Accepted Version

A Novel Biological Role of α-Mangostin in Modulating Inflammatory Response Through the Activation of SIRT-1 Signaling Pathway

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Abstract

Several studies have shown that xanthones obtained from Garcinia Mangostana (GM) have remarkable biological activities. α-Mangostin (α-MG) is the main constituent of the fruit hull of the GM. Several findings have suggested that SIRT-1, a nuclear histone deacetylase, could influence cellular function by the inhibition of NF-kB signaling. ROS can inhibit SIRT-1 activity by initiating oxidative modifications on its cysteine residues, and suppression of SIRT-1 enhances the NF-kB signaling resulting in inflammatory responses. The goals of the present study were to evaluate the quantity of α-MG in the methanolic extract of GM (Vithagroup Spa) and to investigate the activity of this xanthone in U937 cell line and in human monocytes from responsive to inflammatory insult analyzing the possible changes on the activation of SIRT-1 protein via NF-Kb. Cells were treated with the methanolic extract of GM and/or LPS. The chromatographic separation of α-MG was performed by an HPLC analysis. EX 527, a specific SIRT-1 inhibitor, was used to determine if SIRT-1/NfkB signaling pathway might be involved in α-MG action on cells. Our results show that α-MG inhibits p65 acetylation and down-regulates the pro-inflammatory gene products as COX-2, iNOS via SIRT-1 activation. Cells treated with EX 527 showed an up-regulation of NFkB acetylation and an over expression of inducible enzymes and their product of catalysis (NO and PGE2). These results suggest that α-MG may be useful for the development of alternative pharmacological strategies aimed at reducing the inflammatory process.

Introduction

Inflammation is a major condition associated with various diseases and is a natural host defensive response to invading pathogens and tissue injury with the involvement of phagocytic cells such as macrophages, mast cells, dendritic cells, granulocytes, and innate lymphocytes (Speranza et al., 2012c; Pesce et al., 2014). In many cases, inflammation is demonstrated to be an important contributor to diseases such as cancer, diabetes, and cardiovascular diseases (Speranza et al., 2012a; Khuda-Bukhsh et al., 2014). The Silent

Information Regulator-1 (Sirtuin1, SIRT-1), a member of NADþ dependent class III histone deacetylase, has been shown to be involved in a variety of pathophysiological processes, such as inflammation, the modulation of cell growth and metabolism, and carcinogenesis. SIRT-1 regulates inflammation by modulating various pro-inflammatory mediators; in particular it inhibits NF-kB signaling which is a major inducer of inflammatory responses (Hubbard and Sinclair, 2014). Moreover, SIRT-1 acts against oxidative stress and can stimulate the expression of antioxidants via the Foxo pathway (Hori et al., 2013; Wang et al., 2015). The Reactive Oxygen Species (ROS) can inhibit SIRT-1 activity by initiating oxidative modifications on its cysteine residues, and the suppression of SIRT1 enhances the NF-kB signaling resulting in inflammatory responses and further, the modulation of many of its downstream mediators (Stein and Matter, 2011; Kauppinen et al., 2013). The sources of free radicals and ROS during inflammation vary and can be the secondary product of certain cellular metabolic reactions or can be to the action of specific enzymes that synthesize them actively as the NADPH oxidase, xanthine oxidase, a lipoxygenase, cyclooxygenase (COX), nitric oxide sinthase (NOS), and cytochrome p450 oxygenase (Speranza et al., 2007). It has been reported and shown that the genes for the majority of the pro-inflammatory proteins are regulated by the nuclear transcription factor NF-kB. The over expression of catabolic enzymes by pro-inflammatory mediators is initiated by a set of pro-inflammatory signaling pathways, such as Tumor Necrosis Factor (TNF)-a, Interleukin (IL)-1, and inflammatory molecules, such as Nitric Oxide (NO), Prostaglandins (PG) (Speranza et al., 2009; Korbecki et al., 2013).

In clinics, several anti-inflammatory drugs are commonly prescribed for pain relief in inflammatory conditions. However, their continual use is associated with serious adverse effects like damage of the whole gastrointestinal disease, salt and water retention, elevation of serum hepatic transaminases, and also anemia and blood loss (Sostres et al., 2013). In light of these observations patients as well as health care providers prefer to use alternative therapeutic agents as they are considered to be safe and effective in alleviating inflammation. Several medicinal plants have been reported as an important source of new chemical moieties with potential therapeutic effects. The studies on plants with substantial folkloric use as anti-inflammatory agents are viewed as a productive and logical research strategy in the search for new anti-inflammatory drugs (Ortega-Ramirez et al., 2014; Ramalingum and Mahomoodally, 2014). The attention of pharmacologists throughout the world have focused on finding safer and strong anti-inflammatory natural drugs. Today, natural products symbolize safety in contrast to the synthetic drugs that are regarded as unsafe to humans and to the environment. Therefore, people are going back to using natural products, with the hope of being safer and secure. Plants that contain active components, namely phenolics and polyphenolics, have raised public interest in their potential to act as antioxidants and as an anti-inflammatory (Daglia et al., 2014; Pesce et al., 2015b). Hence, this study focuses on evaluating the anti-inflammatory activity of the whole plant of Garcinia Mangostana (GM), Mangosteen is a tropical fruit available in South-East Asia. It has been used as traditional medicine to treat skin infections, wounds, and diarrhea. Mangosteen, Garcinia mangostana L. (Guttifereae), is a tree that is fairly widespread in Thailand, India, Srilanka, and Myanmar and is known for its medicinal properties. The fruit hull of this plant has been widely used for many years in South-East Asia as a traditional anti-inflammatory agent (Obolskiy et al., 2009; Gutierrez-Orozco and Failla, 2013). The GM has been shown to contain a variety of secondary metabolites such as prenylated and oxygenated xanthones that have been classified in five groups: (a) simple oxygenated xanthones, (b) xanthone glycosides, (c) prenylated xanthones, (d) xanthonolignoids, and (e) miscellaneous xanthones (Ee et al., 2014; Mohamed et al., 2014). Several studies have shown that xanthones obtained from the Mangosteen-fruit have remarkable biological activities (including anti-infectious, anti-oxidative, anti-inflammatory, anti-proliferative, pro-apoptotic, and anti-metastatic). a-Mangostin (α-MG) is the main constituent of the fruit hull of the Mangosteen (Ngawhirunpat et al., 2010). Two studies by Jang et al. (2012) and Higuchi et al. (2013) have established that the crude extract of GM has a very potent anti-inflammatory effect on animal model. The compounds a- and g-Mangostin may be responsible for this effect. The goals of this study were to evaluate the quantity of α-MG in the extract of GM (Vithagroup Spa) and to investigate the activity of this xanthone on the U937 cell line and peripheral

blood monocytes from responsive to inflammatory insult analyzing the possible changes on the activation of SIRT-1 protein via NF-Kb.

Materials and Methods

Materials

The GM methanolic extract was purchased from Vithagroup Spa (Italy). α-MG, Lipopolysaccharides, DMSO, fetal calf serum, streptomycin, penicillin, L-glutamine, 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide, Nitro Blue-tetrazolium, potassium phosphate, catalase, xanthine, xanthine oxidase, Griess reagent, formic acid, acetonitrile, and methanol were obtained from Sigma–Aldrich (US/Canada). The primary antibody acetylNFkB was obtained from Abcam, (Cambridge, UK). The primary antibodies: anti-sirtuin 1, p65-NFkB, iNOS, and COX2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The secondary antibodies actin and tubulin were obtained from Sigma– Aldrich. The U937 mononuclear cells were purchased from American Type Culture Collection (Manassas, VA) and RPMI 1640 medium was obtained from GIBCO (Invitrogen, Carlsbad).

