IOSR Journal Of Pharmacy And Biological Sciences (IOSR-JPBS)
e-ISSN:2278-3008, p-ISSN:2319-7676. Volume 15, Issue 6 Ser. III (Nov. –Dec. 2020), PP 08-13
www.Iosrjournals.Org

The Ethanolic Extract of *Maranthes Glabra* (Oliv.) Prance (Chrysobalaceae) Bark Reduces the Inflammation Through The Modulation Of Pro-Inflammatory Cytokines Level

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Abstract
Maranthes glabra (oliv.) prance (Chrysobalaceae) is used in Congolese folk medicine to treatinflammatory diseases.We aimed to investigate toxicity and mechanisms of actionregarding the use of the bark ethanolic extract of M. glabra.
Materials and methods*: Toxicity was determinate in vitro using the keratinocytes HaCaT line without LPS. Cellswere treated with 0.5 to 250µg/mL of the plant extract. Cell viability was assessed by MTT colorimetricassay. The production of cytokines by HaCaT cells, in the LPS presence and after treatment by plant extract, was also determined in the supernatantsto elucidate the impact of the plant extract on the expression of proinflammatory cytokines TNFα and IL-1β.*
Results*: Low toxicity were observed with the two extract concentrations were used in this study (0.5 and 1,56 µg/ml). The cell viability was superior at 70%.For the extract concentration superior at 1,56 µg/ml, the cell viability was inferior at 70%. Regardless the concentration used in vitro, theextract exhibited a significant anti-inflammatory activity, as perceived by the reduction of the inflammatorycytokines IL-1β and TNF-α on cell supernatants.*
Conclusion:*Our results demonstrated that the bark ethanolic extract of M. glabrapossess ananti-inflammatory activity influenced by a downregulation in proinflammatory cytokines.*
Keywords*: Maranthes glabra, proinflammatory cytokines, Keratinocytes, Lipopolysaccaride (LPS)*

Date of Submission: 05-12-2020 Date of Acceptance: 20-12-2020

I. Introduction

Popular use of plants in traditional medicine represents a great source of discovering molecules with therapeutic effect. In this context, natural molecules isolated from medicinal plants have long been used to treat different inflammatory conditions.*Maranthes glabra*, is a plant found almost everywhere in Africa to treat many diseases. In Congo-Brazzaville *M. glabra*is widely used in the form of maceration or decoction/infusion for inflammatory diseases, dysentery, swelling, anaemia and other physiological dysfunctions (Bouquet, 1969; Abena et al., 1996).

Experimental studies conducted with extracts of different parts from this plant have presented promising results regarding the anti-inflammatory effect (Epa et al., 2019b). The ethanolic extract of *M. glabra* bark has demonstrated the occurrence of polyphenolic compounds and flavonoids (Epa et al., 2019a). In general, these compounds are frequently found in plants, fruits and vegetables besides of presenting anti-inflammatory, anti-infectious and anti-tumoral effects (Allouche et al., 2009; During et al., 2012; Singh et al., 2014).

Despite the beneficial effects of the ethanolic extract of M. glabra bark, its cells toxicity and especially the mechanism of action, have never been addressed before. Thus, the aim of this study was to evaluate cytotoxicity and the anti-inflammatory mechanisms related to the compounds in the ethanolic extract of *M. glabra* bark using *in vitro* approach.

II. Material And Methods

Plant material and preparation of the extract

Samples of *M. glabra* used in this study were collectedfrom the western-Cuvette department in Congo-Brazzaville in February and March 2019. Samples wereidentified by reference to the herbarium of the Exact andNatural Sciences Research Institute of Congo(IRSEN). Voucher specimens are preserved at theherbarium of IRSEN.*Moranthes glabra* barks were dried at room temperature,reduced in powder, and stored until extraction procedure.

The dried powder from the dried barks (50 g) wasextracted by the maceration technique 90% ethanol undermagnetic stirring for 72 hours. The solutions obtainedwere concentrated under reduced pressure (BÜCCHIrotavapor) and then preserved at +4 °C until in vitro tests.

