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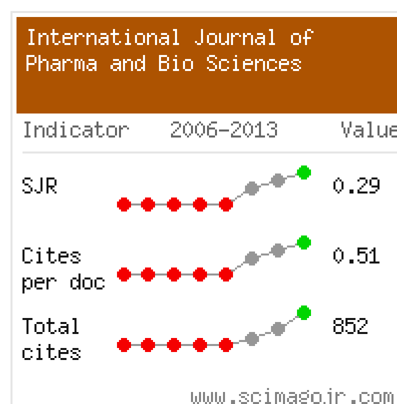

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GREEN COFFEE EXTRACT INDUCES ACTIVATION MARKERS IN J774.1 MACROPHAGES AND PROTECTS THEM FROM DOXORUBICIN INDUCED APOPTOSIS

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ABSTRACT

Green Coffee Extract (GCE) is a popular health supplement known for its anti-obesity & anti-diabetic effects. Here we tested GCE for its capacity to activate J774.1 mouse macrophages and protect against Doxorubicin induced apoptosis. J774.1 cells treated for 24 h with 100 and 500 µg/ml GCE showed increased production of IL-6, TNF-α and nitric oxide (NO) in a dose dependent manner. GCE upregulated production of these molecules by inducing transcription of the IL-6, TNF-α and iNOS genes. In addition, GCE was able to restore viability of J774.1 cells treated with Doxorubicin. Pre-treatment of cells with GCE for 1 h abrogated Dox induced loss of cell viability. While Dox robustly induced caspase 3/7 activity within 6 h of treatment, pre-treatment with GCE for 1 h was enough to attenuate this induction. The results we report here suggest that GCE activates J774.1 macrophages and is cytoprotective against Dox induced apoptosis.

KEYWORDS:*Green Coffee Extract,Doxorubicin, J774.1, macrophages, Caspase 3/7, cytokines, NO, iNOS*

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INTRODUCTION

Macrophages are important effectors of innate and adaptive immunity and need to be activated to perform this function. The hallmark of macrophage activation is the secretion of inflammatory molecules like IL-6 and TNF-α as well as cytotoxic molecules like nitric oxide.[1,2] Activating macrophages results in increased immune function and the body's ability to fight pathogens.[3] Instances of lowered immune function are common in various situations. It is well known that one of the serious side effects of doxorubicin (Dox), a potent chemotherapeutic drug, is immune suppression and Doxtreated macrophages show increased apoptosis.[4,5] Cytokines like IL-6 and TNF-α are pleiotropic molecules and can be cytoprotective. IL-6 has been shown to protect macrophages from viral infection,[6] promote alternative activation for establishing inflammatory homeostasis[7] and protect from toxic free cholesterol accumulation.[8] More pertinently, there is evidence that IL-6 signaling through the STAT3 pathway sends pro-survival signals to macrophages under ER-stress and thus helps in macrophage survival and health.[9] TNF-α can be pro or anti-apoptotic, depending on the cellular context and receptor subtype engagement.[10] Macrophage apoptosis is observed in arthritic patients treated with anti-TNF-α antibodies[11] and Lo et al[12] showed that treatment with TNF-α prevents methotrexate induced macrophage apoptosis. NO is a molecule that is known to have dual effects[13] and low concentrations of this molecule are cytoprotective to macrophages while high concentrations are cytotoxic.[14,15] It has been demonstrated that increase in NO production systemically as well as in the lungs and liver restores heart function in doxorubicin induced cardiac toxicity.[16]Coffee is the most popular beverage consumed all over the world. It is a complex mixture of about a thousand different components.[17] While roasted coffee is consumed as a beverage, green unroasted coffee is very popular as a health supplement with putative anti-diabetic,[18] anti-hypertensive[19] and neuroprotective[20]and even anti-bacterial properties[38]. While there are reports of anti-inflammatory effects of coffee,[21] there has been no strong epidemiological evidence that coffee consumption is associated with lower inflammatory marker levels.[22] Thus there seems to be no consensus as to the nature of the relationship between coffee and inflammation. We have already reported that a hydroalcoholic green coffee extract (GCE) protects H9C2 cardiomyocytes from Doxorubicin induced cell death.[23] In this study we provide evidence that GCE upregulates activation markers in J774.1 macrophages, increases production of cytokines TNF-α and IL-6 at the level of transcription and protein. GCE increases production of NO from J774.1 cells and induces transcription of the enzyme iNOS. GCE also protects them from Doxorubicin induced apoptosis by preventing Caspase activation. These hitherto unknown activities of GCE pave way for new applications for green coffee as a strongly cytoprotective functional food.

