4-fold decrease when compared to BPA induced cells. In contrast, the expression of glucocorticoid receptor encoding gene, NR3C1 was significantly decreased ($P < 0.05$) when cells were induced with BPA when compared to untreated control cells (Figure. 3c). Treatment with G 3′-gly, showed a 3-fold significant increase ($p<0.01$) in the expression of GR indicating protective effect of the compound in AtT20 cells.

**3.6 G-3′gly decreases the expression of corticosterone levels in AtT 20 cells**

The level of corticosterone, a major stress marker is assessed in AtT20 cells after treatment with G-3′gly. As displayed in figure 4, the corticosterone level significantly increased ($P < 0.01$) 413 folds in BPA induced group when compared to the control group (150 folds). However, after treatment with G-3′gly, the levels markedly decreased ($P < 0.01$) by 265 folds indicating the protective role of the compound. Also, the standard flx comparatively showed the decrease in expression similar to the treated compound (205 folds).

**3.7 Effects of G-3′gly on pro-inflammatory cytokines**

Since our results showed that G-3′gly potentially scavenged intracellular ROS, inhibited lipid peroxidation and scavenged nitric oxide production to a greater extent and also owing to the potent anti-depressant activity of G-3′gly, we demonstrated the influence of G-3′gly on the expression of pro-inflammatory cytokines TNF-α, IL-1β and IL-6.

Our results showed that the BPA induced cells showed a significant ($p< 0.01$) 2-fold increase of TNF- α, a 3-fold increase of IL-1β and 6-fold increase of IL-6 in AtT20 cells. On contrast when treated with G-3′gly, the expression of TNF-α, IL-1β and IL-6 decreased by 1-fold, 2- fold and 3 folds ($p< 0.01$) respectively (Figure 5). The positive control flx also showed similar decrease when compared to G-3′gly treated cells.

**3.8 Analysis of pro-inflammatory cytokine protein expression**

To understand the mechanistic regulation of inflammation associated cytokines and since our results indicated a possible correlation of stress system genes with the release of pro-inflammatory cytokines, we investigated the protein expression of TNF-α, IL-1β and IL-6 in BPA induced AtT 20 cells upon treatment with G 3′-gly.

Our results showed that BPA induced significant upregulation of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 by 57 folds, 45 folds and 72 folds ($p< 0.01$) respectively. However, upon treatment with G 3′-gly, the increased protein levels induced by BPA reduced significantly for TNF-α by 32 folds ($p< 0.01$), IL-1β by 38 folds ($p< 0.01$) and IL-6 by 57 folds ($p< 0.01$) (Figure 6). The standard control flx showed similar expression as that of the treatment drug G 3′-gly.

**3. Discussion**

In this study, our findings report the protective effect of the compound Gossypetin 3′-glycoside (G-3′gly). This compound was previously reported from the plants, *Talipariti elatum S* and *Abelmoschus manihot*.[13] For the first time our work revealed the presence of G-3′gly from an endophyte. Previous studies have reported the anti-oxidant, anti-inflammatory and anti-pyretic activities of the floral extracts containing G-3′gly but the studies showing the activity of the pure compound is very limited. Our study focuses on elucidating the protective mechanism of G-3′gly involving the HPA axis stress pathway. Many approaches towards neuroprotection are currently focusing on alleviating neuro inflammation via antioxidant defense mechanisms.

The isolated compounds were checked for cell viability in AtT 20 mouse pituitary cells using MTT assay. All the compounds showed potent cell viability. Further, the $IC_{50}$ values of compounds α-NF, SA, GATE, 2-FA, G-3′gly were calculated as 70 µg, 125 µg, 64 µg, 88 µg and 141 µg/ml respectively. These $IC_{50}$ concentrations were used for further *in vitro* assays.

To understand the influence of isolated compounds on intracellular oxidative stress, DCFDA stained AtT 20 cells were used for FACS analysis. Among the five compounds, G-3′gly exhibited most promising intracellular radical scavenging activity (approx 80%). These results confirmed that the isolated compounds exhibited the potential to reduce the intracellular oxidative stress in AtT 20 cells. Studies show that reducing the excess intracellular ROS is essential in maintaining homeostasis as increased ROS leads to the disruption of the negative feedback loop of the

HPA axis.[21,22,23] Hence the ability of the compounds to scavenge the intracellular ROS serves as an indicator for identifying potential anti-inflammatory and neuroprotective activity of the isolated compounds.