Apparatus and chromatographic conditions

The chromatographic analysis was carried out using a HPLC system (Waters, Milford, MA) combined with a pump (Model 600), absorbance detector (Model PDA 2996), thermostat column compartment (Model Jetstream2 Plus), and a degasser (Model 1260). A Rheodyne model 7125i injector (Rheodyne, Cotati, CA) equipped with 20ml loop was used. The entire configuration was operated by an Empore Software System Manager. A Visiprep SPE Vacuum Manifold equipped with a Visidry Drying Attachment (Supelco, Bellefonte) was used for solvents evaporation. The chromatographic separation was performed on a Kinetex C18 (100 4.6 mm I.D., 2.6 mm particle size) column that was maintained at 25°C, protected by a disposable Security Guard C18 pre-column (3.0 4.0 mm) (Phenomenex, Torrance, CA). The mobile phase consisted of a mixture of formic acid solution (0.1% v/v), acetonitrile and methanol in a ratio of 30:60:10, v/v/v. All separations were performed in an isocratic mode at a flow rate of 1.0 ml/min. The ultraviolet detection wavelength was set to 316 nm. The acetonitrile and methanol were filtered before use through a 0.45mm WTP membrane (Whatmann, Maidstone, UK). The total run time was 15 min.

Preparation of stock and standard solutions

The Stock standard solutions of α-MG or 1,3,6-trihydroxy-7- methyoxy-2,8-bis-(3-methyl-2-butenyl)-9H-xanthone-9-one, were prepared in methanol at a concentration of 1.0 mg/ml and further diluted with methanol to make a series of working solutions from 5.0 to 80mg/ml. All stock and working solutions were kept at 20°C in glass vials and brought to room temperature before use. Independent stock solutions were prepared for calibration and quality control. Standards for the calibration curves and quality control samples were then prepared using serial dilutions of this stock solution. Pooled Quality Control (QC) samples of α-MG were prepared to determine the limit of quantitation, the intra and inter-assay precision and accuracy of the method and to evaluate the stability of the analyte when stored under several conditions. All control pools were aliquoted into glass vials and stored at 20 °C until analysis.

Method validation

To demonstrate the feasibility of the present approach for determination of α-Mangostin and to test its practicability, the method was validated in according to the ICH Guidelines (FDA, Guidance for Industry, 2003). The calibration curves were constructed by adding aliquots of 25 ml of analyte standard solutions at seven different concentrations and injected into the LC system. This procedure was done in triplicate for each point. The analyte peak area obtained were plotted against the corresponding concentrations of the analyte expressed as mg/ml in the range of 5–80 mg/ml. The calibration curves were constructed by means of the last-square method. One stock solution was used for each replicate: different working solutions were prepared from the stock solutions and added to the blank plasma samples to obtain the different

concentrations. The values of limit of quantification (LOQ) and limit of detection (LOD) were calculated as the analyte concentrations which give rise to peaks whose heights are 10 and 3 times the baseline noise, respectively.4

Cell culture

The U937cells were cultured in a 5% CO2 atmosphere in RPMI 1640 medium containing 10% fetal calf serum, 100 ng/ml streptomycin, 100 U/ml penicillin, and 2 mM L-glutamine. Peripheral blood mononuclear cells (PBMCs), supplied from donors, were separated by Ficoll gradient as described previously (Speranza et al., 2012b). Aliquots of PBMC were incubated at 10 106 cells/ml in RPMI 1640 supplemented with 10% fetal calf serum for 1 h at 37°C in a 5% CO2 incubator in plastic tissue culture flasks. The cells were allowed to adhere for 1 h, and not more, to avoid further activation. After that time the supernatants, which contained non-adherent cells, were discarded and the remaining adherent monocytes (about 10% of the original population) were removed from the flask by vigorous washing with ice-cold phosphate-buffered saline (PBS) and scraping of the flask. The isolated cells were washed and their viability tested. U937 and human monocytes cells were seeded (at 2 105 cells per well) onto six-well tissue culture plates and cultured in medium with LPS (10mg/ml) for 24 h, in the presence or absence of the GM, α-MG, and/or Sirtuine Inhibitor (EX527) which were added to the culture medium 30 min prior to the LPS treatment. Control cells did not contain GM, α-MG, and/or LPS. In all experiments, equal volumes of PBS or DMSO were added to the medium of control cultures (controls were performed using non-stimulated cells). Both concentrations of LPS, GM, α-MG, and Sirtuine Inhibitor were chosen according to our preliminary optimization studies. After incubation, cells were harvested by centrifugation to assess cellular viability, gene, and protein expression. Media of U937 cells were collected in order to evaluate the PGE2 and NO release.4

MTT assay for cell viability and cytotoxicity

The MTT assay was used to assess cell damage by the oxidants and cell viability protection by the extracts (Franceschelli et al., 2014). Briefly, the U937 cells were seeded on 96-well plates at a density of 86103 cells/well, cultured and treated according to the method described above. A total of 20ml of MTT was added at a concentration of 0.5 mg/ml after medium (200 ml) was added to each well. The plates were incubated at 37°C for 4 h to dissolve the formazan that had formed. The solution (220 ml) was removed from each well and 150 ml of DMSO was added. The reduced MTT was measured on an ELISA reader (Bio-Rad, Hercules, CA, USA) at a wavelength of 570 nm. The percentage cell viability was calculated according to the equation below:

% = (Absorbance of treated cells/Absorbance of control cells)x1004

NitroBlue-tetrazolium (NBT) assay

This assay is used to detect the superoxide dismutation potential of the extract. As described previously (Patruno et al., 2015), the following were added to each well of a 96-well plate: 100ml potassium phosphate buffer, pH 7.8 (50 mM), 5ml catalase, 25ml NBT (5.6 10 9 M), 50ml xanthine (0.1 mM), 50ml xanthine oxidase (0.1 mM) and herbal extract in the concentration range between 0.01 and 10 mg/ml protein. Following the addition of NBT the plates were allowed to stand at room temperature for 1 h until the blue color had developed and the absorbance was measured at 560 nm.4

Quantitative real-time PCR

A Quantitative Real-time PCR assay was carried out in an Eppendorf Mastercycler EP Realplex (Eppendorf AG) as described previously (Pesce et al., 2014). Briefly, the preliminary PCR were run to optimize the concentration and ratio of each primer set. Primers NFkB, iNOS, COX2, and GAPDH as control were designed using GeneWorks software (IntelliGenetix, Inc., Mountain View, CA). The primer pairs used were as follows: acetyl NFkB forward-TGGCGTGGCAGGAGAACAG and reverse-GC AGTT GGTCACAGAGTAGGGC; GADPH forward-GAAGGT GAAGGTCGGAGTC and reverse-GAAGATGGTGATGGGA TTTC; COX2 forward-

TGATGGCGAAGCGAGTGAAG and reverse-ACTCATCCATACACAGGACCC; iNOS forward-TT CCCTCTCGCCAAAGAGTTT and reverse AAGTGCTAGTG GTGTCGATCT; IL1B-forward ATG ATG GCT TAT TAC AGT GGC AA and reverse IL1b GTC GGA GAT TCG TAG CTG GA; IL10-forward GAC TTT AAG GGT TAC CTG GGT TG and reverse TCA CAT GCG CCT TGA TGT CTG; TNFa forward CCT CTC TCT AAT CAG CCC TCT G and reverse GAG GAC CTG GGA GTA GAT GAG. Similar amplification procedures and data computation were followed as described above. No PCR products were generated from genomic versus the cDNA template. The melting curve analysis was performed to confirm the purity of the PCR products. The relative expression of NFkB, iNOS and COX2 was normalized to GAPDH using the DCT Method. Data are representative of three different experiments each run in triplicate and are presented as the mean

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SEM of triplicates. DNA was denatured at 95°C for 2 min. followed by 40 cycles of 30 sec at 95°C together with 30 sec at 60°C. The experiments were repeated twice with consistent results.

Western blot analysis

Total protein extracts were prepared by treating cells with the lysis buffer (RIPA). Nuclear extracts were prepared as previously described (Pesce et al., 2014). Proteins were quantified using the Bradford Method. The Western Blot analysis was performed as described previously (Speranza et al., 2008) using the following primary antibodies: acetyl-NFkB, anti-SIRT 1, p65-NFkB, iNOS, and COX2 (diluition 1:500). Protein levels were normalized to the housekeeping proteins actin and tubulin to adjust for the variability in protein loading, and expressed as a percentage of the vehicle control.