MATERIALS AND METHODS

All cell culture reagents like Dulbecco's Mimimal Essential Medium (DMEM), glutamine, fetal bovine serum (FBS), penicillin, streptomycin, trypsin, were obtained from Invitrogen. DMSO and Doxorubicin were products of Sigma Aldrich. All reagents were of cell culture or analytical grades.

(i) Cells and cell culture

J774.1 mouse macrophage-like cell line and A549 lung cancer cell line were obtained from ATCC. J774.1 cells were maintained in DMEM supplemented with 10% FBS and with penicillin and streptomycin. A549 cells were maintained in DMEM supplemented with 2 mM Glutamine, 10% FBS, penicillin and streptomycin. The cultures were incubated at 37^0C under humidified 5% CO_2 and 95% air. Adherent cells were gently detached using 0.25% trypsin and 0.02% EDTA.

(ii) Preparation of GCE

The decaffeinated hydroalcoholic Green Coffee Extract (GCE) used in this study was kindly supplied by Sanat Products (Delhi, India). Green coffee beans from the plant *Coffeaarabica* were milled and extracted for 24 h with 80:20 aqueous alcohol, concentrated to a thick liquid in vacuum, filtered, dried and pounded to a fine powder. The extract was weighed out freshly for each experiment and dissolved in incomplete cell culture medium (DMEM) at the required concentration before cell treatment.

(iii) Analysis of cytokine and NO production

J774.1 cells (10,000 cell/well) cultured overnight in a 96 well cell culture plate were treated with GCE (100 and 500 µg/ml) or LPS (1 µg/ml). After 24 h of incubation, cell free supernatant was used to determine the amount of nitrite by using Griess reagent (Invitrogen) as per manufacturer's instructions. The amount of IL-6 and TNF-α were also quantified in these supernates using commercially available ELISA kits (R&D Systems) according to manufacturer's instructions. Cell pellets were used for real-time RT-PCR.

(iv) Analysis of gene expression

J774.1 cells (10,000 cells/well) were cultured overnight in 96 well cell culture plate and treated with GCE (100 and 500 µg/ml) for 6 h. Total RNA was extracted fromcells using Trizol Reagent (Ambion) according to manufacturer's instructions. RT-qPCR was performed using SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen) employing the PikoReal 96 real time PCR instrument (Thermofisher). Primer pairs used are listed in Table 1. GAPDH gene expression was used as an endogenous control and relative gene expression was calculated based on the comparative CT method. Data were shown as the fold difference normalized to GAPDH.

(v) Alamar Blue Viability Assay

J774.1 cells (10,000 cell/well) or A549 cells (4,000 cells/well) cultured overnight in a 96 well black walled clear bottom plate (Corning) were pre-treated for 1 h with GCE (50, 100 and 500 µg/ml) with subsequent addition of 1 µM Doxorubicin for 72 hrs. Following this, cells were incubated with 1X Alamar Blue (Invitrogen) for 3 h at 37^0C and fluorescence was read in a Tecan Infinite M1000 Pro instrument at 544/590nm (Excitation/Emission). All estimations were performed in triplicate.

(vi) Caspase activity assay

J774.1 cells (4,500 cells/well) were cultured overnight in a white-walled opaque 384-well plate (Corning). The cells were pretreated for 1 h with GCE at 100 and 500 µg/ml and then Doxorubicin was added to a final concentration of 1 or 3 µM for 6 h. Caspase 3/7 activity was measured using the Caspase 3/7 Glo kit (Promega) according to manufacturer's instructions. Luminescence was quantified with a Tecan´s Infinite M1000 Pro instrument. The assay was performed in triplicate.

(vii) Statistical analysis

Each result was expressed as the mean ± standard error of mean (SEM). One way ANOVA followed by Tukey's test was applied.

