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Original Article

Anti-inflammatory sesquiterpene lactones from *Tithonia diversifolia* trigger different effects on human neutrophils

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ABSTRACT

The tagitinins isolated of *Tithonia diversifolia* (Hemsl.) A. Gray, Asteraceae, are the most studied sesquiterpene lactones due to their wide spectrum of pharmacologic activities, especially related with nuclear factor-kappa B inhibition. Nevertheless, detailed studies about the mechanism of action of its active compounds are still lacking. Neutrophils perform a fundamental role in the inflammatory response to several etiologic factors. However, the effect of tagitinins on human neutrophil is not yet clearly known. We investigated the role of tagitinin C (**1**), tagitinin F (**2**) and tagitinin A (**3**) in activation and survival of human neutrophils to establish possible effects in their mechanisms of inflammation. Human neutrophils were purified from the peripheral blood and cultivated with tagitinins C (**1**), F (**2**) and A (**3**) in the presence or not of *Escherichia coli* lipopolysaccharide. The enzymatic activity, apoptosis and secretion of cytokines rate were determined after 18 h. Lipopolysaccharide-induced myeloperoxidase activity of human neutrophils was significantly inhibited only by tagitinin F (**2**). Apoptosis of neutrophils was increased in the presence of tagitinin C (**1**), and it occurred independently of the presence of lipopolysaccharide or dexamethasone. Tagitinins C (**1**), F (**2**) and A (**3**) decrease lipopolysaccharide-induced interleukin-6, interleukin-8 and Tumor necrosis factor alpha production by human neutrophils. Together, these results indicate that tagitinins exhibit anti-inflammatory action on human neutrophils. However, tagitinin F (**2**) was the only sesquiterpene lactone that decreased secretion of inflammatory products by neutrophils without inducing neutrophil apoptosis.

Introduction

Tithonia diversifolia (Hemsl.) A. Gray, Asteraceae, is a medicinal plant that is known worldwide and several biological properties have been reported for this species, such as anti-parasitic, antimicrobial and anti-inflammatory, among others (Chagas-Paula et al., 2012). *T. diversifolia* is an important source of biologically active natural products such as terpenoids and phenolic compounds (Chagas-Paula et al., 2012). Among the terpenoids, the sesquiterpene lactones (STL) comprise the most studied class because they are frequently the major compounds present in active extracts and fractions from leaves and flowers (Chagas-Paula et al., 2012). Although STL are plant-derived compounds with remarkable anti-inflammatory and antitumor activity, they are usually known for their toxic properties (Chagas-Paula et al., 2011, 2012; Merfort, 2011; Ghantous et al., 2010; Schmidt, 1999). Among all compounds from *T. diversifolia* already tested for biological activity, the tagitinins (Tags), STL, have shown relevant activity in diverse pathologic situations (Chagas-Paula et al., 2012; Gu et al., 2002; Liao et al., 2011; Sánchez-Mendoza et al., 2011; Zhao et al., 2012). At the molecular level, at least partially, the anti-inflammatory effect of STL can be explained by the inhibition of the activation of the nuclear factor-kappa B (NF-κB) (Merfort, 2011; Siedle et al., 2004). NF-κB family comprises a group of transcription factors that regulates inducible gene expression in various physiological contexts but it is best known for its functions in immunity, inflammation, and oncogenesis (Ghosh and Hayden, 2008; Suganuma et al., 2002; Tornatore et al., 2012). Bacterial products, such as lipopolysaccharide (LPS), induce neutrophil activation, and it is largely known to result in myeloperoxidase (MPO) release and, through its binding with receptors on neutrophil membrane to activate NF-κB (El Kebir and Filep, 2013; Xiao et al., 2014). Then, resulting NF-κB activation

