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Turnera subulata Anti-Inflammatory Properties in Lipopolysaccharide-Stimulated RAW 264.7 Macrophages

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ABSTRACT In South America, particularly in the Northeastern regions of Brazil, *Turnera subulata* leaf extract is used as an alternative traditional medicine approach for several types of chronic diseases, such as diabetes, hypertension, chronic pain, and general inflammation. Despite its widespread use, little is known about the medicinal properties of the plants of this genus. In this study, we evaluate the antioxidant and anti-inflammatory of *T. subulata* leaf extract in an *in vitro* model of inflammation, using lipopolysaccharide-stimulated RAW-264.7 macrophage cell line. We observed that cotreatment with *T. subulata* leaf extract was able to reduce the oxidative stress in cells due to inflammatory response. More importantly, we observed that the leaf extract was able to directly modulate inflammatory response by altering activity of members of the mitogen-activated protein kinase pathways. Our results demonstrate for the first time that *T. subulata* have antioxidant and anti-inflammatory properties, which warrant further investigation of the medicinal potential of this species.

KEYWORDS: • ***inflammation*** • ***oxidative stress*** • ***RAGE*** • ***TLR-4*** • **Turnera subulata**

INTRODUCTION

The health benefits of plant compounds acquired through the ingestion of tea, juice, fruits, and derivatives are an area of active research and have been approached by many different studies. In fact, the biological composition of these beverages and foods has been regarded as an important factor to reduce risk of chronic diseases.[1–3] Moreover, with the development of new technologies to produce industrialized foods, the interest in consuming natural compounds that prove to be beneficial to human health has increased in last few years. Of particular note are the compounds with anticancer, anti-inflammatory, and antioxidants properties, which have been extensively investigated in plant extracts and derivatives.

Turnera subulata is a tropical plant that occurs mainly in Northeastern Brazil.[4] It is used in traditional medicine for treatment of different diseases, such as diabetes, hypertension, chronic pain, and inflammation.[5] Some authors have also reported aphrodisiacs and anxiolytic properties in plants of the genus *Turnera*. Characteristically, phenolic compounds, flavonoids, alkaloids, and tannins have been related as the main biological molecules of genus *Turnera,* which can be responsible for mediating biological activities in organisms (Table 1).[6] Some reports have demonstrated the antioxidant effects of extracts obtained from *Turnera* species.[7–9] However, the biological properties and effects of *Turnera subulata* leaf extract remain unknown.

Recently, studies have associated positive effects attributed to genus *Turnera* with higher concentrations of arbutin, a biological compound found in different parts of the plant.[10–12] The decrease of cytokine secretion, such as TNF-α, IL-1, and IL-6, observed in treatments with *Turnera diffusa* or *Turnera ulmifolia* in different models of *in vivo* inflammation was reported as positive effects of those plants.[5,7,13,14] Moreover, the antioxidant properties of those extracts were demonstrated through the decreased lipoperoxidation and modulation of antioxidant enzymes such as glutathione peroxidase, superoxide dismutase (SOD), and catalase (CAT) in the liver of CCl4-treated rats.

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Table 1. Chemical Constituents Found in Extracts of Genus *Turnera*

	Chemical constituent
Phenolic	*Arbutin*
Flavonoid	*Luteolin*
Flavonoid	*Quercetin*
Flavonoid	*Apigenin*
Flavonoid	*Pinocembrin*
Flavonoid	*Syringetin*

Principal chemical compounds found in different species of genus *Turnera*.

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The role of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in mechanisms that lead to pathological diseases are largely studied. It is known that ROS/RNS is involved in normal signaling pathways and metabolic processes. ROS/RNS products, such as superoxide radical anion ($O_2^{-}\cdot$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH\cdot$), and nitric oxide, can trigger cells to proliferate and differentiate, and cell apoptosis and death.[15–19] However, an imbalance in the organism between ROS/RNS production and antioxidant defenses can lead to a condition known as oxidative stress.[20–22] Cancer, neurodegenerative diseases, diabetes, and cardiovascular diseases are pathological conditions that present oxidative stress in its etiology. Moreover, ROS/RNS also plays central involvement in activation and progression of inflammatory pathways.[23,24] Due to the complex biological composition, the consumption of plants extracts has been largely recommended to prevent risk of diseases. In part, this is suggested as a result of antioxidant properties that extracts exhibit.

In this study, we investigated the antioxidant and anti-inflammatory effects of leaf extract from *Turnera subulata* in an *in vitro* model of inflammation. We observed that leaf extracts exhibit antioxidant activities. Moreover, the extract was able to inhibit phosphorylation in mitogen-activated protein kinases (MAPKs) p38, ERK ½, and JNK, which was mediated by lipopolysaccharide (LPS)-stimulated macrophages. Markers of macrophage response for inflammation such as toll-like receptor 4 (TLR4), receptor for advanced end glycation products (RAGE), and CD40 were also investigated. Secretion of cytokines TNF-α and IL-1β in the medium was measured. Leaf extract of *Turnera subulata* inhibited the effect mediated by LPS in our model of inflammation. Our data demonstrate for the first time the potential anti-inflammatory and antioxidant effects of *Turnera subulata*.