Table 1

Sequences of primers for Real-time RT-PCR

Primer	Sequence (5'-3')
iNOS	GTAAACTGCAAGAGAACGGAGAAC (Forward) GAGCTCCTCCAGAGGGGTAG (Reverse)
TNF-α	CCCTCACACTCAGATCATCTTCTForward) GCTACGACGTGGGCTACAG (Reverse)
IL-6	GACAAAGCCAGAGTCCTTCAGAGAG (Forward) CTAGGTTTGCCGAGTAGATCTC (Reverse)
GAPDH	ATGTTTGTGATGGGTGTGAA (Forward) ATGCCAAAGTTGTCATGGAT (Reverse)

RESULTS AND DISCUSSION

1. Effect of GCE on cytokine gene expression and cytokine production by J774.1 cells

Activation of macrophages leads to the production of pro-inflammatory cytokines like TNF-α and IL-6. Figure 1 (A) and (C) shows that GCE stimulated the expression of the cytokines TNF-α and IL-6 at mRNA level within 6 h of treatment. The induction of these genes increased with increase in dose of GCE as well as time of incubation with GCE (data not shown). To understand if gene expression translated to protein production, the levels of TNF-α and IL-6 protein were measured after 24 h of GCE treatment. As can be seen from Figure 1 (B) and (D), GCE treatment resulted in a dose-dependent increase in TNF-α and IL-6 release within 24 h.Green coffee, or certain purified components of green coffee have been reported to be anti-inflammatory by several groups, with reports of decreased cytokine levels in inflammatory conditions like carrageenan induced paw edema, diabetes [24-27]etc. But in this study we observed induction of IL-6 production by J774.1 macrophages by GCE.These differences in results can be due to differences in the mode of extract preparation, the species of *Coffea* used and possibly even due to combining treatment with extracts from other plants. For example, in the study by Moriera et al [24] the green coffee extract is shown to decrease cytokine levels in carrageenan induced paw edema, the extract was prepared by boiling the coffee beans in water, while the extract used in this study was a hydroalcoholic extract. In the study by Chang et al [27] where treatment showed decrease in cytokine levels, green coffee was combined with several other extracts from cocoa, green tea and garcinia. While IL-6 and TNF-α have a reputation for being pro-inflammatory, a lot of evidence exists to show that these molecules can be cytoprotective towards immune cells.[9,12] We later sought to understand if this cytokine production due to GCE could be cytoprotective towards J774.1 macrophages treated with Doxorubicin treatment.