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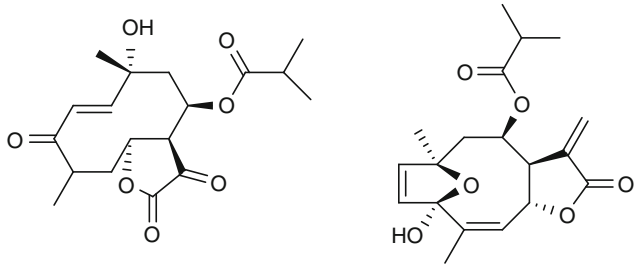
in these cells could generate pro-survival signals and decreased apoptosis being important to trigger defense strategies of the immune system (Chen and Chen, 2013; Kebir and Filep, 2013). Neutrophils are the first leukocytes to be recruited to an inflammatory site, and they have diverse roles in infection, inflammation and cancer immunology (Borregaard, 2010; Galli et al., 2011; Futosi et al., 2013). Although neutrophils have long been considered exclusively pro-inflammatory and their life span is prolonged by inflammatory cytokines, there is increasing evidence that some types of neutrophils may exhibit anti-inflammatory or healing characteristics (Kolaczkowska and Kubes, 2013). Considering such information it is possible to state that the depletion of neutrophils could result in harmful levels of immunosuppression (Kolaczkowska and Kubes, 2013). For these reasons, the idea of manipulating neutrophils as a form of immunotherapy during inflammatory processes should be evaluated carefully. Therefore, effects of STL as attempting to control inflammation through inhibition of NF-κB activation may negatively influence neutrophil function resulting in immunodeficiency.

In modern medicine, the use of plants as medicinal treatment for several diseases has been stimulated. The use of plants in order to medicate disease is almost universal among non-industrialized societies since pharmaceuticals are prohibitively expensive for most of the world's population (Da Silva et al., 2002).

Because the pharmacological properties observed for *T. diversifolia* extracts might be useful for manipulation of neutrophils (Chagas-Paula et al., 2012), we investigated the effects of the Tags C (**1**), F (**2**) and A (**3**) on human neutrophils.



Materials and methods

Material

LPS from *Escherichia coli*, dimethyl sulfoxide (DMSO) and dexamethasone (Dexa) were purchased from Sigma–Aldrich (St. Louis, Missouri, MO). Aposcreen Annexin V-FITC was purchased from R&D Systems (Minneapolis, MN, USA).

Plant material

Leaves from *Tithonia diversifolia* (Hemsl.) A. Gray, Asteraceae, were collected by D.A. Chagas-Paula in March 2008, in Ribeirão Preto, SP, Brazil (S 21°10.681′; W 047°51.541′; altitude 538 m). A voucher specimen (R.B. Oliveira #497) was deposited in the herbarium SPFR of the Department of Biology FFCLRP, USP, Ribeirão Preto, SP, Brazil. Entire leaves were airdried at 40 °C for a week and kept in humidity and light free conditions until the extraction process was started.