MATERIALS AND METHODS

Chemicals

Culture analytical grade reagents were purchased from Sigma Chemical Co. (St.Louis, MO, USA). Antibodies against total and phospho-SAPK-JNK, total and phospho-p38 MAPK, total and phospho-ERK, TLR4, and β-actin were all purchased from Cell Signaling (Beverly, MA, USA). Anti-RAGE and CD40 antibodies were purchased from AbCam (Cambridge, United Kingdom).

Cell culture

The mouse macrophage cell line RAW 264.7 was grown in RPMI-1640 and 10% FBS, and maintained at 37°C in an atmosphere containing 5% CO_2. The media were supplemented with 1% penicillin/streptomycin.

Extract

The leaves of *Turnera subulata* were collected in Parnamirim (5° 55′21.0″ S – 35° 11′ 50.5″ W)–Rio Grande do Norte -Brazil. The plant material was identified by the Pharmacy Faculty Center of Federal University of Rio Grande do Norte–UFRN–Brazil. Specimen from *Turnera subulata* was deposited at the Herbario do Departamento de Botânica, Ecologia e Zoologia of Federal University of Rio Grande do Norte–UFRN–Brazil. To prepare extract, the leaves of *Turnera subulata* were air-dried at 40°C, powdered, and extracted by infusion with boiling water (95°C, plant solvent 1:10, g/mL) for 10 min. The aqueous extract was filtered, lyophilized, and stored at −80°C until tested.

Treatments

The extract was dissolved in a medium. Concentrated stocks were prepared immediately before experiments by dissolving extract into the medium and solution was kept protected from light and high temperatures during all procedures. Cells were treated with different concentrations of extract (0.5, 5, 500 μg/mL). All treatments were initiated by adding concentrated solutions to reach final concentrations in the well.

Thiobarbituric acid-reactive species

The cells were plated onto flasks of 25 cm^2.After 24 h of treatment, the cells were collected and homogenized. As an index of lipid peroxidation, we measured the formation of thiobarbituric acid-reactive species (TBARS) during an acid-heating reaction, which is widely adopted for measurement of lipid redox state, as previously described.[25]

Measurement of protein carbonyls

Cells were plated onto flasks of 25 cm^2. After 24 h of treatment, the cells were collected and homogenized. The oxidative damage to proteins was measured by the quantification of carbonyl groups based on the reaction with dinitrophenylhydrazine as previously described.[26]

Measurement of protein thiol content

The cells were plated onto flasks of 25 cm^2. After 24 h of treatment, the cells were collected and homogenized. Protein thiol content in samples was analyzed to estimate oxidative alterations in proteins.[27]

Estimation of antioxidant enzyme activities

The cells were plated onto flasks of 25 cm^2. After 24 h of treatment, the cells were collected and homogenized. The

catalase (EC 1.11.1.6) (CAT) activity was assayed by measuring the rate of decrease in H_2O_2 absorbance in a spectrophotometer at 240 nm, and the results are expressed as units of CAT/mg of protein.[28] The SOD (EC 1.15.1.1) activity was assessed by quantifying the inhibition of superoxide-dependent adrenaline autoxidation in a spectrophotometer at 480 nm, as previously described, and the results are expressed a units of SOD/mg of protein.[29]

Determination of intracellular RS production (real-time dichlorofluorescein oxidation assay)

Intracellular reactive species production was determined by the DCFH-DA-based real-time assay using intact living cells. Briefly, RAW 264.7 cells were plated onto 96-well plates and incubated for 1 h with DCFH-DA 100 μM (stock solution in DMSO, 10 mM) in 1% FBS culture medium at 5% CO_2 and 37°C.[30] Cells were then washed and treatments were carried out. During treatment, changes in the fluorescence by the oxidation of DCFH into the fluorogen DCF were monitored in a microplate fluorescence reader (F2000, Hitachi Ltd., Tokyo, Japan) for 1 h at 37°C.

MTT assay

The cells were plated onto 96-well plates. When the culture reached 60% confluence, the culture medium of the RAW 264.7 cells was removed and the treatments were added. After 24 h of leaf extract treatment, RAW 264.7 cell viability was assessed by the MTT assay.[31] H_2O_2 300 μM was used as positive control for cell death.