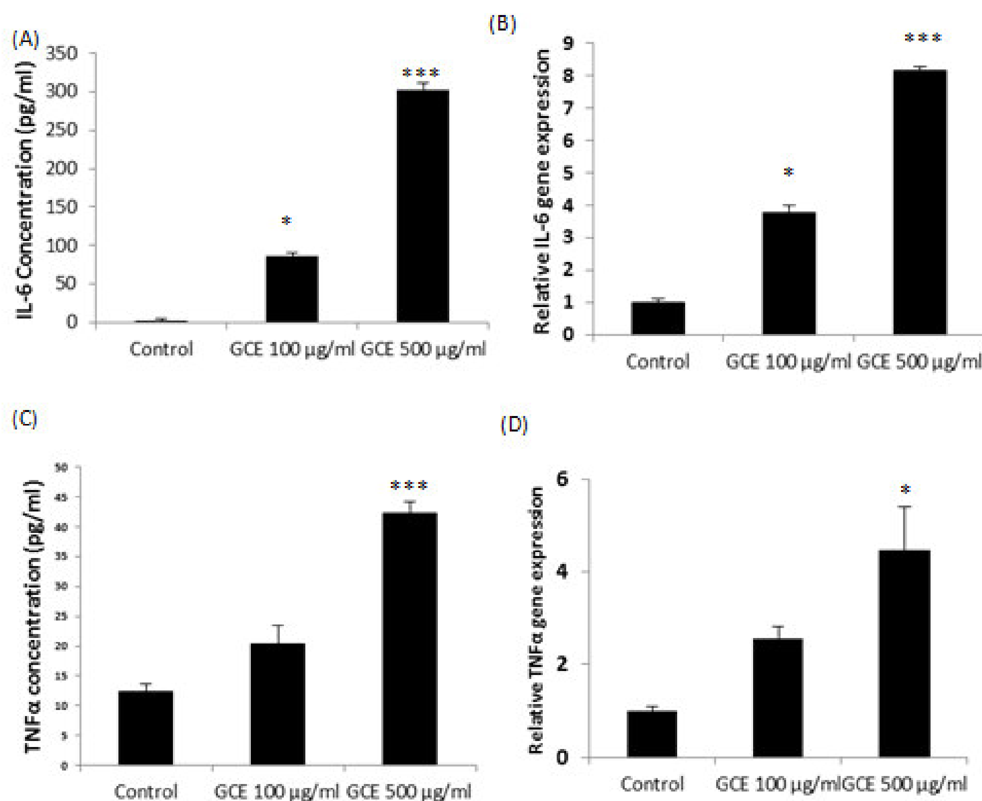

Figure 1

GCE upregulates cytokine production and cytokine gene expression in J774.1 cells. (A) & (C) Production of IL-6 and TNF-α by J774.1 cells after 24 h of treatment (B) & (C) Relative gene expression of IL-6 and TNF-α after 6 h of treatment, normalized to GAPDH used as internal control. Significant differences compared with control are indicated with $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

2. Effect of GCE on NO production and iNOS gene expression

Nitric Oxide (NO) is a free radical produced by activated macrophages,[1] and helps macrophage function by inhibiting replication of pathogens like bacteria and viruses, and also of tumor cells.[28] Since inducing NO production is a hallmark of immunomodulatory activity, we evaluated if GCE possesses this capacity. As can be seen from Figure 2, GCE was able to induce statistically significant production at 500 µg/ml from J774.1 macrophages, though there was a definite trend of induction even at 100 µg/ml. LPS was used as a positive control and the level of induction of NO by GCE was a 2.5 fold lower than by LPS. Yoshioka et al[14,15] have demonstrated that low NO concentrations are cytoprotective to macrophages while high concentrations are cytotoxic. GCE induced NO production at a lower level,suggestingcytoprotective activity. NO is synthesized from L-arginine by inducible nitric oxide synthase (iNOS), and upregulation of (iNOS) gene expression is predominantly responsible for macrophage NO production.[29]To understand if induction of NO production by GCE is affected at the level of gene expression, iNOS gene expression was quantitated in GCE treated J774.1 cells. Figure 2 (B) shows that GCE indeed upregulated the expression of iNOS within 6 h of treatment dose dependently. This suggests that GCE regulates NO production at transcription level.

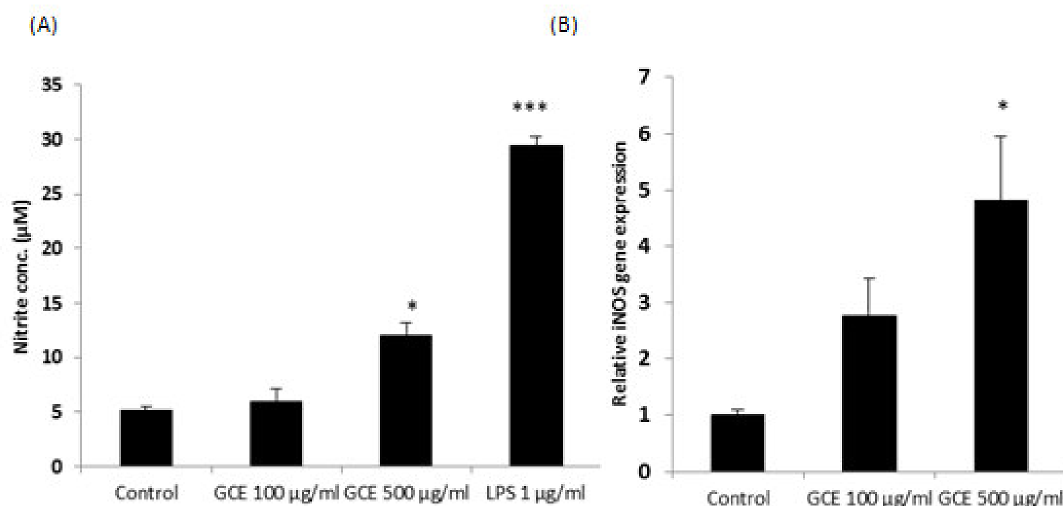

Figure 2

GCE induces NO production and iNOS gene expression in J774.1 cells. (A) Production of NO by J774.1 cells after 24 h of treatment (B) Relative gene expression of iNOS gene in J774.1 cells after 6 h of treatment, normalized to GAPDH used as internal control. Significant differences compared with control are indicated with $^{*}p < 0.05$ and $^{***}p < 0.01$.