Similarly, overproduction of another major inflammatory marker NO could result in tissue damage and activation of proinflammatory mediators associated with acute and chronic inflammation.[24] The best inhibitory activity against NO was seen in G-3′gly. These results were in line with our previous results supporting the antioxidant and anti-inflammatory activity of the isolated compounds. Hence, the anti-inflammatory activity of these compounds targeting NO inhibition might serve as potential candidates for the treatment of neuroinflammatory diseases caused due to the overproduction of Nitric oxide.

Lipid peroxidation is an essential marker of oxidative stress induced inflammation which can be assessed by the measurement of malondialdehyde (MDA).[18] Our study showed a decrease in the MDA levels which indicates that the compounds α-NF, SA, GATE, 2-FA, G-3′gly are effective inhibitors of lipid peroxidation in AtT 20 cells and this potential reveals the efficacy of compounds as anti-inflammatory agents.

Owing to the potent anti-inflammatory and scavenging activities of G-3′gly, the efficacy of G-3′gly was demonstrated on the regulation and hyperactivation of HPA axis associated genes. The hypothalamic hormone CRH stimulates the production and secretion of ACTH in the hypothalamic-pituitary-adrenal axis. ACTH, in turn, stimulates the secretion of GCs from the adrenal cortex; GCs exert a negative feedback action on hypothalamic neurosecretory cells and downregulate CRH. Hence GR plays a critical role in the regulation of negative feedback mechanism and thus controlling chronic stress. NR3C1 is a glucoocrticoid receptor encoding gene which acts as a transcription factor that binds to the glucocorticoid response elements and regulates gene expression upon stress.[25]

The mRNA expression of the stress system genes CRHR1, POMC and NR3C1 were assessed using RT-PCR. The increase in the expression of CRHR1 and POMC mRNA, suggested an induction of stress response and thereby activation of HPA-axis and from the results obtained, it was evident that treatment with G-3′gly normalized the BPA induced changes in AtT 20 cells. Studies show that the hyperactivation of HPA axis is closely related to the pathology of depression and this could be reversed during clinical therapies using anti-depressant drugs.[26] Several medicinal plants and plant derived microorganisms have been shown to possess anti-depressant like effects by modulating the HPA axis activation.[27]

Our results implicated that G-3′gly decreased the expression of CRHR1 and POMC while significantly increasing the expression of GR encoding gene NR3C1 indicating the possible mechanism as an anti-depressant and modulating the functional status of HPA axis. Also, flx a standard anti-depressant drug showed similar effect on the BPA induced cells which also indicates the efficacy of G-3′gly in modulating the stress pathway.

Also, the level of corticosterone, a major glucocorticoid harmone which releases in response to stress was assessed using ELISA showed an increased expression when cells were induced with stress. Evidences show the elevated levels of corticosterone were associated with depression like behavior thereby disrupting the HPA axis.[28] Treatment with G-3′gly effectively reversed the corticosterone level in AtT20 cells which was in accordance with the previous studies. As a standard anti-depressant agent, flx could effectively prevent the elevation of corticosterone levels.

Studies also report that apart from these neuroendocrine mediators (CRHR1,POMC, NR3C1), several other parameters especially the immune cells get directly influenced by the varying concentrations of these HPA axis genes resulting in the secretion of cytokines.[28,29] During chronic stress, the immune cells get activated and produce cytokines in response to stress thereby provoking neuroinflammation.[29] Studies show that many medicinal plants extracts and compounds have been shown to possess antidepressant activity by inhibiting the pro-inflammatory cytokines.[28] In line with this, the mRNA levels of TNF-α, IL-1β and IL-6 were demonstrated in BPA induced and G-3′gly treated AtT 20 cells. Our results clearly showed the alterations in the levels of these pro-inflammatory cytokines when treated with G-3′gly. Also, the protein expression of pro-inflammatory cytokines assessed using ELISA showed increase in the levels of cytokines when AtT 20 cells induced with BPA which upon treatment with G-3′gly showed altered expression. Hence our results correlated with the RT-PCR mRNA expression levels indicating a possible association of pro-inflammatory cytokines with the activation of HPA axis.