Measurement of PGE2 release

The measurement of the release of PGE2 was performed using an enzyme immunoassay (Arbor Assays, Ann Arbor, MI). The assay was carried out as described previously (Pesce et al., 2015a). Briefly, 5 105 cells were seeded in 2 ml of medium, both control and samples were added to each well and incubated for 15 min at room temperature and overnight at 4°C with a primary antibody and a conjugate. After washing, the substrate solution was added to each well for 30 min at room temperature. Finally, the “stop solution” was added, and the optical density of each well was determined within 30 min using a microplate reader (wavelength 450 nm).

Measurement of NO release

Briefly, 2 106 cells were seeded in six-well/plate and nitrite was measured in culture supernatants as an indicator of the NO production. The assay was carried out as described previously (Patruno et al., 2012a). Aliquots of the culture supernatant were mixed with an equal volume of the Griess reagent and absorbance was determined at 540 nm using a microplate reader. Sodium Nitrite, at concentrations of 0–100mM, was used as a standard to assess nitrite concentrations

Statistical analysis

All results were expressed as means

SD. Repeated-measure ANOVA was performed to compare means between samples. A probability of null hypothesis of < 0.05) was considered as statistically significant5.

Results

GM extract protects against LPS induced cytotoxicity

Several results show that monocyte/macrophages are a sensitive in vitro model to analyze potential anti-inflammatory activity of different substances, so that it may be a reasonably accurate model to study the LPS-dependent inflammatory response (Patruno et al., 2012b; Pesce et al., 2013). To examine the protective effects of GM against LPS-induced cytotoxicity we selected the U937 cell line. Various

concentrations of the GM extract (50 ng/ml, 100 ng/ml, 500 ng/ml, 1mg/ml, 5mg/ml, 10mg/ml, 50mg/ml, 100 mg/ml, 500mg/ml, 1 mg/ml) were added to the culture medium with and without LPS for 24 h up until the end of recovery. The reduction of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide] to formazan were used as indicators of cytotoxicity as this reaction is dependent on the mitochondrial respiration. As shown in Figure 1A, the treatment of the cells with only the extract of GM does not adversely affect cell viability in respect to the control cells. The viability of the cells exposed to 24 h LPS was reduced to 64

4.5% compared to the control. The concentration of 1 mg/ml pre-treatment with the GM extract does not alleviate the cytotoxic effects of LPS, while at concentrations higher than 5 mg/ml, the cytoprotective effect of the GM extract is present. Furthermore, the superoxide anion radical-scavenging activity was also measured in a non-enzymatic method. The phenazine methosulphate–NADH mixture at pH 7.4 generates superoxide anions, which can be measured by its ability to reduce NBT measured as an increase in absorbing change. The generation of superoxide anions was markedly inhibited (50%) from the concentration of 5 mg/ml in respect to cells stimulated with LPS (Fig. 1B). In our experiments, this concentration led to a down-regulation of the expression and the activity of the inducible enzyme, iNOS (Fig. 1C).

Chemical characterization of α-MG

As shown in the bibliography, α-MG was found to be the xanthone which was more present in the GM methanolic extract (Ngawhirunpat et al., 2010). Identification was accomplished after the HPLC analysis by comparison to the retention time and UV spectra of the analytical standard with the GM extract as shown in Figure 2. The α-MG concentration in the GM extract was determined by the interpolation of the peak area of the analyte in the calibration curve previously obtained from the less square regression data in the range of 5–80mg/ml1 , determination was performed at 315 nm using External Standard Method. Through HPLC analysis of 1 mg/ml1 GM methanolic extract, the concentration of α-MG resulted to be 41.45mg/ml1 , more than the other xanthones present in minutes.

Superoxide radical scavenging activity of α-MG

Given the reported beneficial effects of the xanthone compound present in the GM extract (Pedraza-Chaverri et al., 2008) we determined the potential cytoprotective effects of aMG in cells exposed to LPS. α-MG at concentrations of 10, 50, and 100mM exerted a cytoprotective effect against the LPS induced toxicity (Fig. 3A). The superoxide anion radical scavenging activities of α-MG were evaluated (Fig. 3B). α-MG showed the dose-dependent scavenging activity on Superoxide Anion Radical (O_2^-); this was in agreement with previous observations (Ngawhirunpat et al., 2010). α-MG was previously reported to exert an anti-inflammatory effect on murine RAW 264.7 macrophage-like cells (Chen et al., 2008). In order to select cytoprotective concentrations of α-MG on our cells (U937) we evaluated the impact of α-MG at increasing concentrations on U937 cells activated with LPS on iNOS expression and activity (Fig. 3C and D). Exposure to LPS increased the NO production above that is in control cultures. Concentrations of 10 mM α-MG inhibited the LPS-induced production of NO by 40%, significantly ($P < 0.05$).

α-MG affect the SIRT-1/ NF-kB signaling in U937

We investigated the NF-kB signaling pathway. The protein levels of NF-kB subunit p65 and acetylated p65 were detected. Interestingly, the p65 acetylation was significantly blocked by α-MG treatment, whereas the total protein level of p65 showed no significant changes compared with the baseline (Fig. 4A). The activation of SIRT-1 proteins, known to be involved in the regulation of NF-kB, was further investigated to determine if there was any link between these signaling molecules and the anti-inflammatory mechanism of α-MG. Figure 4B shows that α-MG significantly increased SIRT-1 expression in U937 cells, suggesting that the anti-inflammatory effect of α-MG is mediated by the NF-kB regulation through SIRT-1. To support this

hypothesis, we analyzed the expression and activity of one of the main enzymes expressed by monocytes activated with LPS, the COX2 (Fig. 4C). The Western Blot Analysis and PGE2 assay of the cell culture supernatant revealed that the α-MG significantly inhibited the LPS-induced inducible protein expression.

The involvement of SIRT1 in the inhibition of NF-kB signaling exerted by α-MG, via the p65 acetylation

We verified whether the anti-inflammatory effect of α-MG was mediated through the acetyl-p65–NF-kB inactivation at the cellular level. The U937 cells were treated with EX 527, a specific SIRT-1 inhibitor. As shown in Figure 5A, the inhibitory effect of α-MG on the LPS-induced activation of NF-kB was counteracted by EX 527, suggesting a role of α-MG on NF-kB signaling via SIRT-1. Similarly, the pharmacological inhibition of SIRT-1 by EX-527, increased the levels of the acetylated p65 protein. In order to confirm the close relationship of α-MG on the activation of SIRT-1 and the subsequent deacetylation of NFKB, we checked the expression of the downstream molecules already identified above (iNOS and COX2). As shown in Figure 5B and C, SIRT-1 inhibition, significantly abolished the inhibitory effect of α-MG on the expression of iNOS and COX2, revealing that α-MG inhibited the pro-inflammatory signaling of NF-kB through SIRT-1 induction in vitro. This data are confirmed by the analysis of the pattern of pro and antiinflammatory cytokines by real-time PCR. As we expected, exposure to LPS led to a significant increase in IL-1b (Fig. 6A), and TNF-a (Fig. 6B) gene expression which decreased following the treatment with α-MG. Treatment with α-MG revealed the inhibition on pro-inflammatory cytokines production but enhancement in IL-10 level (Fig. 6C) in LPS-induced cells. Therefore, we demonstrated from the first time that α-MG, via the SIRT-1/ NF-kB Signaling pathway, down-regulated the mRNA expression of iNOS and COX 2 in U937 cells stimulated with LPS.