Sulforhodamine B assay

This colorimetric assay was performed to assess growth. It estimates cell numbers indirectly by staining total cellular protein with sulforhodamine B (SRB).[32]

Immunoblot

To perform immunoblot experiments, RAW 264.7 cells were lysed in Laemmli sample buffer (62.5 mM Tris–HCl, pH 6.8, 1% [w/v] SDS, 10% [v/v] glycerol) and equal amounts of cell protein (30 μg/well) were fractionated by SDS-PAGE and electroblotted onto nitrocellulose membranes. Protein loading and electroblotting efficiency were verified through Ponceau S staining, and the membrane was blocked in Tween–Tris-buffered saline (TTBS: 100 mM Tris-HCl, pH 7.5, containing 0.9% NaCl and 0.1% Tween 20) containing 5% albumin. Membranes were incubated overnight at 4°C with antibodies in the presence of 5% skim milk and then washed with TTBS. Anti-rabbit IgG peroxidase-linked secondary antibody was incubated with the membranes for an additional 1 h (1:5000 dilution range), washed again, and the immunoreactivity was detected by enhanced chemiluminescence using the ECL Plus kit. Densitometric analysis of the films was performed with Image J software. Blots were developed to be linear in the range used for densitometry.

Indirect ELISA

TNF-α was quantified by indirect ELISA. A 96-well ELISA plate was coated with 200 μL of culture medium and purified recombinant TNF-α protein (Abcam—Cambridge, United Kingdom) diluted in 50 mM carbonate buffer, pH 9.0, for standard curve calculation. After 24 h of incubation, plates were washed thrice with TTBS (100 mM Tris–HCl, pH 7.5, containing 0.9% NaCl, and 0.1% Tween-20). Subsequently, 200 μL of anti-TNFα (1:8000 dilution range) was added and incubation was carried for 24 h at 4°C. The plate was washed thrice with TTBS and incubated with 200 μL of a rabbit IgG peroxidase-linked secondary antibody (1:7000 dilution range) for 2 h. After washing the plate thrice with TTBS, 200 μL of substrate solution (TMB spectrophotometric ELISA detection kit) was added to each well and incubated for 15 min. The reaction was terminated with 50 μL/well of 12 M sulfuric acid stopping reagent and the plate read at 450 nm. IL-1β was detected by Abcam IL-1 beta Mouse ELISA (Enzyme-Linked Immunosorbent Assay) kit Immunoassay Kit following the manufacturer's instructions.

Statistical analysis

Results are expressed as mean value ± standard error of the mean; *P*-values were considered significant when $P < .05$. Differences in experimental groups were determined by one-way ANOVA followed by the *post-hoc* Tukey's test whenever necessary.

RESULTS

We first analyzed the viability of cells treated with leaf extracts at the concentration of 0.5, 5, and 500 μg/mL (Fig. 1). We observed that all treatments did not alter cell viability, as indicated by MTT and SRB-based assays. We also analyzed the effects of leaf extract when used in cotreatment during inflammatory response mediated in macrophage cells through LPS treatment at 1 μg/mL. At all concentrations of 0.5, 5, and 500 μg/mL, cotreatment with leaf extract blocked the effect mediated by LPS in viability of macrophages (Fig. 1).

The antioxidant properties were evaluated by measuring parameters of oxidative stress in macrophage cells treated with different concentrations of leaf extract for 24 h. The extract, when used alone in macrophage cells, did not alter the levels of lipid peroxidation. However, in LPS-induced macrophage cells, we observed decreased lipid peroxidation levels in macrophage cells that received leaf extract cotreatment (Fig. 1). Moreover, these effects observed were dose dependent. The protein carbonylation levels also were evaluated. The treatment with extract did not alter the levels of protein carbonylation in macrophage cells. Similar to effects observed in lipid peroxidation, the cotreatment with extract in LPS-induced macrophage cells blocked the increase in protein carbonylation levels (Fig. 1). Interestingly, protein thiol content was not altered by leaf extract treatment. However, cotreatments in LPS-induced macrophage cells

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blocked the effect mediated by LPS in protein thiol content (Fig. 1).

The activity of antioxidant enzymes was altered by leaf extract treatment. We observed a decrease in CAT and SOD activities in cells treated with leaf extract (Fig. 1) alone. In LPS-induced macrophage cells, the cotreatment with leaf extract blocked effects in CAT and SOD activities (Fig. 1). In addition, the effects observed in antioxidant enzymes activities were dose dependent (Fig. 1). Together, these data indicate that leaf extract of *Turnera subulata* presents antioxidant properties that result in the modulation of redox parameters induced by LPS.

To confirm antioxidant properties of leaf extract, we evaluated the production of ROS/RNS in macrophage cells treated with leaf extract using real-time DCFH oxidation assay. We observed that leaf extract at all concentrations inhibited cellular ROS/RNS production (Fig. 1). To confirm these antioxidant properties of the leaf extract, we cotreated LPS-induced macrophage cells and the rate of intracellular reactive species production was evaluated by DCF fluorescence. Once more, leaf extract treatment exhibits antioxidant properties (Fig. 2H), which were responsible for decreased ROS/RNS detection in the DCFH assay. Together, these results demonstrate that leaf extract was efficiently able to reduce cellular ROS/RNS production and prevent increase in ROS/RNS levels when the cells are exposed to oxidative stress conditions.