## 4. Conclusion

In the present investigation, it can be hypothesized that G-3′gly acts as a potential anti-inflammatory agent by scavenging the intracellular ROS, nitric oxide production and inhibiting lipid peroxidation thereby decreasing the expression of pro-inflammatory cytokines in the peripheral tissues. This in turn influences the down regulation of

stress system genes thereby maintaining homeostasis. Hence the anti-depressant like action of G-3′gly is directly correlated with the downregulation of pro-inflammatory cytokines thereby behaving as a neuroprotective agent. Overall, the current finding provides an insight into the pharmacological activities and molecular mechanism of G-3′gly and highlights its significance in the prevention of stress induced neurological disorders.

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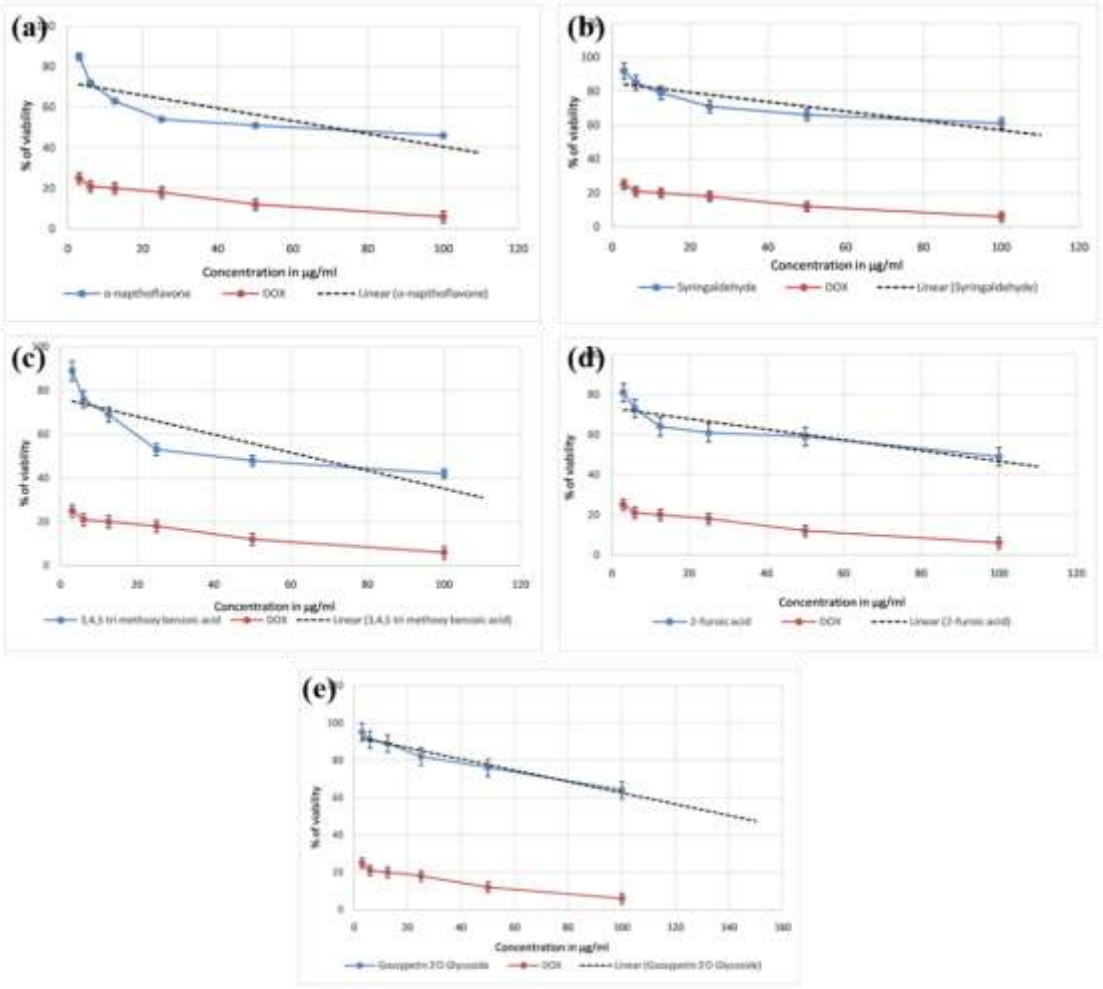