Extraction and isolation of the Tags A, C and F

The extract was obtained from 2.0 kg of entire and dried leaves individually rinsed for 20 s with acetone. The fresh extract was filtered through common filter paper, and after solvent evaporation under reduced pressure, the dried residue was resuspended in 10 ml of MeOH–H_2O (7:3, v/v) to precipitate lipophilic material. The precipitate was discarded, and the solvent from the supernatant was evaporated under reduced pressure. The extract (20 g) was partitioned with n-hexane following by EtOAc partition. The EtOAc fraction (5 g) was submitted to vacuum liquid chromatography using 500 g of silica gel 60 H (Merck, Brazil, art. no. 7736) on a glass column (115 mm of diameter) and eluting with n-hexane followed by increasing concentrations of EtOAc, furnishing 30 fractions [fractions 1–2, 500 ml of *n*-hexane/fraction; fractions 3–6, 250 ml of *n*-hexane/EtOAc (75:25, v/v)/fraction; fractions 7–8, 250 ml of *n*-hexane/EtOAc (1:1, v/v)/fraction; fractions 9–12, 125 ml of *n*-hexane/EtOAc (1:1, v/v)/fraction; fractions 13–18, 125 ml of *n*-hexane/EtOAc (25:75, v/v)/fraction; fractions 19–24, 125 ml of *n*-hexane/EtOAc (1:9, v/v)/fraction; fractions 25–30, 125 ml of EtOAc/fraction]. The fractions were evaluated and further combined by TLC (pre-coated aluminum sheets with silica gel 60 F254; Merck, Brazil, art. no. 5554) eluting with *n*-hexane/EtOAc 1:1 and 1.5% of HOAc and revealed with vanillin–sulfuric acid. The fractions 17–20 (1.1 g) were grouped and further purified by RP-HPLC (ODS C18 column 20 mm × 250 mm, Shimadzu, Japan; Proeminence Shimadzu chromatograph linked to a CBM 20 controller, UV/visible detector SPD-20, LC 6 AD pumps and automatic fraction collector FCR-10, Shimadzu) on isocratic mode with MeCN–H_2O (45:55, v/v), flow rate of 10 ml/min, to give pure Tag C (**1**) (20.8 mg) and F (**2**) (6.1 mg). One gram of the EtOAc fraction (see above) was fractionated by flash chromatography (20 mm × 140 mm glass column, 17 g of silica gel 0.040–0.063 mm, Merck, Brazil, art. no. 9385) using the following solvents as mobile phase (flow rate of 5 cm/min): 30 ml of $CHCl_3$ (fractions 1–3), 120 ml of diethyl ether (fractions 4–15), and 100 ml of EtOAc (fractions 16–25). The fractions were evaluated by TLC (see above). The fractions 14–17 (42.3 mg) were combined and further purified by RP-HPLC (see above) to furnish 18.2 mg of pure Tag A (**3**). The structural elucidation of the three compounds was carried out by ^1H and ^{13}C NMR and the spectral data were compared with those from authentic material and data from the literature (Baruah et al., 1979; Zdero et al., 1987).

Sample preparation

Prior to the bioassays, the compounds were dissolved in DMSO (0.1% in RPMI 1640 medium).

Neutrophil isolation

Human neutrophils were isolated from the peripheral blood of healthy donors by Histopaque 1119 and 1083 gradients (Sigma–Aldrich, Germany), as described by Dalboni et al. (2013). Neutrophils purity was assessed by Giemsa staining (phase-contrast microscopy) and viable by Trypan blue exclusion.

Neutrophils culture

Human neutrophils were suspended at a density of 1×10^6 cells per ml and incubated at 37 °C in 5% CO_2. For STL stimulation studies, 1, 10 and 100 μM of Tag C (**1**), F (**2**) or A (**3**) was added to neutrophils

for 21 h in the presence or absence of LPS (10 ng/ml), DMSO (1 μl) or Dexa (100 μM). Dexa was used as positive control because it has been largely related to an anti-inflammatory mechanism involving neutrophils (Calou et al., 2008; Vigil et al., 2008; Tsuchihashi et al., 2002; Chin et al., 2000).

MPO activity

MPO activity was determined by enzymatic reaction as described by Dalboni et al. (2013). Neutrophils were harvested after culture and centrifuged at 350 × *g* for 15 min, and the pellet was frozen at −20 °C. The pellet was then liquefied and centrifuged twice at 10,000 × *g* for 15 min at 4 °C. The MPO activity in the suspended pellet was assayed by measuring the change in absorbance at 450 nm using tetramethylbenzidine (1.6 mM) and H_2O_2 (0.5 mM).

Detection of apoptosis

Apoptotic cells were identified by staining with annexin V–fluorescein, as previously described (Tessarolli et al., 2010). In addition, the viability of neutrophils was also analyzed by fluorescence microscopy (Axiostar plus HBO 50/AC, Carl Zeiss, Germany). The percentage of apoptotic cells was calculated from the proportion of neutrophils positivity for Annexin-V-FITC or propidium iodide (green and/or red cells) in relation to the total number of neutrophils (Tessarolli et al., 2010).

Cytokines and chemokine detection

Interleukin-6 (IL-6), interleukin-8 (CXCL8), tumor necrosis factor alpha (TNF-α) production were quantified by the quantitative ELISA using commercial KITS (BD Pharmingen Corp., San Diego, CA, USA) according to the manufacturer's instructions.