Effect of α- mangostin in human monocytes from peripheral blood stimulated with LPS and modulation of SIRT 1/NFkB signaling

Primary human macrophages are sentinels of the immune system and following infection, circulating monocytes migrate from the peripheral blood to tissues where they differentiate into resident tissue macrophages. To further investigate our findings in a more physiopathological context, we have proposed the same experimental set in human monocytes from peripheral blood stimulated with LPS assessing the influence of α-MG on SIRT-1/NFkB signaling. First we have analyzed the cytotoxicity of α-MG, in terms of cell viability, on monocytes by MTT assay. The results are comparable to those obtained in the experiments carried out on U937 cells (data not shown). Subsequently, we assessed whether in this cellular system there is a modulation of SIRT-1 protein. Figure 7 shows that α-MG significantly increased SIRT-1 expression in monocytes from peripheral blood respect to cells activated with LPS. To address the molecular mechanism involved in the response of human monocytes to α-MG, the experimental set was repeated adding the inhibitor of sirtuin, EX-537, and contextually we examined if the treatment with α-MG could suppress the LPS-induced COX-2 and iNOS expression. When we treated the cells with LPS and α-MG, expression of this enzymes was repressed to the similar level with that of the baseline control (Fig. 8B and C). Importantly, the Western blot analysis performed nuclear extract shows that α-MG carries out its activity via acetylp65–NF-kB inactivation (Fig. 8A). As the last part of the study, to further validate these data, we have measured the levels of cytokines both pro-inflammatory (IL-1b and TNF-a) and antiinflammatory (IL-10). The data of real time-PCR (Fig. 9) confirmed that, in human monocytes from peripheral blood, aMG through the regulation of SIRT-1/ NF-kB Signaling is involved in the control of the cellular response to inflammation LPS-induced.

Discussion

Epidemiological evidence suggests that a diet rich in fruit and vegetables prevents or may delay the onset of various diseases such as cardiovascular diseases, cancer, diabetes, and dementia (Alissa and Ferns, 2015). These effects have been attributed, in part, to plant-derived poly-phenolic compounds that are good inducers of anti-inflammatory pathways (Barbagallo et al., 2013). Xanthones from the Mangosteen fruit, for instance, exhibit anti-inflammatory and anticarcinogenic activities in vitro but the molecular mechanisms

have not been well elucidated. The powerful antioxidant activity as well as the anti-inflammatory linked to the identity of the specific Mangosteen as referenced and as well documented, from its own composition (Pedraza-Chaverri et al., 2008). Several inducible proteins are involved in alteration of redox cellular signaling as heme oxygenase-1, iNOS, and COX2 (Kushida et al., 2002; Li Volti et al., 2002). The part of the Mangosteen from where the drug is taken, is due to dried pericarp and its preparation. From the data of the studies, pharmacological properties are indeed attributed to the dry extract obtained from the standardized pericarp in active constituents (Pothitirat et al., 2010; Asasutjarit et al., 2014). In this study, for the first time, we demonstrated that the xanthone α-MG of GM is able to significantly reduce the response to inflammation via SIRT-1. In fact, in our experimental model, we proposed in vitro an inflammatory condition, by treating the monocytic cell line U937 with LPS. This state created a evident change in the redox state of the cell with an over expression of inducible proteins as iNOS and COX-2. As studies have revealed, an important antioxidant and antiinflammatory action of the pericarp of GM, we treated our cells LPS-stimulated with the GM extract. Our results (Fig. 1B) show how the concentration of the GM extract, 5mg/ml is able to induce a down-regulation of the ROS production, as well as the expression of inducible iNOS protein (Fig. 1C). Also, through the Griess assay, the same concentration of extract results in a reduction of NO, which is one of the main mediators of inflammation associated with an increased activity of iNOS (Fig. 1C). As mentioned above, the pericarp is the main source of a particular family of xanthones (such as α-Mangostinone, g-mangostins, methylxanthine A, megastigmane sulphoglycoside etc (Jung et al., 2006; Tran et al., 2016). Scientific studies on a and g Mangosteen xanthones revealed important functions due to these two molecules and the most important is certainly their ability to limit the action of cancer cells and induce them to apoptosis and the premature death before these multiply. The same capacity in tests carried out on other thypes of cancer cells at the origin of leukemia and colon cancer was highlighted (Khuda-Bukhsh et al., 2014). α-MG possesses a wide spectrum of biological activities, which includes anti-inflammatory, cardio-protective, anti-tumor, antidiabetic, anti-bacterial, anti-fungal, antioxidant, anti-parasitic, and anti-obesity properties (Pedraza-Chaverri et al., 2008). The xanthone α-MG is the major compound found in the pericarp of the Mangosteen (Ngawhirunpat et al., 2010). Through HPLC analysis (Fig. 2), we have shown that the concentration of α-MG in 1 mg/ml of the GM methanolic extract is 41.45 mg/ml. Our goal of this study was to investigate a possible new molecular target of α-MG in modulating an inflammatory condition. The NF-kB is the central regulator of inflammation and it has been shown to activate more than 500 genes, most of which are involved with inflammation (Gupta et al., 2010). Therefore, NF-kB is a target for many antiinflammatory drugs and it is regulated by various molecules. Our study is focused on α-MG that would act as activators of SIRT-1, a nuclear enzyme from the Class III Histone Deacetylases modulating the expression of genes (Liu and McCall, 2013). It has been demonstrated and was originally discovered in a screen for gene silencing factors in yeast, and therefore, given the name Sir2 (Silent Information Regulator2) (Hubbard and Sinclair, 2014). Several recent studies have confirmed that SIRT1-mediated deacetylation of p65/RelA inhibited the NF-kB signaling which in turn regulates genes encoding several pro-inflammatory molecules (Risitano et al., 2014). These suggest that SIRT1 plays a pivotal role in the regulation of the NFKB-dependent pro-inflammatory mediator expression such as iNOS and COX-2 enzymes that are important mediators of inflammatory processes and have been associated with the pathogenesis of certain types of human cancers, as well as inflammatory disorders (Murakami and Ohigashi, 2007; Maccallini et al., 2009). The U937 cell lines, stimulated with LPS and treated with α-MG, demonstrate a surprising up-regulation of SIRT-1, resulting in a reduction not only of the COX 2, but also of its product of catalysis, PGE 2, known to be molecule strongly up-regulated in the inflammatory process (Fig. 4C and D). To confirm the association between a-Mangosteen and activation of SIRT-1, U937 cells were then treated with EX 527, a specific Sirtuin inhibitor. As shown in Figure 5A, the inhibitory effect of α-MG on the LPS-induced activation of NF-kB was counteracted by EX 527, suggesting a role of SIRT1 in the modulatory effect of α-MG on NFkB signaling. Subsequently, we were prompted to investigate our findings in a more physiopathological context, we so repeated the same sperimental set in human monocytes from peripheral blood stimulated with LPS. The

analysis of acetyl-NFKB (Fig. 8A), of inducible proteins (iNOS and COX; Fig. 8B and C), and of the cytokines level (IL-1b, TNF a, and IL-10; Fig. 9) confirm our results obtained on U937 cell line. Our study suggests that the modulation of SIRT 1 by α-MG may be important in the prevention of inflammation and may contribute to its anti-inflammatory effect. Together, our findings indicate that GM, in particular α-MG, protect in vitro against endotoxininduced response activating directly SIRT-1 negatively modulating the NFkB and consequently the iNOS/NO and the COX2/PGE2 pathways. In summary, our findings demonstrate that the natural product α-MG inhibited signaling cascade of a pro-inflammatory gene expression in LPS-stimulated U937 cell line by suppressing the NF-kB activation, through SIRT-1 activation. Therefore, the activation of SIRT1 could alleviate a multitude of NFkB-driven inflammatory and metabolic disorders. This implies that SIRT1 activators could exert significant benefits in the treatment of inflammation. Although several determinants of SIRT-1 regulation have been identified and the involved mechanisms have begun to be delineated, further studies are required.

Conflicts of interest:
We state that there is no conflict of interest, and declare that we have no financial and personal relationship with other people or organizations that could influence this work.

Contract grant sponsor: Italian Ministry for the University and Research.

Acknowledgments
This work is supported by the Italian Ministry for the University and Research. We thank Marco Reato (Vithagroup Spa) for providing GM extract for these experiments and a study grant for young researcher.