**Figure 1.**Determination of cell viability of compounds against AtT 20 cells by MTT assay. Cells were treated with different concentrations of compounds α-NF, SA, GATE, 2-FA, G-3′gly (3, 6, 12.5, 25, 50 &100 µg/ml) respectively. Doxorubin was used as positive control. Data are represented as means ± SD (n =3) **p< 0.01

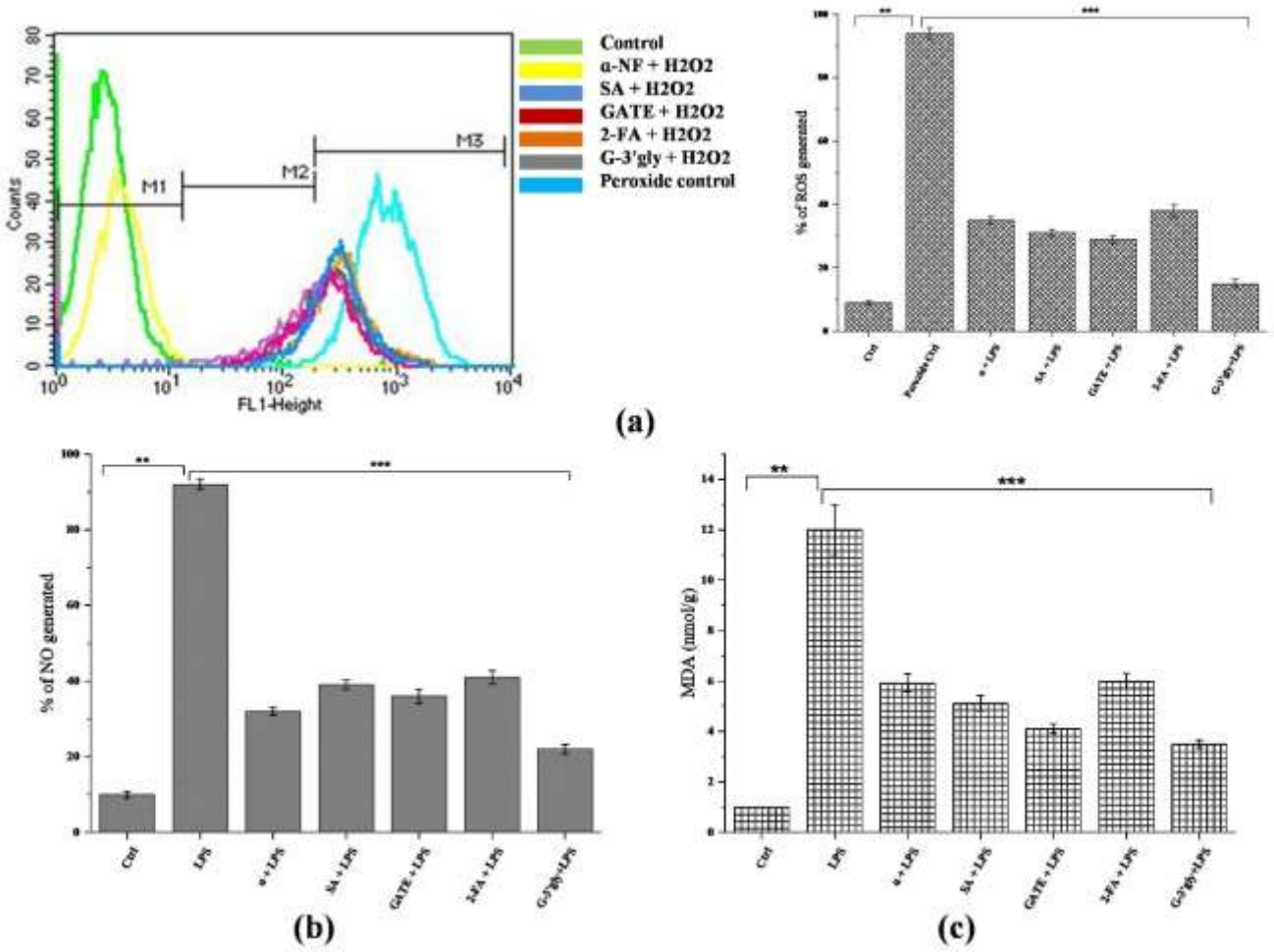


**Figure 2.** Graphical representation of (a) Intracellular ROS scavenging activity of compounds α-NF, SA, GATE, 2-FA, G-3′gly in AtT 20 cells. Overlay histogram plot showing the shift in fluorescence by DCFDA and Graphical representation of % of ROS generated (b) LPS induced nitric oxide scavenging activity. All the compounds showed significant reduction in the production of NO (c) LPS induced lipid peroxidation. All the compounds showed significant reduction in the production of the product MDA. Levels of MDA are represented in nmol/g. Data are represented as means ± SD (n =3) **p< 0.01, ***p< 0.001