Statistical analysis

Statistical analysis and graphical representations were performed using GraphPad Prism (version 5 for Windows, GraphPad Software, San Diego, CA, USA). One-way ANOVA followed by Bonferroni's test was used for the analysis.

Results

*Tag F (**2**) negatively influenced MPO activity on LPS-stimulated neutrophils*

MPO is a heme-containing peroxidase highly expressed by neutrophils and released when they are stimulated (Malle et al., 2007). We then investigated the direct effects of Tags C (**1**), F (**2**) and A (**3**) at 100 μM in neutrophils' MPO activity. Neutrophils activation with LPS induced significantly increased MPO activity (Fig. 1A). Importantly, Tag F (**2**) and Dexa significantly inhibited MPO activity by LPS-stimulated neutrophils (Fig. 1A). In contrast, treatment with Tags C (**1**) and A (**3**) increased the MPO activity. These data therefore demonstrated that Tag F (**2**) may affect enzymatic activity of neutrophils modifying their inflammatory machinery. As expected, Dexa significantly decreased MPO activity.

To determine if the reduction of MPO activity after Tag F (**2**) stimulation could be consequence of neutrophil death, we analyzed the apoptosis rate (Fig. 1B). We did not detect significant differences in apoptosis rate in unstimulated neutrophils and LPS or Dexa-stimulated neutrophils. In addition, neutrophils apoptosis was not altered after stimulation with Dexa plus Tags (Fig. 1B). More importantly, the stimulation of LPS-stimulated neutrophils with Tag F (**2**) did not induce increase in apoptotic rates (Fig. 1B). On the other hand, Tag C (**1**) significantly induced neutrophil apoptosis (Fig. 1B). Such an apoptosis rate after Tag C (**1**) stimulation occurred even when LPS or Dexa were added into the cultures (Fig. 1B). Tag A (**3**) did not alter apoptotic rates in LPS-stimulated neutrophils (Fig. 1B). No substantial difference was found in relation to MPO activity and apoptosis rate when neutrophils were cultivated with Tags at 1 or 10 μM (data not shown).

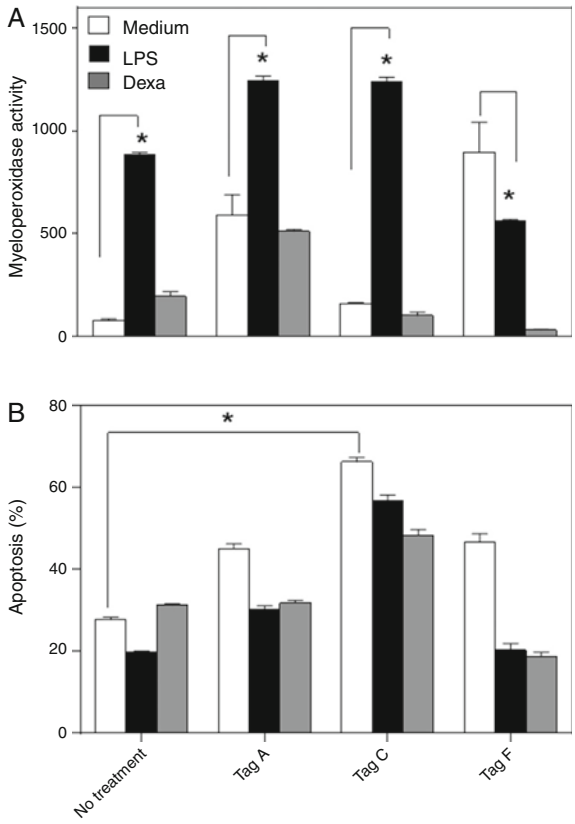


Fig. 1. MPO activity and apoptosis percentage of tagitinins-treated human neutrophils. Purified neutrophils (1×10^6) were cultivated with 10% SFB RPMI 1240 (white bars), LPS (10 ng/ml) (black bars), dexamethasone (100 μM) (gray bars) only (no treatment) or associated with tagitinin A (**3**), C (**1**) or F (**2**) (100 μM) and, after 12 and 21 h, respectively, to apoptotic cells (A) and MPO (B) were measured as described in Materials and Methods. The results are expressed as mean ± SEM for each volunteer tested individually. The experiments were performed in triplicate. The results were evaluated by one-way ANOVA followed by Bonferroni's post-test. *$p < 0.05$ when treated groups were compared with no treatment data.