3. Effect of GCE on Dox induced loss of cell viability

The data presented above suggests that GCE can function as an immunomodulator in J774.1 macrophages. To ascertain whether it is cytoprotective, J774.1 cells were treated with Doxorubicin in the presence and absence of GCE. Doxorubicin is a chemotherapeutic drug that is known to be immunosuppressive and cytotoxic to macrophages. There are many studies that report reduced macrophage viability in the presence of Dox.[4,5] As can be seen in Figure 3 (A), Dox reduced the cell viability of J774.1 macrophages to 57.5%. GCE was able to restore cell viability to control levels in a dose dependent manner. To understand if GCE interferes with Dox anti-cancer capability, we treated the lung cancer cell line A549 with Dox in the presence and absence of GCE. The viability of J774.1 cells in the presence of Dox was restored to Vehicle Control levels by pretreatmentwith 100 µg/ml of GCE, and at the same dose, GCE did not interfere with the ability of Dox to kill A549 lung cancer cells (Figure 3 (B)).

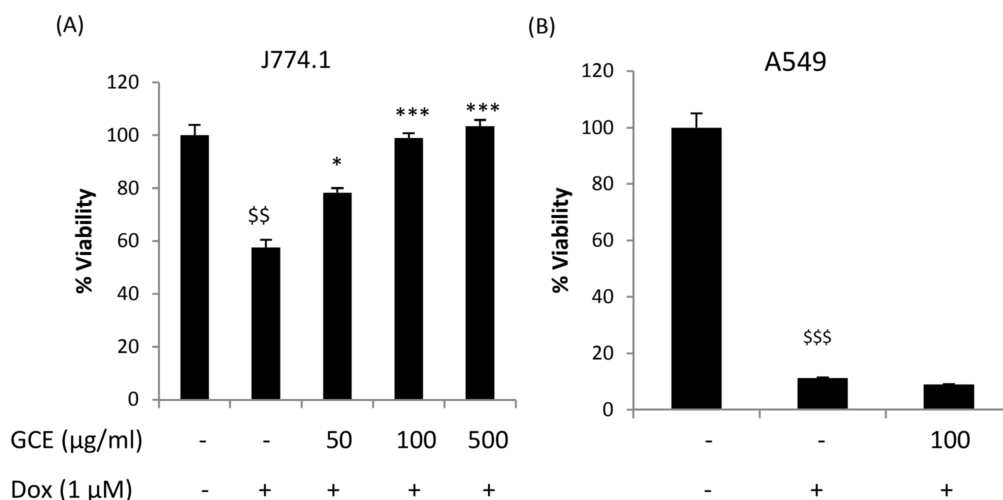

Figure3

GCEattenuates Dox induced loss of viability in J774.1 cells but does not interfere with Dox ability to kill A549 lung cancer cells. (A) Effect on viability in J774.1 cells. (B) Effect on viability in A549 cells.Significant differences compared with control are indicated with $^{\$\$}p < 0.01$, $^{\$\$\$}p<0.001$; significant differences compared with Dox treatment are indicated with * p<0.05; *** p<0.001.

4. GCE effect on Caspase 3/7 activation by Dox

Caspase 3 and Caspase 7 belong to the caspase family of proteases that play key roles in the apoptotic process. These enzymes are known as the executioner caspases, and are essentially the effector proteins of the cellular apoptotic process.[30] Activation of Caspase-3 has been identified as a key step in Dox mediated apoptosis in non-tumor cells.[31] Both the intrinsic and extrinsic pathways of apoptosis converge at the point of Caspase 3 activation and Dox is known to activate both these pathways.[31] There is extensive evidence that suggests that suppression of activation of Caspase 3 rescues cells from Dox-induced apoptosis in a variety of cell types.[32,33,34,35] Figure 4 shows that in our study, Dox treatment of J774.1 cells at 1 and 3 μM activated Caspase 3 by 1.7 and 9.6 fold respectively, within 6 h. Pre-treatment with GCE for 1 h attenuated Caspase 3 activation in a dose-dependent manner. This demonstrates that GCE protects J774.1 macrophages from Dox induced apoptosis robustly and dose-dependently.