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Figures and Legends

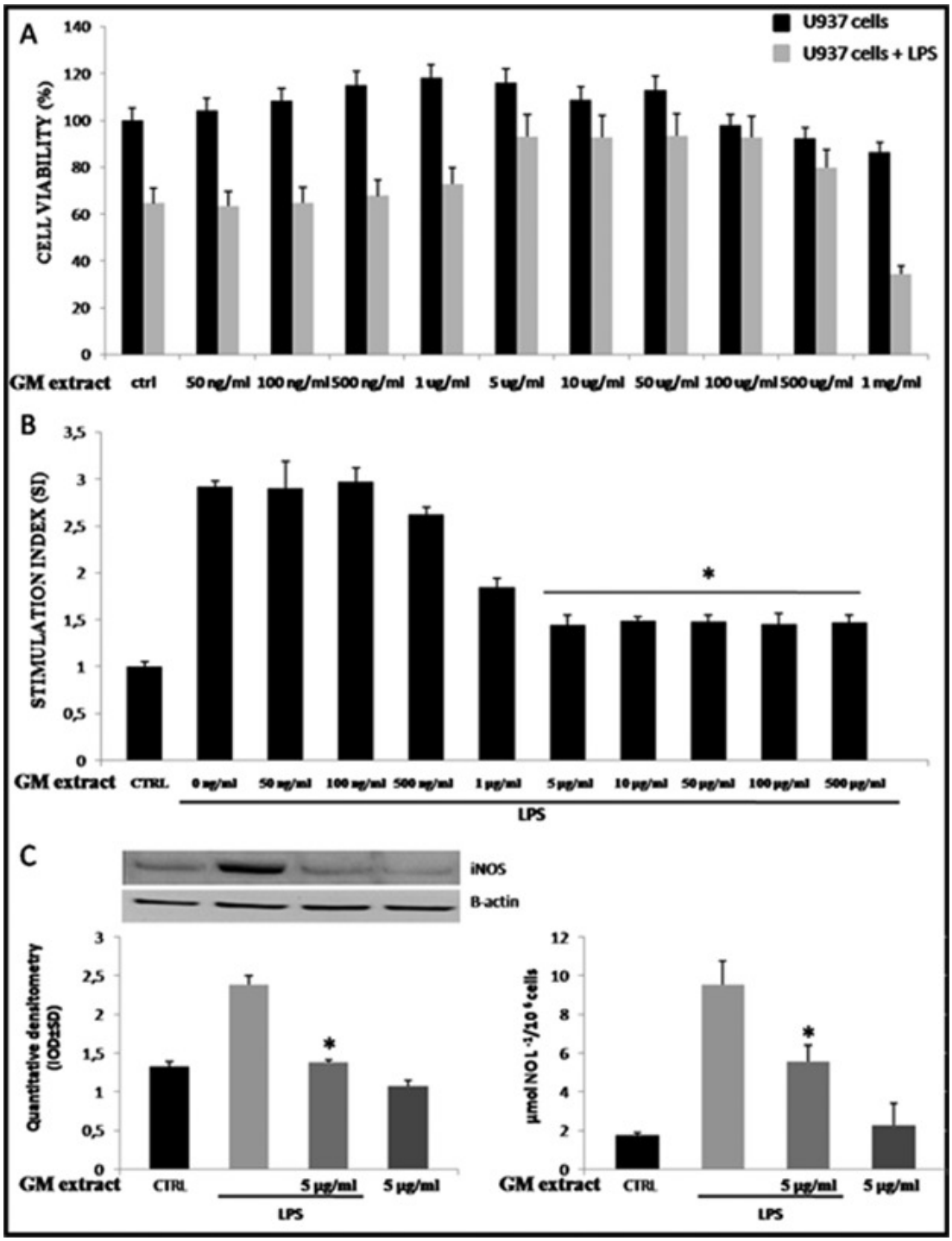

Figure 1

(A) Cytotoxic effect of GM extract tested on U937 cells line. Cells were treated with GM extract (50 ng/ml, 100 ng/ml, 500 ng/ml, 1 µg/ml, 5 µg/ml, 10 µg/ml, 50 µg/ml, 100 µg/ml, 500 µg/ml, 1 mg/ml) for 24 h and subjected to MTT assay to analyse cell cytotoxicity. Data are presented as means ± SD for triplicate experiments. *P ≤ 0.05 versus control cells. (B) Antioxidant activity of GM extract against oxidative stress measured by NBT test. Results were registered as stimulation index (SI). SI value of 1 was assigned to control cells. (C) Effect of GM extract on preventing LPS-induced iNOS expression in U937 cells. To the left, there is a representative image of western blotting experiment for iNOS from 6 gels using 3 separate pools of protein extracted from U937 cells. Averaged band density of iNOS from U937 cells normalized versus β-actin. To the right NO levels were quantified by the accumulation of nitrite in the cell culture medium and are expressed as µmol NO L−1/106 cells. Cells were treated or not treated with LPS and/or GM extract. *P < 0.05 versus LPS treated cells; **P < 0.05 versus control cells.

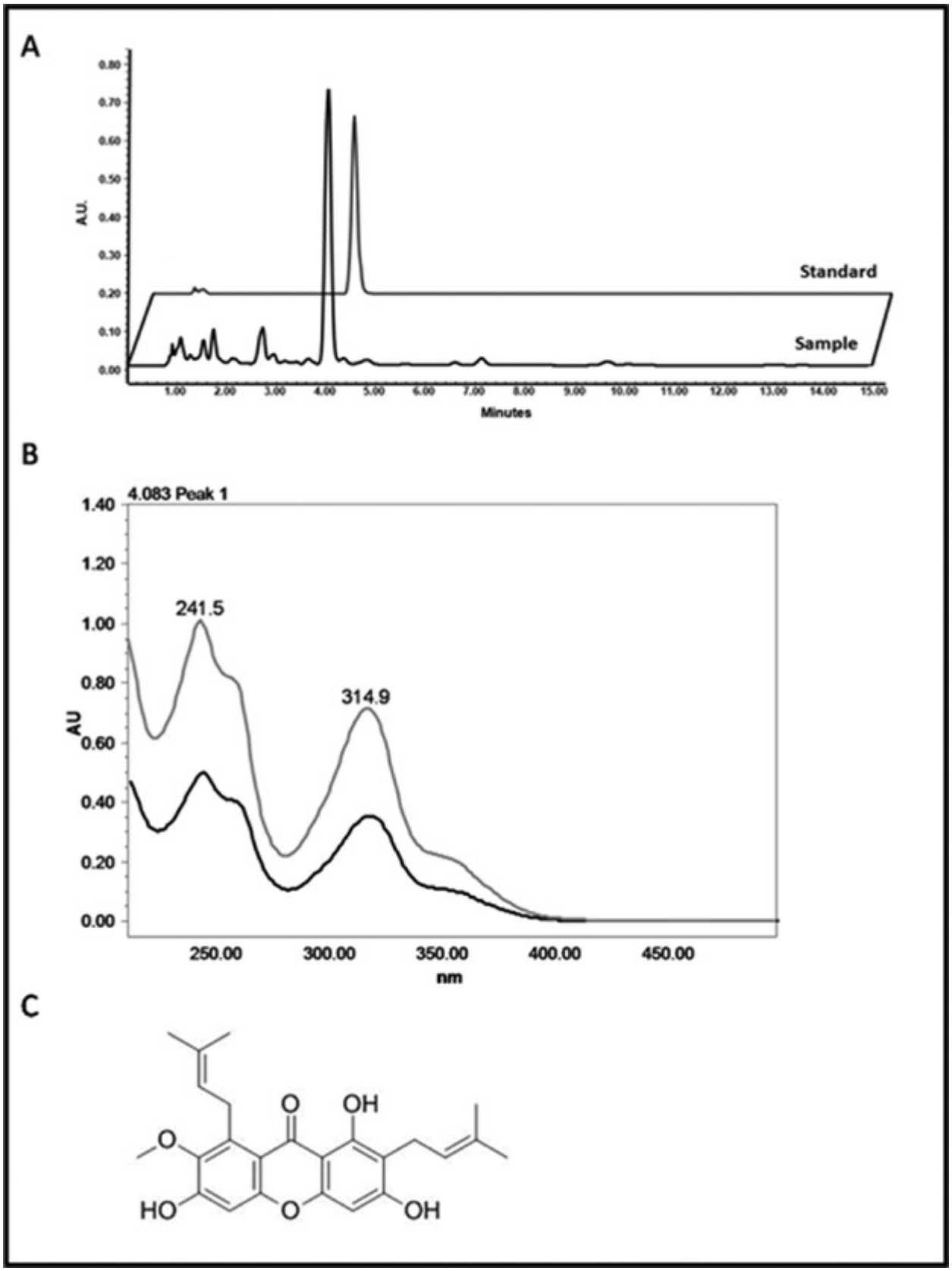

Figure 2

(A) Chromatogram of the α-MG concentration in the GM extract. (B) UV spectra of standard (black) versus sample (gray) and (C) chemical structure of α-MG.