Our results indicate that the decreased MPO activity of neutrophils was not a consequence of their apoptosis. Together these observations also indicate that the impact of Tag F (**2**) upon MPO activity did not influence neutrophil survival.

*CXCL8, IL-6 and TNF-α release from human neutrophils are reduced in the presence of Tags C (**1**), F (**2**) and A (**3**)*

Activated neutrophils secrete a variety of pro-inflammatory cytokines, for example IL-1, IL-6, CXCL8, and TNF-α (Cassatella et al., 1997; Dalboni et al., 2013). To ascertain if stimulation with Tags C (**1**), F (**2**) and A (**3**) altered human neutrophils activation, we isolated human neutrophils and incubated them with LPS in the presence or absence of Tags for 18 h. We detected CXCL8, IL-6, and TNF-α in supernatants from LPS-stimulated neutrophils. Tags C (**1**), F (**2**) and A (**3**) at 100 μM significantly decreased CXCL8, IL-6 and TNF-α production by LPS-stimulated neutrophils (Fig. 2A–C). Unexpectedly, Tag A (**3**) induced TNF-α production by human unstimulated neutrophils (Fig. 2C). The results showed no significant difference when compared to negative (medium) or positive (Dexa) groups

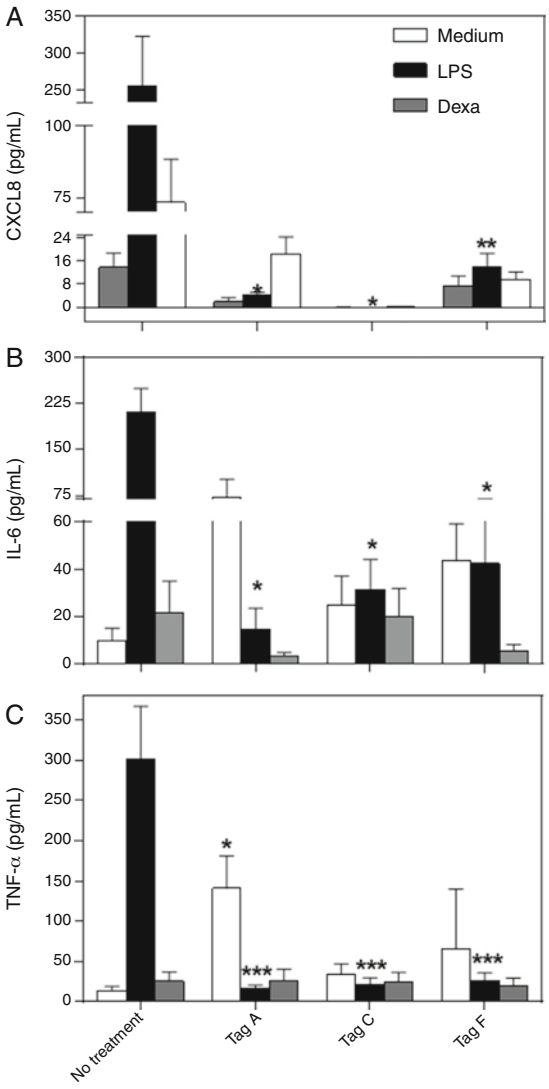


Fig. 2. Detection of cytokines produced by tagitinin-treated human neutrophils. Purified neutrophils (1×10^6) were cultivated with 10% SFB RPMI 1240 (white bars), LPS (10 ng/ml) (black bars), dexamethasone (100 μM) (gray bars) only (no treatment) or associated with tagitinin A (**3**), C (**1**) or F (**2**) (100 μM) and, after 21 h, CXCL8 (A), IL-6 (B) and TNF-α (C) were quantified by ELISA. The results are expressed as mean ± SEM for each volunteer tested individually. The experiments were performed in triplicate. The results were evaluated by one-way ANOVA followed by Bonferroni's post-test. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ when treated groups were compared with no treatment data.