ROS/RNS is involved in the genesis of inflammatory response. Due to the cellular signaling pathways that could be affected by the oxidative stress during inflammatory response, we decided to investigate the effects of leaf extract in MAPKs, which are a family of proteins involved in inflammatory response signaling. We analyzed the effects of *Turnera subulata* leaf extract in inflammatory response, mediated in macrophage cells through LPS treatment, on the activation state of MAPK ERK ½, SAPK/JNK, and p38. MAPK phosphorylation is generally triggered within few minutes after cell stimulation; so we preincubated macrophage cells with leaf extract at different doses for 60 min and then LPS at 1 μg/mL was added to cells. Subsequently, we performed immunoblots to detect the phosphorylated (*i.e.*, active) forms of these protein kinases. It is known that LPS treatment stimulates ERK ½, JNK, and p38 phosphorylation within few minutes. ERK ½ phosphorylation steadily increased with time and peaked at 60 min, while p38 and JNK activation peaked at 30 min of incubation. *Turnera subulata* leaf extract cotreatment at the concentration 500 μg/mL inhibited the LPS-induced phosphorylation of ERK ½ (Fig. 2). In p38 (Fig. 2), no changes were observed in the activation states in macrophage cells that were cotreated with leaf extracts. Similar behavior was observed in SAPK/JNK activation (Fig. 2). These results indicate that *Turnera subulata* leaf extract was able to block effects in LPS-induced cells only through ERK ½ activation states. The other members of MAPK proteins evaluated in this study were not modulated by *Turnera subulata* treatments.

During cellular response induced by LPS treatment, there occurs secretion of cytokines, such as TNF-α and IL-1β. These cytokines regulate critical cellular processes, such as apoptosis, inflammation, and proliferation. In this study, we observed that cotreatment at 500 μg/mL of *Turnera subulata* leaf extract reduced the secretion of both TNF-α and IL-1β (Fig. 3) in LPS-induced macrophages cells. The other concentration used in cotreatment did not block TNF-α and IL-1β secretion mediated by LPS. In macrophages, TLR4 can be modulated by LPS treatment. In addition, it is also known that RAGE and CD40 have their expression modulated during an inflammatory process induced by LPS. Therefore, we decided to investigate the immunocontent of these receptors in cells treated with LPS and cotreated with *Turnera subulata* leaf extract. We found that cotreatment with leaf extract did not inhibit the increase of TLR4 mediated by LPS treatment (Fig. 3). However, the effects mediated by LPS in RAGE and CD40 immunocontent were inhibited through cotreatment with *Turnera subulata* leaf extract at 500 μg/mL of concentration. Taken together, these data indicate that the *Turnera subulata* leaf extract used in our study have anti-inflammatory properties. In addition, the results found in this study suggests that the action of the leaf extract could be triggered by ERK ½ inactivation.

DISCUSSION

The role of plants extract exerts in traditional medicine has been largely studied. The anticancer, anti-inflammatory, and antioxidant properties of diverse extracts have been associated to innumerous secondary metabolites found in different parts of the plants. The beneficial effects described for these molecules have stimulated the use of natural plant products in nutritional supplements. In addition, innumerous authors have suggested the positive relationship in consumption of natural products and decrease of risk of chronic diseases.[33–35] In these lines, the use of plant extracts has represented along the years, a promising tool to prevent and decrease the onset of chronic diseases.

In this study, our work demonstrates antioxidant and anti-inflammatory effects that leaf extract of *Turnera subulata* exhibited when used in a model of *in vitro* inflammation. Leaf extract decreased lipid peroxidation and protein carbonylation, which was demonstrated through dose-dependent effects during LPS-induced treatment. In addition, activities of CAT and SOD were also modulated by leaf extract of *Turnera subulata*, principally in LPS-induced model. Authors recently described that the genus *Turnera* is a source of different secondary compounds, such as phenolic, alkaloids, cyanogenic glycosides, steroids, saponins, and flavonoids that are likely associated with the positive effects of plant products.[6,36,37] Some of these compounds can exhibit higher antioxidant activities, which in almost all studies are suggested to be used to reduce ROS/RNS production in biological systems.

The antioxidant properties of secondary metabolites of plants can be exerted by different mechanisms. Authors have shown that compounds found in plants can act as reducing agents, scavengers of free radicals, metal ion chelators, cofactors of enzymes catalyzing oxidative reactions, inhibitors