Cell culture and in vitro experiment design

The HaCathumankeratinocytes lineage was purchased from theCell Line Service GmbH (CLS, Eppelheim, Germany). Thecells were plated in 96-well culture plates (Corning Inc., Corning, NY,USA) at $1x10^4$ cells/well in DMEM medium supplemented with 10%foetal bovine serum (FBS) and 1% penicillin-streptomycin solution, the 50 ml cell culture flask was used.Cells were cultivated in an incubator at 37 °C with 5% carbondioxide. After the formation of a monolayer,the plate was gently centrifuged at 150 g (Beckman, Indianapolis,IN, USA) for 5 min. Then, the supernatant was gently removedand replaced by 200 µL of cells culture medium containing different concentrationsof the ethanolic extract (1.56, 15.12, 31.25, 62.5, 125, 250µg/mL). After24 h of incubation, cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetricassay (Sigma-Aldrich, Hamburg, Germany). Absorbance was measured at 570 nm with a spectrophotometer(Varioskan, Thermo Scientific, Saint-Herbain, France).Cytotoxicity was expressed as a percentage of controls (untreated cells).

For determination of in vitro anti-inflammatory activity, after cytotoxicity assays, HaCaT cellswere treated with two ethanolic extractconcentrations (0.5 and 1.56µg/mL) in accordance with cell viability tests. Assayswere realized on activated and non-activated cells.The culturemediumwas replaced by HBSS 2 h before treatment in orderto avoid the artefact of medium replacement on signalling(Smith et al., 1997).

Cytotoxicity and effect of lipopolysaccharide (LPS) on keratinocyte cells

The cell model used in this study is the one based on lipopolysaccharide (LPS) described by many authors (Niebel et al., 2012; Moulari et al., 2014). In order to determine the non-cytotoxic concentration of LPS which induces the maximum pro-inflammatory cytokine, a cytotoxicity of LPS on HaCaT cell line was assessment.For these assays, cells were seeded on 96-well microplates(Becton Dickinson, Meylan France) at a density of 1×10^4 cells perwell. The cells were fed every two days with culture medium andused day 7 after seeding.

Briefly, the medium was removed, cells were washed with HBSStwice, and the LPS (100 µL) at different concentrations were added to the cells.Microplateswere incubated at 37 °C to evaluate a cellular viability for 4 h.Then, cell viability was assessed using the MTT colorimetric assay. The cell supernatants were used to quantify the pro-inflammatory cytokine.

Investigation of the effect of bark ethanolic extract on cytokine activation by LPS

The humankeratinocyte cell line HaCaT was grown in DMEM supplemented with FCS and antibiotics as described above. The cells were seeded in 96-well plates at a density of 2×10^4 cells per well and were allowed to adhere for 24 h. 10 µg/ml of lipopolysaccharide (LPS) was added to one part of the cells to induce cell inflammation (cytokine secretion). After 4 h the cells were washed twice with DPBS, fresh medium was added, and the cells were (or no) treated with ethanolic extract at 0.5 and 1.56 µg/ml. After incubation for 8 h, 24 h and 48 h cell supernatants were collected, centrifuged and analyzedfor the amount of TNFα and IL-1beta with commercial ELISA kits (Thermo Scientific, Saint-Herbain, France).

Statistical analysis

The resultswere expressed as mean values±S.D. For the analysis ofstatistical significance ANOVA followed by Dunn's test for all pairwisecomparison in case of multiple comparison were applied, exceptedwhen normality and equal variance were passed, it was followed bythe Tukey test. Student's t test was applied to study the significance ofdifference between two treatment groups, however, if normality and/or equal variance was not achieved the Mann–Whitney U test was applied.In all cases, p b 0.05 was considered to be significant.

III. Results

Cytotoxicity of Maranthes glabra barks ethanolic extract

To address the *in vitro* toxicity of the ethanolic extract ofM. glabra, HaCaT keratinocytes were cultivated with different concentrationsof the extract as described above.