in the presence or absence of Tags C (**1**), F (**2**) and A (**3**). Together these results show that Tags C (**1**) and F (**2**) were able to decrease LPS-induced production of inflammatory cytokines by neutrophils. Besides, Tag C (**1**) and F (**2**) did not stimulate neutrophils to secrete CXCL8, IL-6 and TNF-α in the absence of inflammatory stimuli.

Discussion

STL, diterpenes, caffeoylquinic acid derivatives as well as flavonoids are the most common natural products which may be encountered in the aerial parts of *T. diversifolia* (Chagas-Paula et al., 2012). Several biological activities have been described for most of these compounds (Liao et al., 2011, 2013; Chagas-Paula et al., 2011, 2012; Lin, 2012; Zhao et al., 2012; Sánchez-Mendoza et al., 2011; Gu et al., 2002). The tested STL from *T. diversifolia* usually exhibit expected biological activity because, in a general way, they are known for their key medicinal potential (Chagas-Paula et al., 2012; Ghantous et al., 2010). Among all compounds from *T. diversifolia* already tested for any biological activity, the STL Tag C (**1**) has been the most frequently studied (Liao et al., 2011, 2013; Chagas-Paula et al., 2012; Lin, 2012; Lee et al., 2011; Sánchez-Mendoza et al., 2011; Ghantous et al., 2010; Goffin et al., 2002). In the presence of LPS, all Tags were able to decrease the rate of neutrophil apoptosis. However, in the absence of stimuli an increased rate of apoptosis of neutrophils cultivated with Tag C (**1**) was observed. Some studies have shown that Tag C (**1**) induced caspase-dependent apoptosis or even autophagic death of tumor cells, and such role of Tag C (**1**) is thought to be beneficial for the prevention and treatment of cancer (Liao et al., 2011, 2013; Liu et al., 2013). When occurring in the site of infection the exacerbated neutrophil apoptosis would be dangerous because of the late effects in the localized immune response such as modulation or even immunosuppression (Ortega-Gómez et al., 2013). Although macrophage phagocytosis of apoptotic neutrophils avoids neutrophil autolysis accelerating the resolution of the inflammation, macrophages secrete VEGF, IL-10 and TGF-β after efferocytosis, resulting in a poor prognosis (Ortega-Gómez et al., 2013; Kawakami et al., 2013; Gholamin et al., 2009; Ohm and Carbone, 2001). Although apoptosis is a default fate of neutrophils, in the inflammatory microenvironment neutrophils are likely exposed to various pro-survival signals, such as LPS (Kebir and Filep, 2013). The defense mechanisms of neutrophils that destroy invading pathogens are also capable of inflicting damage to the surrounding tissue (Nathan, 2006); however, it is not interesting that a medicine may induce neutrophil death since it could become an individual susceptible to severe infections (Tortorella et al., 2001; Salmen et al., 2004; Aref et al., 2011; Break et al., 2012). Consequently, apoptosis induction of neutrophils would comprise a bad side effect of Tag C (**1**). On the other hand, we found Tag F (**2**) and Tag A (**3**) did not induce neutrophil apoptosis; this fact might indicate better chances to the use of these compounds in an *in vivo* system.