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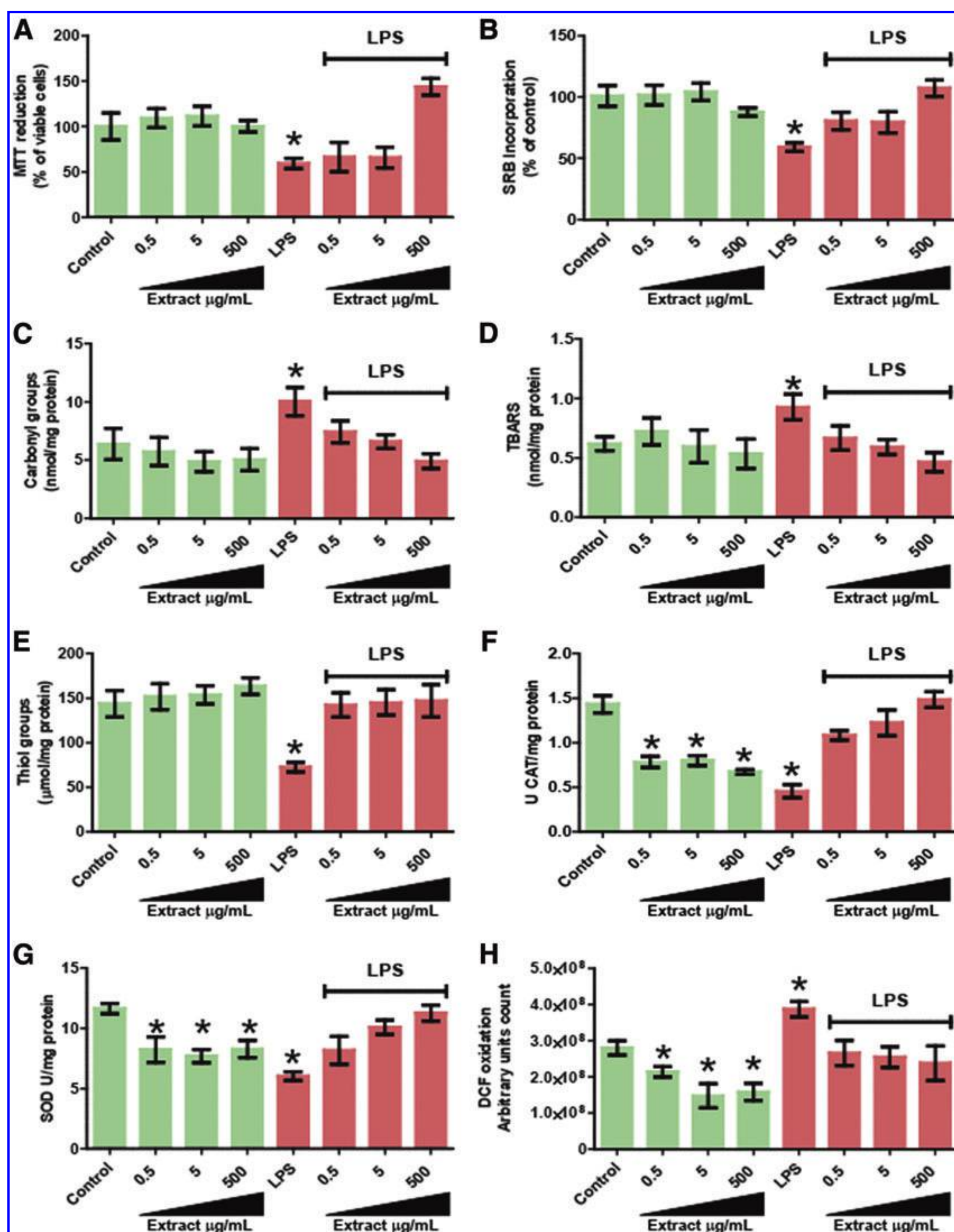

FIG. 1. Parameters of cell viability and oxidative stress in RAW 264.7 cells treated with *Turnera subulata* leaf extract for 24 h and in RAW 264.7 cells lipopolysaccharide(LPS) stimulated (1 μg/mL) and cotreated with *Turnera subulata* leaf extract for 24 h. RAW 264.7 cells were treated with leaf extract at 0.5, 5, and 500 μg/mL. Different assays were performed to evaluate cell viability after incubation; LPS (1 μg/mL) was used as a positive control for loss of viability. **(A)** MTT reduction assay and **(B)** SRB–incorporation assay. Parameters of oxidative stress: **(C)** carbonyl levels were quantified to evaluate cell protein oxidative damage; **(D)** thiobarbituric acid reactive species (TBARS) levels were assessed as an index for cellular lipid peroxidation; and **(E)** thiol levels were assessed to verify protein redox modification. The activities of the antioxidant enzymes **(F),** catalase (CAT), and **(G)** superoxide dismutase (SOD) were also evaluated. Intracellular reactive species production by RAW 264.7 cells subjected to leaf extract treatment was evaluated. **(H)** Cells were treated with different concentrations of leaf extract for 1 h and the total production of reactive species by living cells was evaluated by the real-time DCFH oxidation assay; LPS (1 μg/mL) was used as a positive control for reactive species production and fluorescence intensity was calculated relative to control cells. Control group is represented in all graphs by ''Control.'' Data represent mean ± SEM from three independent experiments ($n = 6$ per group). One-way ANOVA followed by the *post hoc* Tukey's test, *$P < .05$ versus the control group. SRB, sulforhodamine B. SEM, standard error of the mean. Color images available online at www.liebertpub.com/jmf