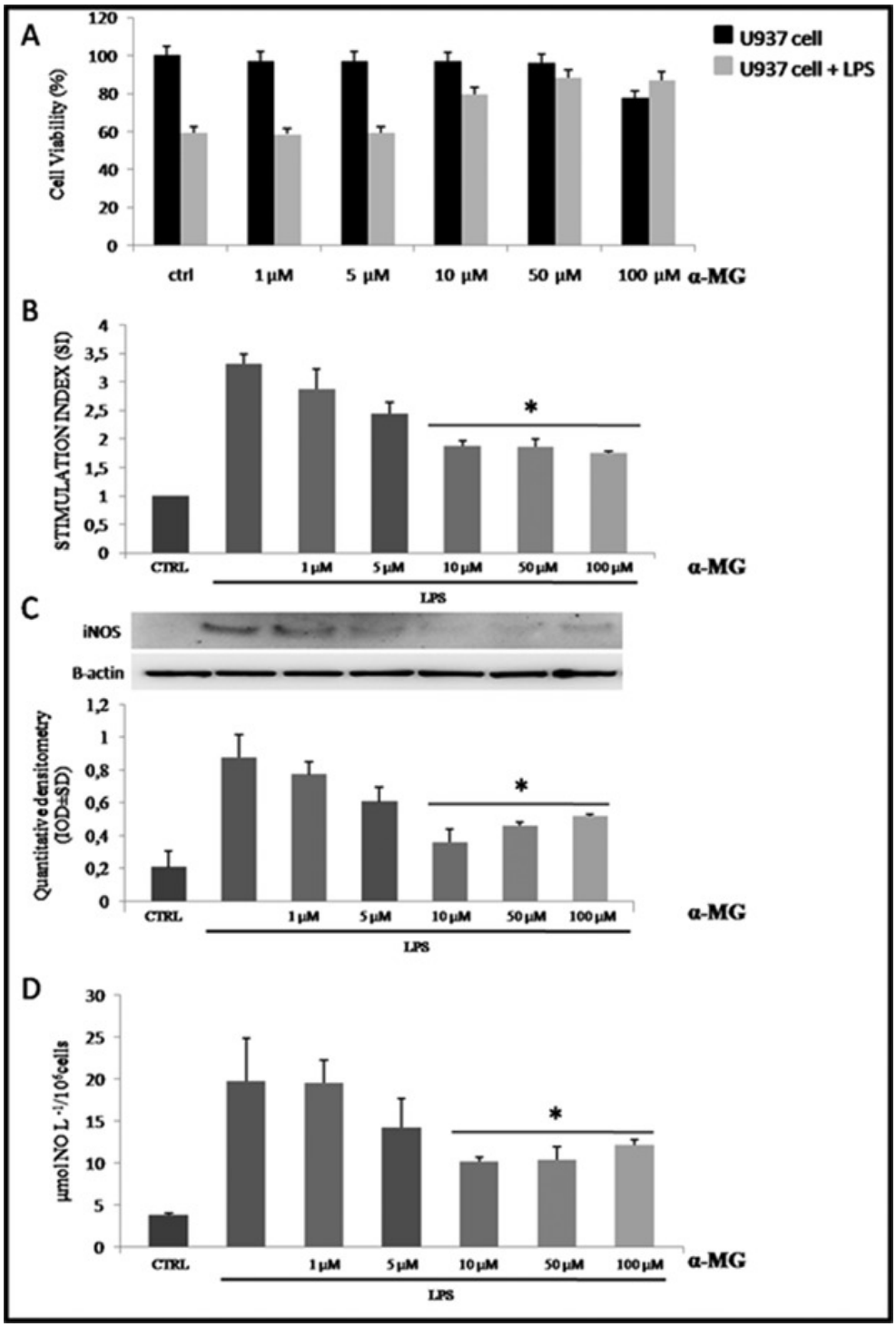

Figure 3

(A) Cytotoxic effect of α-MG tested on U937 cells line. Cells were treated with α-MG (1, 5, 10, 50, and 100 µM) for 24 h and subjected to MTT assay to analyse cell cytotoxicity. Data are presented as means ± SD for triplicate experiments. *$P \leq 0.05$ versus control cells. (B) Antioxidant activity of α-MG against oxidative stress measured by NBT test. Results were registered as stimulation index (SI). SI value of 1 was assigned to control cells. (C) Effect of α-MG (1, 5, 10, 50, and 100 µM) on preventing LPS-induced iNOS expression in U937 cells. Averaged band density of iNOS from U937 cells normalized versus β-actin. Cells were treated or not treated with LPS and/or α-MG.*$P < 0.05$ versus LPS treated cells; **$P < 0.05$ versus control cells. (D) Effects of α-MG after 24 h of incubation with LPS on NO production in U937 cells. NO levels were quantified by the accumulation of nitrite in the cell culture medium and are expressed as µmol $NO/l^{-1}/10^6$ cells. Values are mean ± SD of different experiments performed in triplicate. *$P < 0.05$ versus LPS treated cells; §$P < 0.01$ versus control cells.