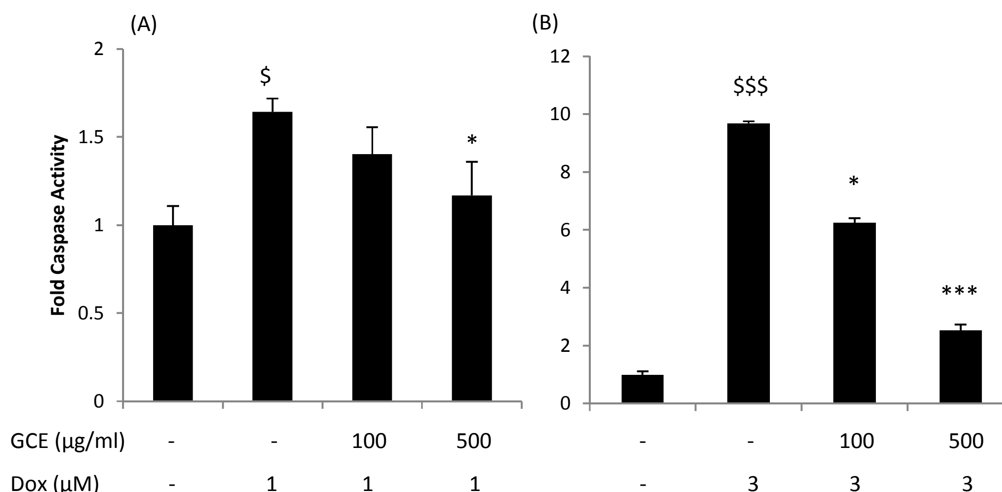

Figure 4

GCE attenuatesDox-induced Caspase 3/7 activity in J774.1 cells dose-dependently.Caspase activity was measured 6 h after Dox treatment. (A) Dox treatment at 1 μM (B) Dox treatment at 3 μM. Significant differences compared with control are indicated with $^{\$}p < 0.05$, $^{\$\$\$}p<0.001$; compared with Dox treatment are indicated with *p<0.05; ***p<0.001.

Taken together, the picture that emerges of GCE is one of an immunomodulatory plant extract that is also cytoprotective. All the three molecules induced by GCE, IL-6, TNF-α and NO are pleotropic in nature, and protective towards macrophages. IL-6 signaling through STAT3 is known to prevent ER-stress mediated apoptosis in macrophages.[9]TNF-α administration prevents macrophage apoptosis induced by a chemotherapeutic agent (methotrexate).[12] NO is a cytotoxic molecule that is produced by macrophages but protects macrophages themselves by inducing catalase expression. Yoshioka et al explain that a balance between NO and its metabolite peroxynitrite decides whether the NO is cytotoxic or cytoprotective.[14,15]Doxorubicin induces apoptosis in macrophages and it is possible that GCE induction of cytokines and NO work towards attenuating Dox induced damage in several ways. NO might work through catalase induction, preventing ROS effects, IL-6 and TNF-α through induction of survival signals to compete with Dox-induced apoptotic signals. Chlorogenic acid has recently been shown to protect H9C2 cardiomyocytes from Dox induced cell damage byMandzuik et al.,[36]Chlorogenic acids form a large component of GCE,[37] and it is a strong possibility that this component is responsible for some or all of the cytoprotective and anti-apoptotic effects of GCE seen here.

CONCLUSION

The results and discussion presented above based on our data suggests that GCE can activate J774.1 macrophages and induce production of NO and cytokines by regulating the expression of their respective genes. GCE also protects J774.1 cells from Dox induced apoptosis. It is possible that the cytokines and NO induced by GCE confer protection against apoptotic signals from Dox treatment. This is the first time that GCE has been demonstrated to have cytoprotective and immunomodulatory activities towards J774.1 macrophages. In continuation with our previous work showing that GCE protects cardiomyocytes from Dox-induced apoptosis, the present study has implications for the application of green coffee in alleviating chemotherapy induced cellular toxicity. An immunomodulatory plant extract that increases immune function and protects macrophages in the presence of chemotherapeutics like Dox would address an important unmet medical need.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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