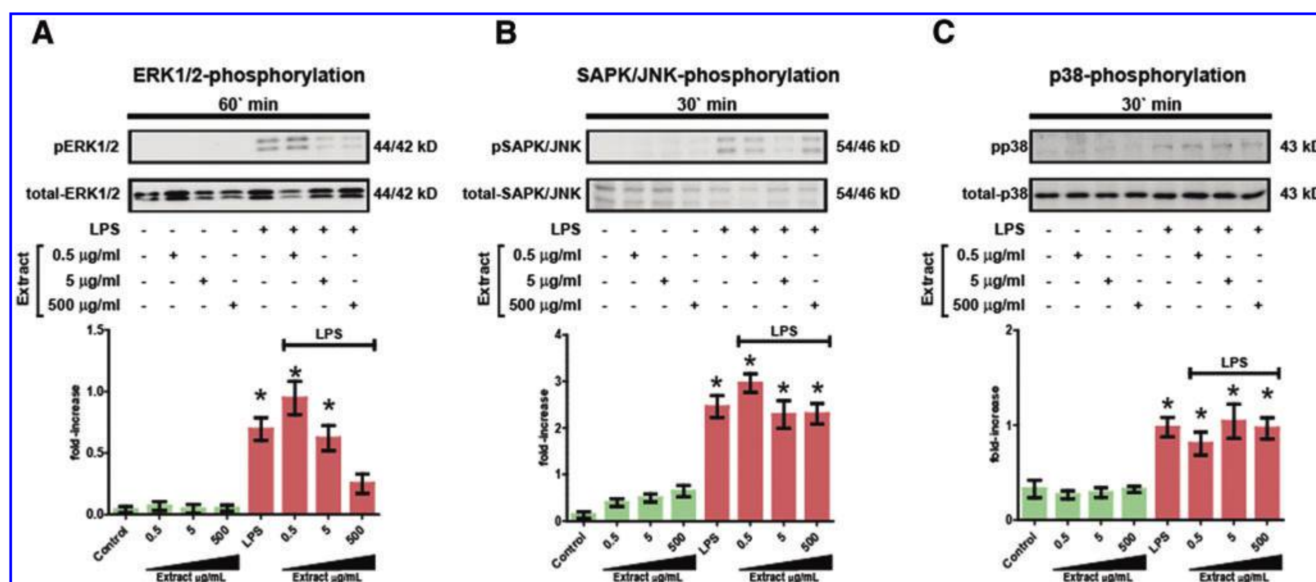

FIG. 2. Effect of *Turnera subulata* leaf extract preincubation (0.5, and 5 μg/mL) on the phosphorylation of **(A)** EKR ½ (60 min), **(B)** SAPK/JNK (30 min), and **(C)** p38 (30 min), in RAW 264.7 cells and RAW 264.7 LPS stimulated. Representative images (Western blots) reveal detection of phosphorylated isoforms of EKR ½, SAPK/JNK, and p38. Graphs exhibit the relative quantification of phosphorylated isoforms of EKR ½, SAPK/JNK, and p38 in relationship to their total immunocontent. Data represent mean ± SEM from three independent experiments ($n = 3$ per group). One-way ANOVA followed by the *post hoc* Tukey's test, $^*P < .05$ versus the control group. Color images available online at www.liebertpub.com/jmf

of oxidases, terminators of radical chain reactions, and stabilizers of free radicals.[38–41] To reinforce these findings, it is known that phenolic compounds are associated with a decrease in $O_2^-\cdot$ production.[42–44] Furthermore, they are able to inhibit $OH\cdot$ formation in a system containing iron and hydrogen peroxide (H_2O_2) through the Fenton reaction.[45,46] In this study, we demonstrated that leaf extract of *Turnera subulata* was able to reduce the lipid peroxidation and protein carbonylation in LPS-induced macrophages. LPS treatment is known for upregulating both $O_2^-\cdot$ and $OH\cdot$ production in macrophage cells. Therefore, our findings suggest that leaf extract exerted an antioxidant effect and these effects could be associated to secondary compounds present in leaf extract. Besides, their actions may be mediated through the synergistic action of these biological compounds.