Regardless theconcentrationsvarying between 0 and 1.56 µg/ml, we observed the cell viability superior or equal 70%. And for concentration superior at 1.56 µg/ml, the cell viability was inferior at 70% (Fig. 1). Thus, in the remainder of this assessment, only the concentrations giving 70% cell viability will be used (0.5 and 1.56 µg/ml).

Cytotoxicity and effect of lipopolysaccharide (LPS) on keratinocyte cells

The figure 2 shows the cytotoxicity effect of LPS on keratinocyte cells at different concentrations. We observed the no-alteration in cell viability for all concentrations tested, the cell viability was superior or equal at 90% (Fig 2). TNFα secretion from non-stimulated HacaT-cells was slightlyincreased.However highest levels were reached with stimulated-HaCaT(Fig. 3A). Similar tendencies were observed with IL-1β (Fig 3B).

Effect of the bark ethanolic extract on pro-inflammatory cytokines activation

TNFα and IL-1 beta secretions from non-stimulated HaCaT-cells was slightly increased for all concentrations. However highest levels were reached with HaCaT-activated by LPS. But on HaCat-activated by LPS and treated with bark ethanolic extract of *M. glabra* (EE), TNF-alpha and IL-1 beta secretion decrease depending the incubation time (Figure 4). At 8 h incubation, only ethanolic extract at 1.56 µg/ml significantly decrease the secretion of both cytokines from HaCaT cells. At 24 and 48 h the wo concentrations tested significantly decrease the secretion of pro-inflammatory cytokines from HacaT cells (Figures 4 and 5).

IV. Discussion

The results presented here demonstratethe anti-inflammatoryproperties of the ethanolic extract of *M. glabra* by an action on TNF-alpha and IL-1 beta secretion in accordance with precedent finding (Epa et al., 2019b). Furthermore, for the concentrations used, plant extractshowed low toxicity onHaCaT cell line. In order we know that to restrain inflammation, a therapeutic drug must beable to modulate different markers involved in this processsuch cytokines. Indeed, cytokines play a crucial role to the onset and maintenance of inflammation and inflammatory diseases (Kawagachi et al., 2011).During inflammatory disease, pro-inflammatory cytokines may induce the productionof NO, especially in immune and non-immune cells. In general,mostly in the presence of infectious agents like bacteria and theirderived products, such as LPS, the production of NO is drasticallyenhanced in phagocytes and other cells in the body, which improvesphagocytosis and microbial destruction (Galkina et al., 2019). It is known that Nitric oxide is an unstable and highly soluble moleculebelonging to the family of reactive oxygen species (ROS), as reviewed by (Mijatovic et al., 2020). Effects of the bark ethanolic extract of *M. glabra*in proinflammatory cytokines could be due to the presence of antioxidant compounds (polyphenols and flavonoids) in this plant as shown previously by Epa et al., 2019a et 2019b.Several authors show that the intake of polyphenols contained in natural sources, such as hydroxytyrosol, tyrosol, oleuropein (olives), naringin and hesperidin (Citrus fruits), resveratrol, procyanidins or oligomeric procyanidin (grapes or grape seed extracts), (-)-epigallocatechin gallate (green tea) and quercetin (grapes, green tea)..., are able to modulate chronic inflammatory diseases, such as type 2 diabetes, rheumatoid arthritis, inflammatory bowel disease in decreasing the TNF-α secretion (Kawagachi et al., 2011).Othercytokine involved in inflammatory disease is IL-1β which can also enhance the production of NO or have itsown production positively influenced by this molecule (Mijatovic et al.,2020). Thus, the bark ethanolic extract of *M. glabra* in decreasing the IL-1β secretion would help reduce inflammation.In addition, our extract could also modulate the synthesis of NO, because it is rich in polyphenols whose role in the modulation of the secretion of inflammation markers has been demonstrated by several authors (Khan et al., 2014; Tangney et Rasmussen, 2013; Hussain et al., 2016). This could explain its anti-inflammatory potential beside its action on the TNF-α and IL-1β modulation. It seems clear that therapies aiming at modulating the production of proinflammatory cytokines such as TNF-α et IL-1β may represent important strategies to control inflammation. Although we have only addressed the in vitro effects of the plant extract in the downregulation of TNF-α et IL-1β, we cannot underestimate its influence in the reduction of proinflammatory cytokines secretion, and therefore the inflammation.