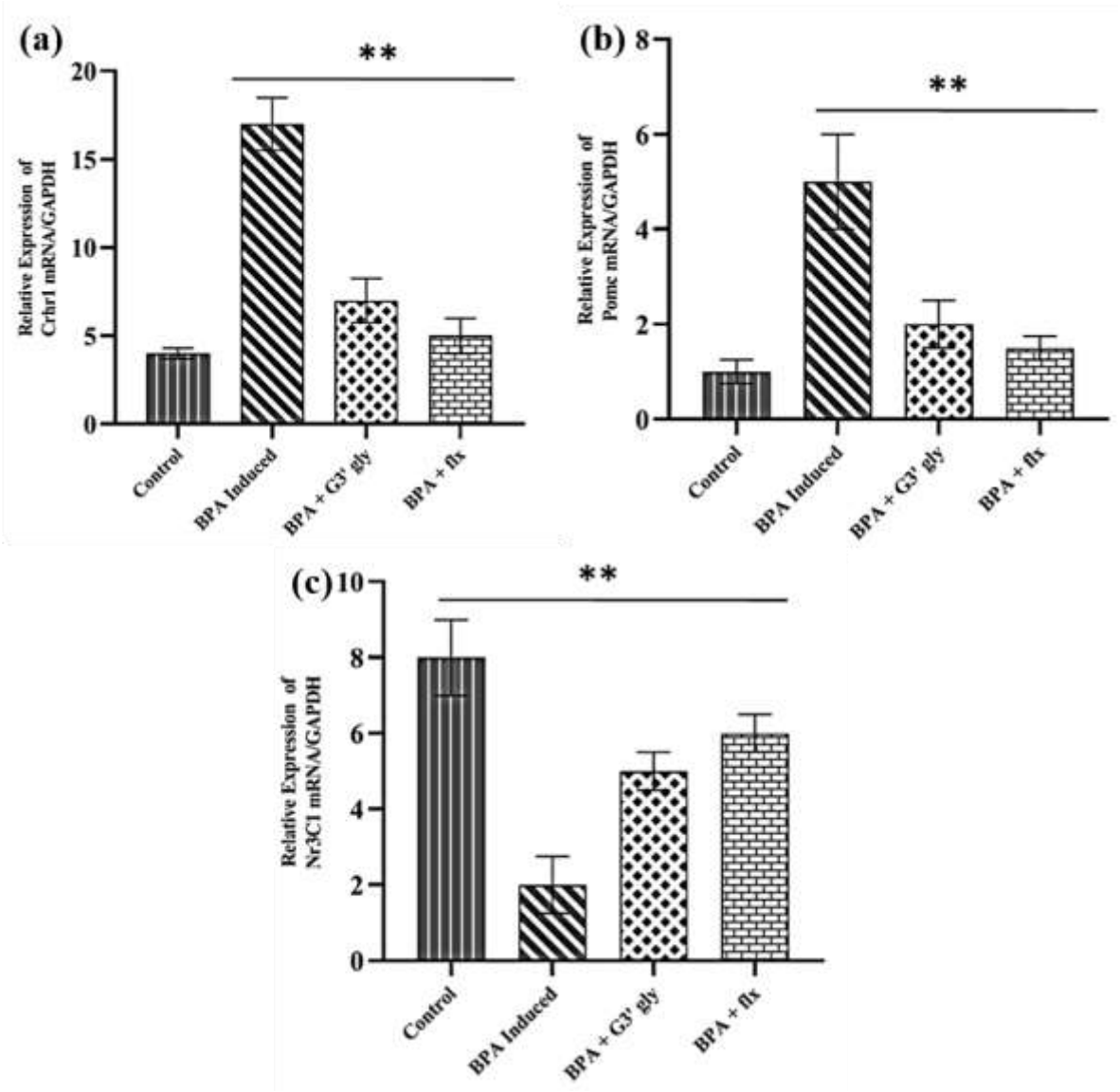


**Figure 3. Graphical representation of RT-PCR Analysis**: Relative mRNA expression of (a) CRHR1 (b) POMC (c) NR3C1 genes of Control, BPA induced, BPA induced and treated with G-3′gly and Flx treated AtT 20 cells. Data are represented as means ± SD (n =3) $^{**}p<0.01$

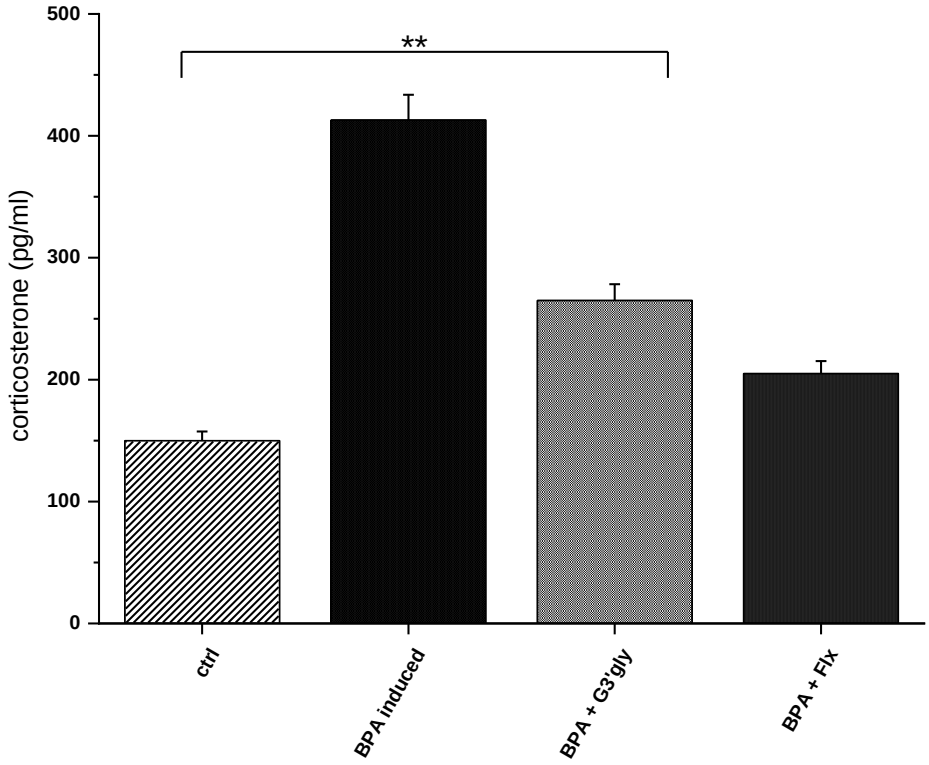


**Figure 4. Graphical representation of corticosterone**: Expression of the stress marker corticosterone of Control, BPA induced, BPA induced and treated with G-3′gly and Flx treated AtT 20 cells. Data are represented as means ± SD (n =3) ** $p<0.01$