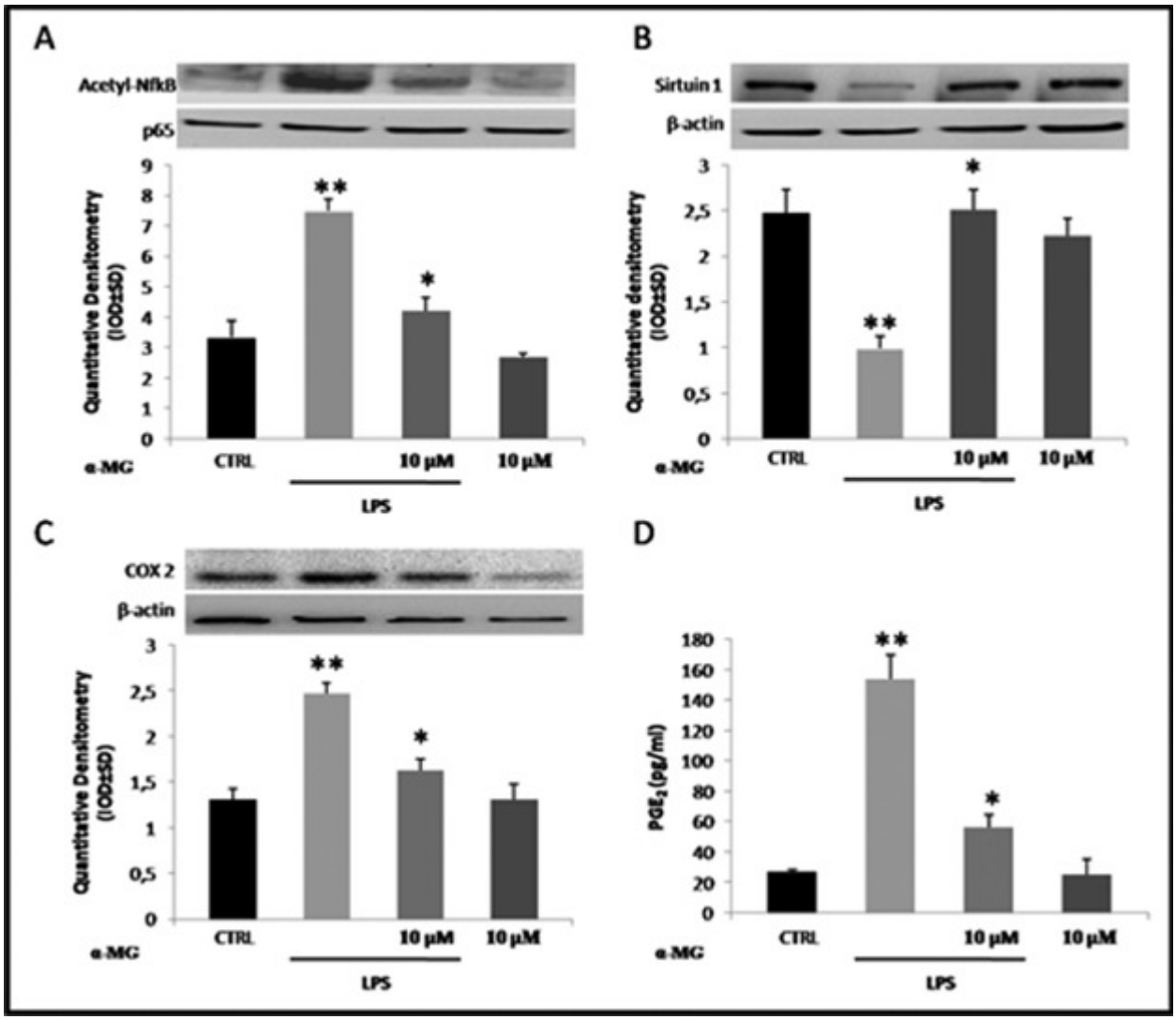

Figure 4

Effects of α-MG at concentrations of 10 µM on SIRT-1/ NF-κB Signaling in U937 cells. Representative image of Western blot analysis for acetyl-NFkB (A), Sirtuin 1 (B), and COX (C). At the bottom, in the densitometric analysis (n = 3), each bar is reported as the intensity of optical density (IOD) ± SD. (D) Secreted PGE 2 was revealed by ELISA kit. Results (n = 3) were reported as means ± SD. *$P < 0.05$, significance with respect to LPS-stimulated cells and **$P < 0.05$ significance with respect to control cells.

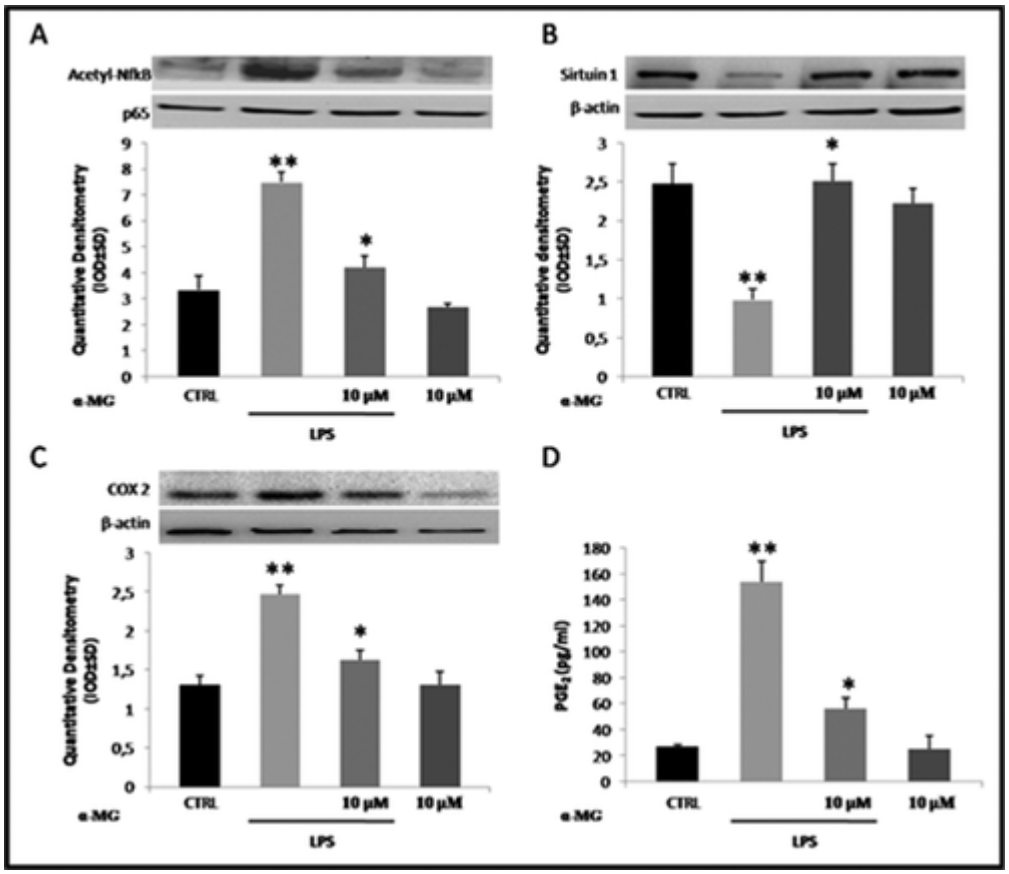

Figure 5

α-MG induced activation of Sirtuin 1 is reversed by treatment with SIRT-1 inhibithor (EX527). (A) Representative western blotting shows how α-MG-blocked NFkB acetylation is inhibited by EX527. The relative expression of acetyl-NFkB is compared with p65 NFkB under each experimental condition. At the bottom, densitometric analysis of the effect of EX527 treatment on acetyl-NFkB protein expression in U937 cells (control cells), in LPS-treated cells, and in EX527 cells treated with LPS + α-MG. *$P < 0.05$ versus LPS + α-MG. (B) The representative western blot analysis shows that iNOS-induced inactivation of α-MG treatment is affected by EX527. Actin shows equal loading protein. Densitometric quantification of Western blots is given as mean values ± SD (n = 3) *$P < 0.05$ versus LPS + α-MG. (C) Western blot analysis was performed using antibodies against COX 2 and actin after the treatment of the cells with α-MG or EX527. Values are means ± SD of three experiments. *$P < 0.05$ versus LPS + α-MG. At the left, real-time RT-PCR quantification of NFkB (A), iNOS (B) and COX 2 (C) mRNA 24 h after the inhibiting procedure. Values are means ± SD of three experiments and are normalized to GADPH *$P < 0.05$ versus LPS + α-MG.

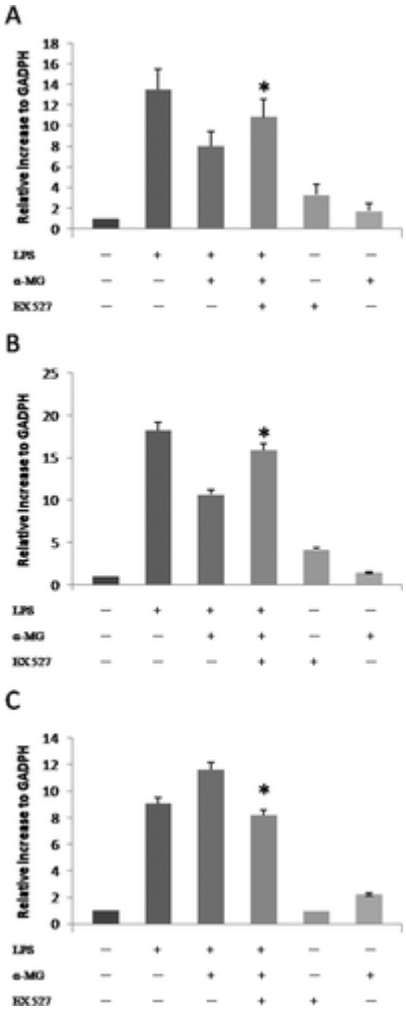

Figure 6

Effect of α-MG on cytokine expression pattern (real-time-PCR) in U937 cells. IL-1β (A); TNF-α (B); IL10 (C) Values are means ± SD of three experiments and are normalized to GADPH *$P < 0.05$ versus LPS + α-MG.