V. Conclusion

In conclusion, our results showed for the first time the modulator effect on proinflammatory cytokines of the ethanolicextract of *M. glabra* bark. The extract was able to constrain the releaseof inflammatory mediators *in vitro*.Further, the mechanism of action seems to be at least partly dependenton downregulation of TNF-α and IL-1β. Finally, our results also suggest that the ethanolicextract of *M. glabra* bark could be a promising candidate to treatimmune-mediated diseases.

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CAPTIONS

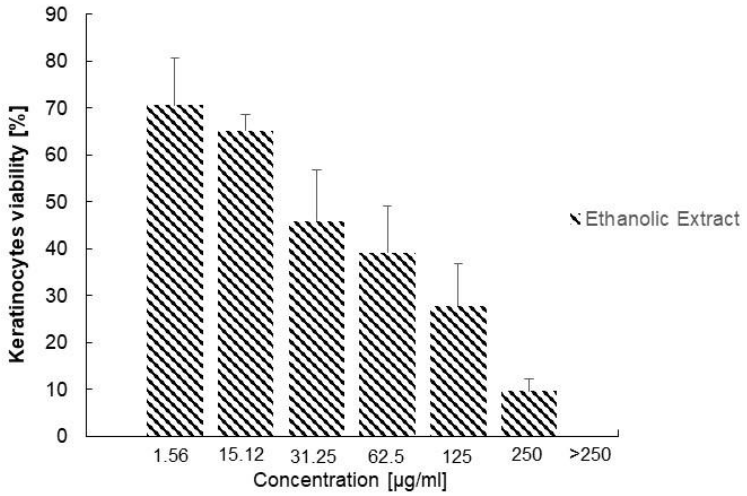

FIGURE 1: Bark ethanolic extract of *M. glabra* (EE) solutions of different concentrations were tested for their toxicity toHaCaT cells after incubation for 8 h. Data are shown as mean±S.D.

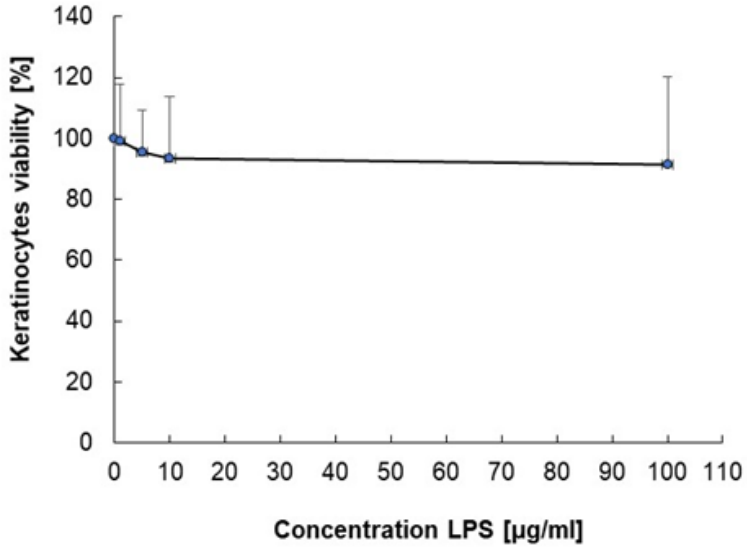

FIGURE 2: :Lipopolysaccaride (LPS) solutions of different concentrations were tested for their toxicity toHaCaT cells after incubation for 4 h . Data are shown as mean±S.D.