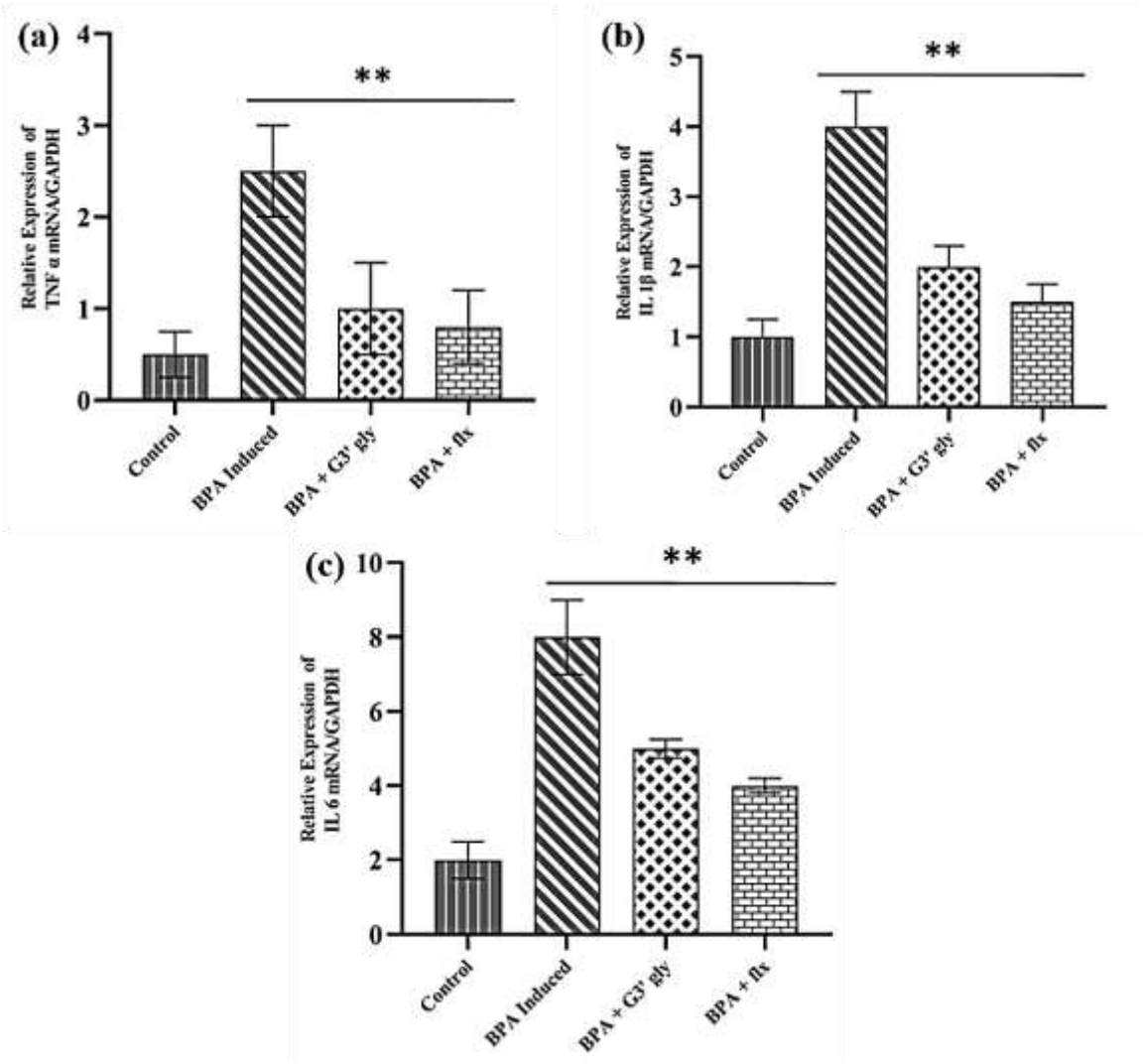


**Figure 5. Graphical representation of RT-PCR Analysis**: Relative mRNA expression of (a) TNF- α (b) IL -1β (c) IL – 6 genes of Control, BPA induced, BPA induced and treated with G-3′gly and Flx treated AtT 20 cells. Data are represented as means ± SD (n =3) $^{**}p<0.01$