In addition, Tag F (**2**) significantly decreased the LPS-induced MPO activity of human neutrophils. LPS is able to strongly activate neutrophils and, as a consequence of their activation, they release MPO. MPO is present in the cytoplasmic azurophilic granules of neutrophils and upon degranulation into the phagosome, MPO can react with hydrogen peroxide to produce various antimicrobial reactive species, also culminating in the hypochlorous acid generation (Amulic et al., 2011). However, studies have suggested that most of these species produced would react with host proteins before reaching the pathogen (Amulic et al., 2011). MPO absence does not necessarily make an individual to be susceptible to the infections (Amulic et al., 2011), and its presence has been correlated to different diseases. Besides, excessive generation of MPO-derived oxidant has been linked to tissue damage and in the initiation and progression of diseases, such as cancer, and cardiovascular illness, which arise from chronic inflammation (Nussbaum et al., 2013; Mika & Guruvayoorappan, 2011). The oxidant activity of MPO is believed to promote the metabolism of chemical carcinogens, cause DNA damage and compromise the repairing process (Nussbaum et al., 2013; Mika and Guruvayoorappan, 2011). Therefore, the control of MPO activity has been thought as a target for new drugs development (Malle et al., 2007). We speculate that Tags could block MPO activity, affecting its action in the inflammatory environment. This hypothesis will have to be investigated in the future.

Activated neutrophils produce several inflammatory cytokines and chemokines, which directly influence in inflammation process at different levels (Thomas and Schroder, 2013; Tazzyman et al., 2013; Fridlender and Albelda, 2012; Kasama et al., 2005; Cassatella et al., 1997). These products have been also pointed as good target for therapeutic against inflammation-based diseases (Kasama et al., 2005). In this work, we found that Tags C (**1**), F (**2**) and A

(**3**) significantly inhibited LPS-induced IL-6, CXCL8 and TNF-α by human neutrophils. However, neutrophils cultivated with Tag A produced TNF-α, a cytokine with effect on neutrophils survival in a concentration-dependent way (Cross et al., 2008; Walmsley et al., 2004). These effects might discourage investigations using Tag A (**3**) as therapeutic agent in inflammation.

With regard to the structural requirements that may be involved in the biological activity of the three compounds, it is interesting to observe that the Tags belong to the germacranolide class and have in common a γ-lactone conjugated with an exocyclic methylene group as well as an isobutanoyloxy side chain group at C8. These common chemical features among the Tags, especially the α-methylene-γ-lactone group, probable are responsible for their common effects on neutrophils, such as inhibition of the production of IL-6 and CXCL8. However, the compounds also show some differences among them. Besides the α-methylene-γ-lactone group, Tag C (**1**) has a carbonyl group (C3) conjugated with two different double bonds while Tag F (**2**) and A (**3**) have an ether linkage between C3-C10 and for that they are called furanoheliangolides. Tag C (**1**) and F (**2**) have a *cis* double bond at C4 while the main skeleton of Tag A (**3**) is completely reduced; finally, besides the hydroxyl group at C3, Tag A (**3**) shows an extra hydroxyl at C1 against only one in Tag F (**2**) (C3) that in turn has a double bond at C1. All these small differences in the structures of the three Tags certainly exert great influence on their effects on the neutrophils (Figs. 1 and 2). For example, the features of Tag C (**1**) (three conjugated elements) could be correlated with its undesirable apoptotic induction on neutrophils and those of Tag F (**2**) should provide its better profile on neutrophils because it was the only that was able to inhibit TNF-α production and MPO activity without apoptotic induction besides the inhibition of production of IL-6 and CXCL8.

Together, these results clearly demonstrate for the first time the action of Tags C (**1**), F (**2**) and A (**3**) from *T. diversifolia* on human neutrophils. However, Tag F (**2**) is the only one that exhibits anti-inflammatory potential on neutrophils without significant side effects.

Further studies are needed to better understand the molecular modes of action of Tag F (**2**) from *T. diversifolia*, as well as to determine its toxicity and activity in co-cultures with immunologic cells and *in vivo* models.

Authors' contributions

APC, FBC, and THG conceived and designed the experiments; AEA, CEO, TMD, and THG for culture-related experiments; BAR, DACP, and RBO for purification and characterization of Tag; AEA, CEO, FBC, APC, and THG contributed to data analysis; APC and FBC contributed with reagents/materials/analysis tools and AEA, CEO, THG, DACP, BAR, RBO, FBC, and APC for paper writing. All authors have read the final manuscript and approved the submission.

Conflicts of interest

All authors have none to declare.

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