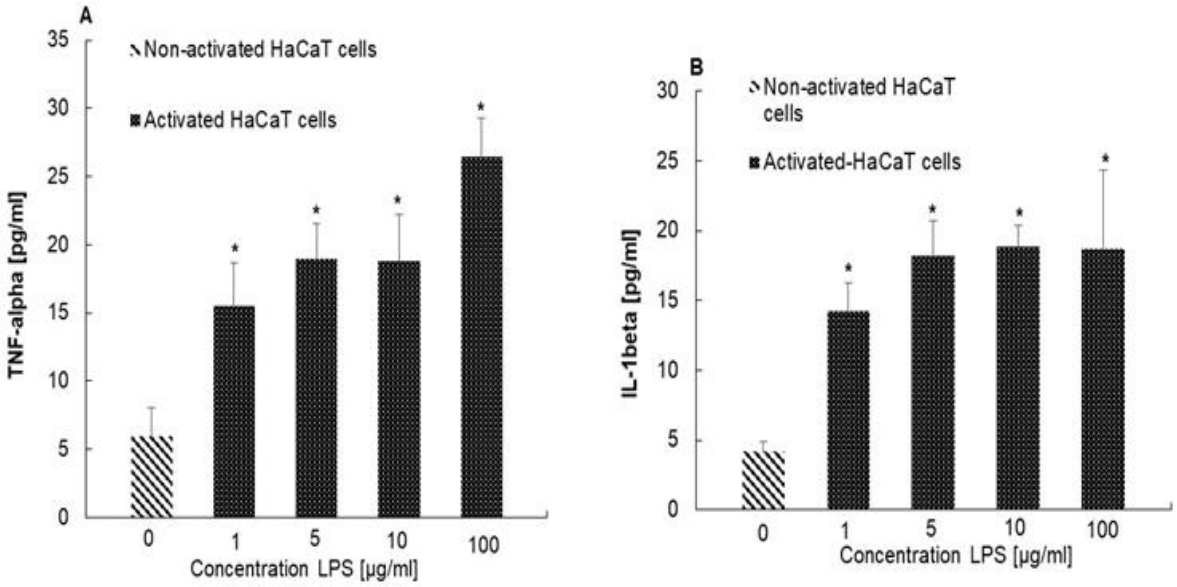

FIGURE 3: TNFα and IL-1β secretion of non-activated and activatedkeratinocytes aftertreatment with different concentrations of LPS; datagiven as mean±S.D., *=P<0.05 compared with non-activatedcells.