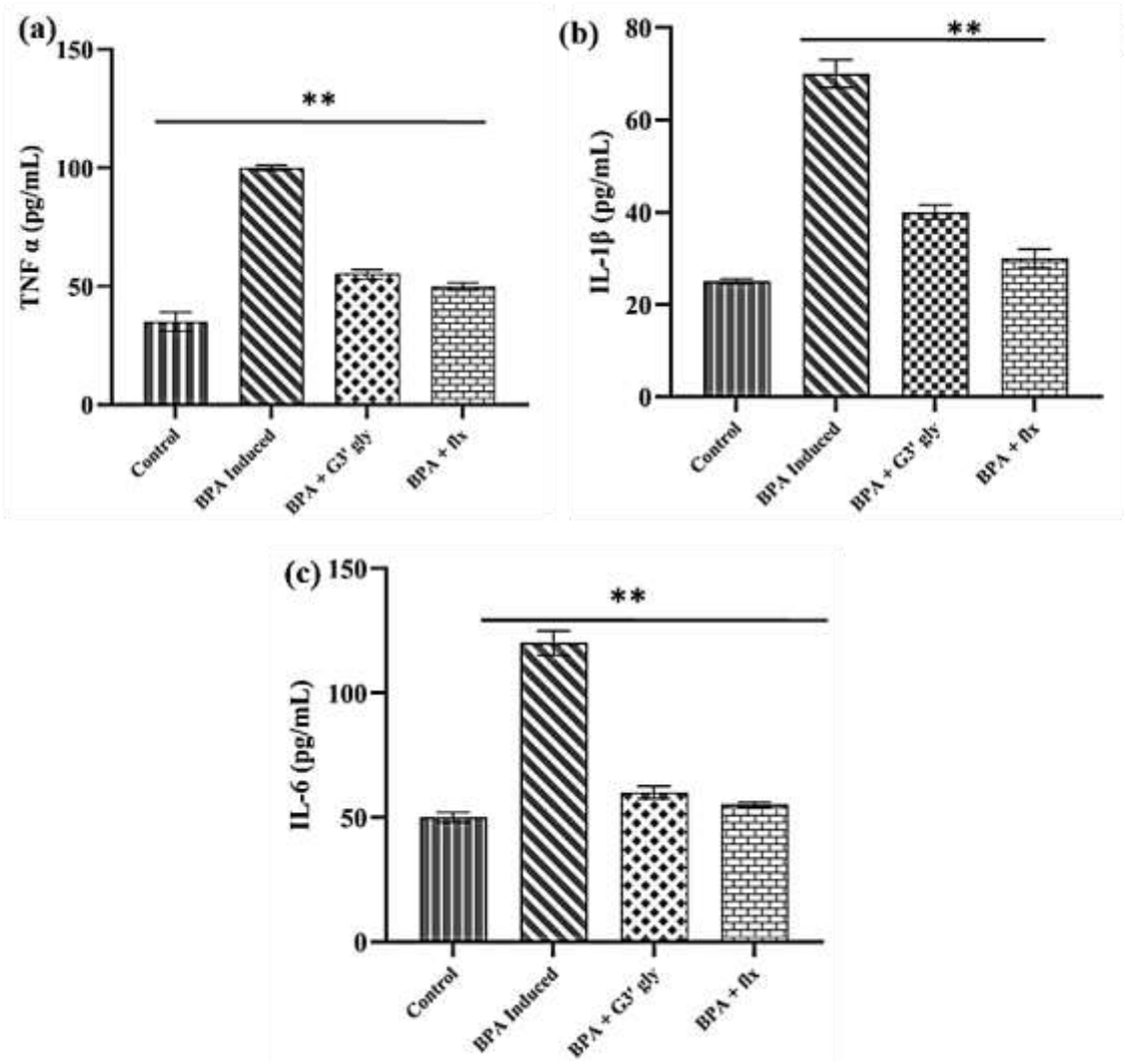


**Figure 6. Graphical representation of protein expression by ELISA**: Protein expression of (a) TNF- α (b) IL - 1β (c) IL – 6 genes of Control, BPA induced, BPA induced and treated with G-3′gly and Flx treated AtT 20 cells. Data are represented as means ± SD (n =3) $^{**}p<0.01$