The capacity of genus *Turnera* to modulate enzymes through inhibition/activation has been reported in different studies. It is known that *Turnera diffusa* extract had properties to inhibit aromatase enzyme. Most of this effect was associated with higher flavonoid content in the extract.[12] However, the effects of genus *Turnera* in modulating antioxidant enzymes responsible for the decrease in ROS/RNS are unclear. Interestingly, we found that leaf extract of *Turnera subulata* was able to modulate antioxidant enzyme activities, such as CAT and SOD activities, in macrophages cells. These effects were more clear in LPS-induced macrophage cells. It is well described that LPS treatment in macrophage cells leads to an impairment of the electron transfer system, thus increasing the rate of $O_2^-\cdot$ production.[47–49] In this study, our results confirmed the increased SOD activity and $O_2^-\cdot$ production during LPS-induced treatment in macrophage cells. Moreover, we observed a decrease in the CAT activity. In these terms, it is well described that $O_2^-\cdot$ is a potent inhibitor of CAT.[50,51] The cotreatment with *Turnera subulata* inhibited the decrease in CAT and SOD activities of LPS-induced macrophage cells. These effects corroborate with data found in lipid peroxidation and protein carbonylation, where leaf extract showed antioxidant properties during cotreatment. It is known that a large portion of biological properties and functions involving protein structure, enzyme catalysis, and redox signaling pathways depends on the redox state of the cells. The latter can trigger the cells during proliferation, differentiation, and/or inflammation.[52,53] Moreover, innumerous reports have demonstrated that the increase in protein carbonylation may be involved in the formation of protein aggregates, which are very likely to culminate in widespread cellular dysfunction.[54] The increased damage to proteins might result in increased free iron, because of its release from damaged ferritin and other iron-containing proteins, favoring the maintenance of the pro-oxidative state. The correlation between protein damage and inflammatory response is related with the increase of ROS/RNS production that occurs during inflammatory response. The decrease in ROS/RNS production is associated with the anti-inflammatory response, and biological molecules that present activities to inhibit the ROS/RNS production are target of recent studies.[55–57] In this study, we found that the leaf extract of *Turnera subulata* was able to reduce damage in proteins and lipids in macrophage cells subjected to LPS treatment. The inhibition of damage in biological molecules might contribute to maintenance of structure and function of proteins and lipids, which in turn leads to the anti-inflammatory response in the organism.[58–61]

During proinflammatory response occurs characteristically the release of cytokines, such as IL-1β and TNF-α.

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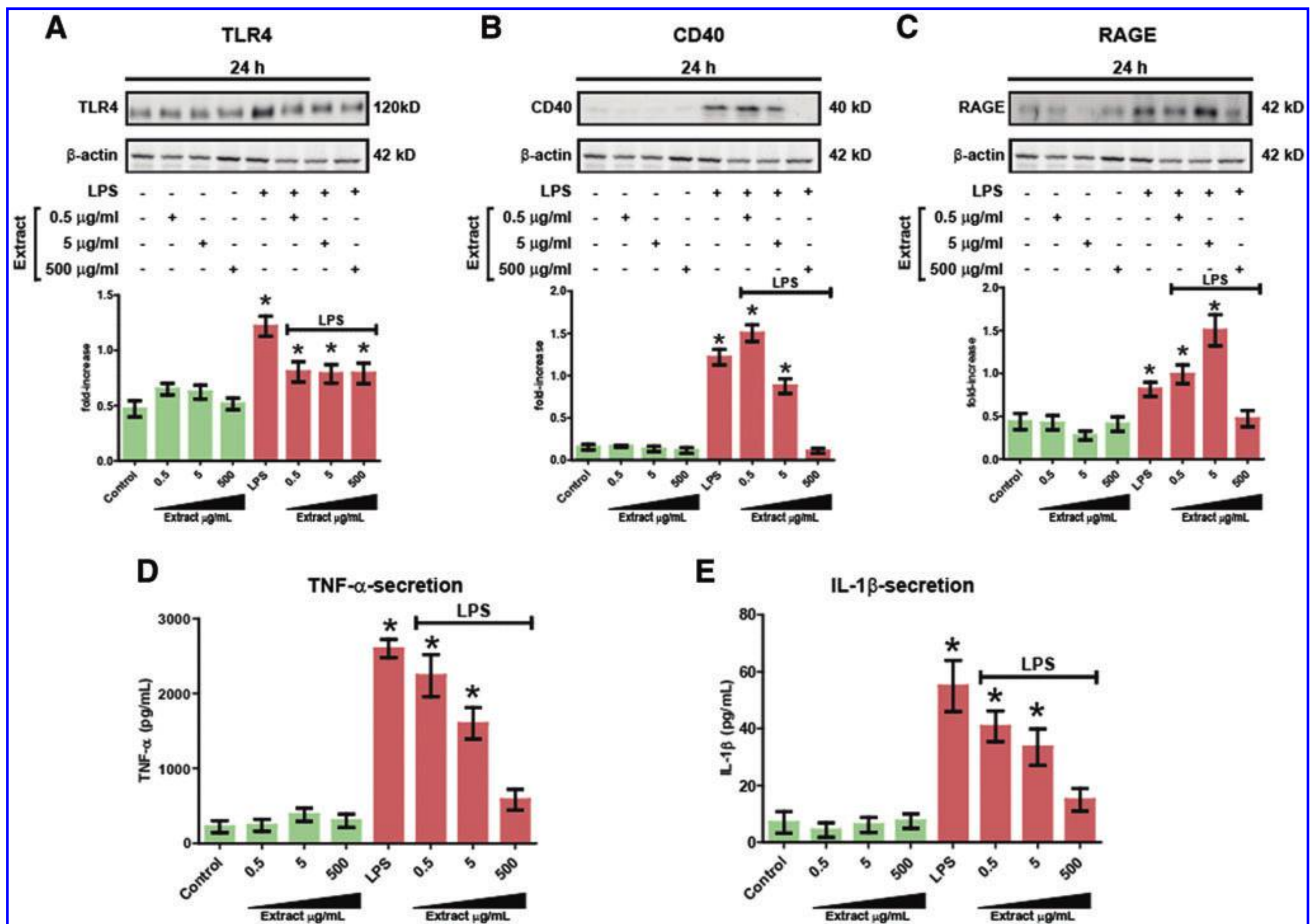