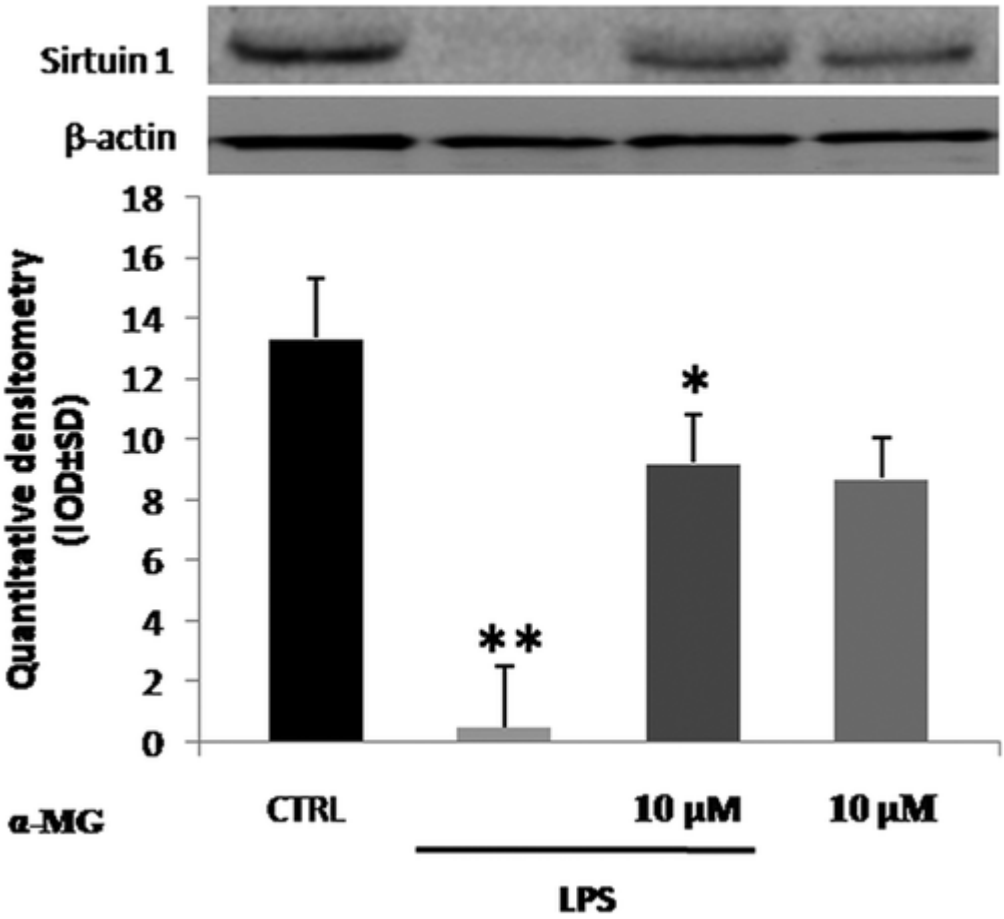

Figure 7

Effects of α-MG at concentrations of 10 µM on SIRT-1 expression in monocytes from PBMCs. Representative image of Western blot analysis for Sirtuin 1. Results (n = 3) were reported as means ± SD. *$P < 0.05$, significance with respect to LPS-stimulated cells and **$P < 0.05$ significance with respect to control cells.

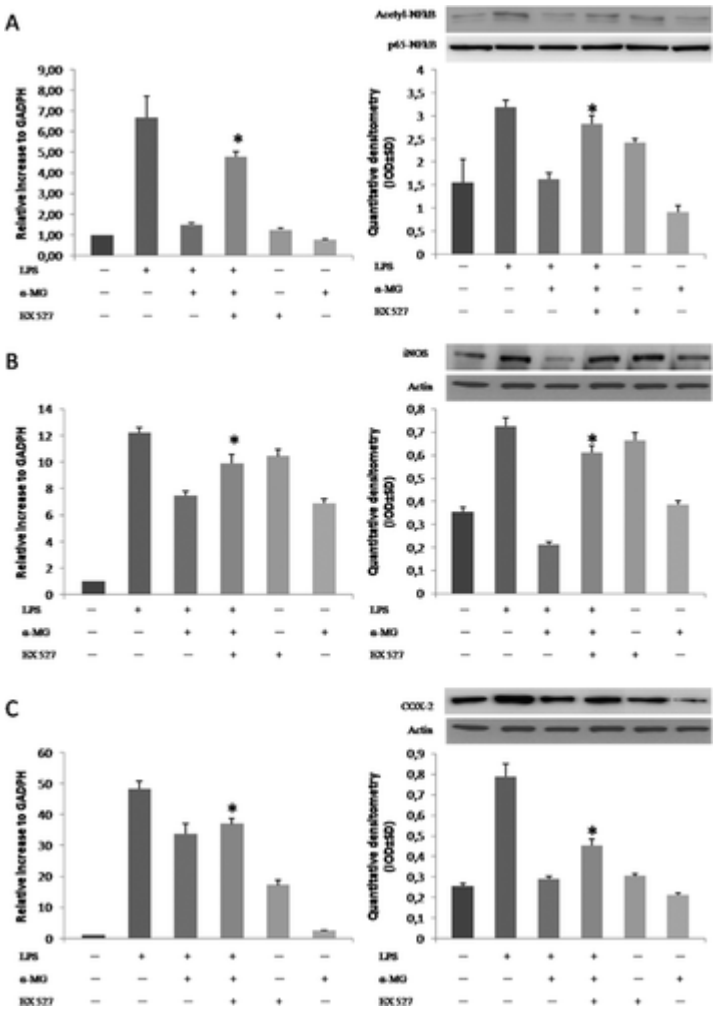

Figure 8

Effect of α-MG on monocytes extracted from PBMCs. (A) Representative western blotting shows that in peripheral blood monocytes α-MG-blocked NFkB acetylation is inhibited by EX527. At the bottom, densitometric analysis of the effect of EX527 treatment on acetyl-NFkB protein expression in peripheral blood monocytes (control cells), in LPS-treated cells, and in EX527 cells treated with LPS + α-MG. *$P < 0.05$ versus LPS + α-MG. (B) The representative western blot analysis shows that iNOS-induced inactivation of α-MG treatment is affected by EX527. Actin shows equal loading protein. Densitometric quantification of Western blots is given as mean values ± SD (n = 3) *$P < 0.05$ versus LPS + α-MG. (C) Western blot analysis was performed using antibodies against COX-2 and actin after the treatment of the cells with α-MG or EX527. Values are means ± SD of three experiments. *$P < 0.05$ versus LPS + α-MG. At the left, real-time RT-PCR quantification of NFkB (A), iNOS (B) and COX 2 (C) mRNA 24 h after the inhibiting procedure. Values are means ± SD of three experiments and are normalized to GADPH *$P < 0.05$ versus LPS + α-MG.

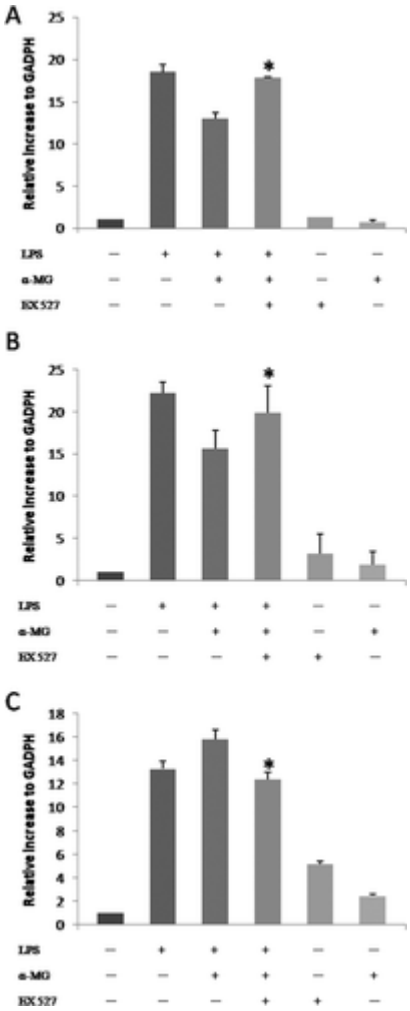

Figure 9

Effect of α-MG on cytokine expression pattern (real-time-PCR) of IL-1β (A), TNF-α (B), and IL10 (C) in monocytes from peripheral blood treated with LPS, α-MG, EX527, or both (LPS, α-MG, EX527). Values are means ± SD of three experiments and are normalized to GADPH **P* < 0.05 versus LPS + α-MG.