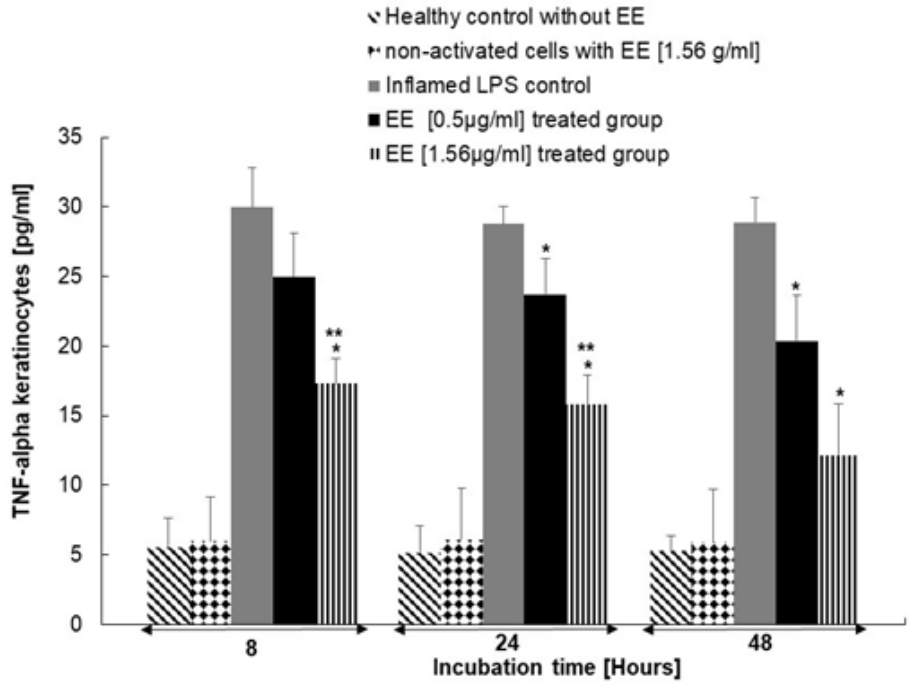

FIGURE 4: TNFαsecretion of non-activated and activatedkeratinocytes aftertreatment with different concentrations of bark ethanolic extract of M. glabra (EE) at different incubation time; datagiven as mean±S.D., *=P<0.05 compared with LPS control; **P<0.05 compared with EE at 0.5 µg/ml.

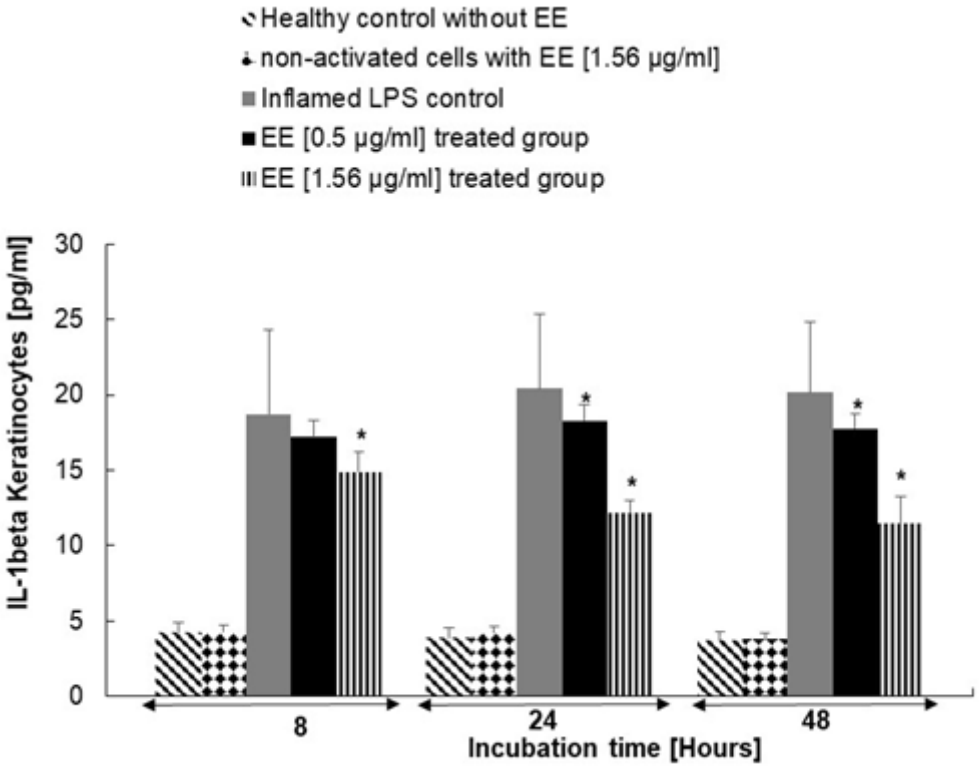

FIGURE 5: TNFα secretion of non-activated and activated keratinocytes after treatment with different concentrations of bark ethanolic extract of M. glabra (EE) at different incubation time; data given as mean±S.D., *= P<0.05 compared with LPS control; **P<0.05 compared with EE at 0.5 µg/ml.

Epa C, Moulari B, Tsati G. "The Ethanolic Extract of Maranthes Glabra (Oliv.) Prance (Chrysobalaceae) Bark Reduces the Inflammation Through The Modulation Of Pro-Inflammatory Cytokines Level." *IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS)*, 15(6), (2020): pp. 08-13.