FIG. 3. Effect of *Turnera subulata* leaf extract (0.5, and 5 μg/mL) on the inflammatory biomarkers and cytokine release. **(A)** TLR-4, **(B)** CD40, and **(C)** RAGE immunocontent in RAW 264.7 cells and RAW 264.7 LPS stimulated after 24 h. Representative images (Western blots) reveal detection of immunocontent of proteins in total cell homogenates. Graphs exhibit the relative quantification of immunocontent of protein in relationship to their β-actin total immunocontent. **(D)** TNF-α and **(E)** IL-1β content in medium of incubation. Release of cytokines was performed by ELISA. Data represent mean ± SEM from three independent experiments ($n = 3$ per group for Western blotting, and $n = 6$ per group to ELISA assays). One-way ANOVA followed by the *post hoc* Tukey's test, $^*P < .05$ versus the control group. RAGE, receptor for advanced end glycation products. Color images available online at www.liebertpub.com/jmf

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IL-1β is an important mediator involved in the inflammatory response of cells.[62,63] Here, our study demonstrated that cotreatment with leaf extract of *Turnera subulata* induces inhibition of IL-1β secretion by LPS-induced macrophages. Interestingly, cotreatment with leaf extract did not inhibit the increase in TLR4 immunocontent. It is known that LPS treatment induces the expression of TLR4 and expression of this receptor is associated with inflammatory response. The class of TLR receptors is involved in activation of different cellular pathways that regulate the expression of cytokines, including IL-1β.[64] TNF-α secretion was also inhibited by the leaf extract cotreatment. TNF-α is known for regulating proinflammatory responses in different cells, such as endothelial cells, and contributes to an increase of ROS/RNS production in immune system cells involved in inflammatory response.[65] The involvement of CD40 in inflammation is well-known, as it is responsible for regulating different pathways involved in the expression of cytokines such as TNF-α and IL-1β.[66] In this study, our results showed that leaf extract cotreatment was able to inhibit the expression of CD40 and RAGE in LPS-induced macrophage cells. Taken together, our results demonstrate that leaf extract of *Turnera subulata* have anti-inflammatory properties.

The leaf extract cotreatment also inhibited the phosphorylation of ERK½. The association of MAPK signaling pathways during inflammatory response has been demonstrated in numerous reports; moreover, transient MAPK activation is associated with cell proliferation, whereas prolonged MAPK activation may be involved in promoting cell death. Previous works have shown the involvement of MAPK activation in regulatory mechanisms of RAGE, CD40, TNF-α, IL-1β, and TLR4 expression in LPS-induced models.[67] In part, LPS treatment effects in MAPKs can be mediated through the involvement of ROS/RNS. MAPK activation can stimulate different transcript factors such as nuclear factor kappa B (NF-κB), nuclear E2-related factor 2 (Nrf2), and p53, which are classically known for their ubiquitous roles in inflammatory, immune, and stress-related responses, and regulation of cell survival in all tissues.[68] Inhibition of MAPK phosphorylation mediated by LPS has been suggested as an anti-inflammatory

mechanism for different biological compounds in numerous studies. These effects, at least in part, are due to MAPK signaling blocking, which in turn leads to inhibition of transcription factor activation such as NF-κB and decrease in the cytokine expression in macrophages and other cells of immune system.[69] Therefore, our results strongly indicate that leaf extract of *Turnera subulata* has the capacity of modulating MAPK signaling pathways. We speculate that inhibition of ERK½ phosphorylation mediated by leaf extract was able to block the inflammatory response in macrophage cells. The same dose of leaf extract that inhibited the ERK½ phosphorylation in LPS-induced macrophages, also blocked the increase in RAGE and CD40 immunocontent and reduced the secretion of TNF-α and IL-1β.

In conclusion, the results presented in this study demonstrate for the first time that the leaf extract of *Turnera subulata* presents antioxidant and anti-inflammatory properties. Moreover, the data reinforce the importance of potential health benefits that the consumption of plants of genus *Turnera* may promote. Our findings may also be useful for better comprehension of the properties and mechanism of action mediated by *Turnera* compounds.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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