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Research article

In vitro Immunomodulatory and Anti-Inflammatory Effects of the *Commelina benghalensis* Plant Extract on RAW 264.7 Murine Macrophage Cell Line

Mmampeule, C.M.[1], *Ndlovu, M.J.[2], Makgatho, M.E.[2] and Matsebatlela, T.[1]

[1]*Department of Biochemistry, Microbiology and Biotechnology, Faculty of Sciences and Agriculture. University of Limpopo, South Africa.*

[2]*Department of Pathology, Faculty of Health Sciences, University of Limpopo, South Africa.*

ABSTRACT

Chronic inflammatory diseases emerge as a response to the disorders of inflammatory processes and excessive production of pro-inflammatory mediators initiating an inflammatory reaction cascade. The plant biomolecules can be used for treatment of inflammatory skin diseases emanating from their influence on different stages of inflammation. Objectives: Immunomodulatory and Anti-inflammatory effects of the *Commelina benghalensis* plant extract on the Raw 264.7 murine macrophage cell line was assessed using different assays. Secondary metabolites detection and ferric reducing antioxidant activity of the plant extract was spectrophotometrically shown using ascorbic acid as a standard. Immunomodulatory and Anti-inflammatory effects on Raw 264.7 cell line was assessed by employing MTT, Annexin-V flous and Griess reagent assay. There were observations of the presence of saponins, flavonoids, glycosides, tannins, carbohydrates and concentration-dependent ferric reducing antioxidant power of the plant extract. The extract was shown to have minimal effects on cell proliferation or induction of apoptosis and production of Nitric Oxide (NO) was inhibited in a concentration-dependent manner. The compounds contained in *C. benghalensis* can be used in the treatment of oxidative stress-related disorders and the potential development of novel immunomodulatory drugs.

Keywords: *Immunomodulatory, anti-inflammatory, antioxidant, nitric oxide*

**Author for correspondence: Email: mxolisi.ndlovu@ul.ac.za; Tel: +27 15 268 2557*

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INTRODUCTION

Anti-inflammatory and antioxidant testing of natural products has attracted increasing interest in recent years, mainly due to growing advocated benefits of traditional medicinal plant products and the eminent fact that an antioxidant-rich diet might provide health benefits. Activated macrophages are a major source of reactive oxygen species, reactive nitrogen species, and peroxynitrite generated through the respiratory burst. Constitutively released pro-inflammatory cytokines, especially tumour necrosis factor-α, triggers nuclear factor-κB, and activator protein-1 translocation leading to the over production of reactive oxygen species and reactive nitrogen species in macrophages. (Castaneda *et al*., 2017).

The adaptive immune response is profoundly dependent on innate immunity, therefore, counteracting some of the cancer-promoting immunosuppressive actions of the innate immune system might heighten the potential of immunotherapies that activate a budding antitumour response. This would reduce the risk of development of chronic inflammatory disorders (Shanthini and Balkwill; 2015).

The inflammatory response is a complex, localized immunological response of well vascularised tissues to injurious stimuli in an attempt of the host to defend against noxious substances, remove damaged tissue components facilitated by the professional phagocytes and ultimately initiate the repair and healing process. Five visual observations characterize the site of inflammation, redness, oedema, heat, pain and loss of organ (Pacher *et al*., 2007). Normally, the inflammatory response is tightly controlled, self-limited and protective. However, in an inflammatory response which is chronic in nature a persistent inflammation can have detrimental effects on the body's systems (Veraldi *et al*., 2015).

Production of reactive oxygen intermediates (ROI) or species (ROS) is central to the progression of many inflammatory diseases. The ROI are produced by cells that are involved in the host-defense response, such as polymorphonuclear neutrophils (PMNs) and promote endothelial dysfunction by oxidation of crucial cellular signaling proteins such as tyrosine phosphatases. The ROS act as both a signaling molecule and a mediator of inflammation.

The ROS such as superoxide can rapidly combine with Nitric Oxide at a diffusion limited rate (k=5 to 10×109M−1s−1) to form reactive nitrogen intermediates (RNI), such as peroxynitrite, and is three to four times faster than the dismutation of superoxide by the superoxide dismutase (SOD) (Mittal *et al*., 2014).

Hyperglycemic conditions increase electron flux through the respiratory chain in mitochondria stimulating the formation of ROS. The key enzyme for ROS production in cells is Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. The NADPH oxidase-mediated release of ROS in macrophages during oxidative respiratory burst leads to the elimination of invading microorganisms. In human monocytes and murine macrophages PI3K and protein kinase C (PKC) pathways are involved in NADPH oxidase stimulation. This mechanism triggers ERK1/2, p38 mitogen-activated protein kinase (MAPK) and nuclear factor (NF)-κB, which terminates in the activation of monocytes and proliferation of macrophages (San Jose *et al*., 2009).

Reactive nitrogen species oxidize proteins and nucleic acids. In addition to producing ROS, the mitochondrial respiratory chain is capable of producing nitric oxide (NO). Inducible NO synthase (iNOS) is a key enzyme in the macrophage that is potently induced in response to proinflammatory stimuli. Macrophages are activated by interferon-γ and microbial products such as lipopolysaccharides (LPS), leading to production of proinflammatory cytokines and high levels of NO. Nitric Oxide is a potent molecule involved in critical macrophages functions such as cytotoxicity against intracellular pathogens, viruses and tumors, and immune regulation (McNeill *et al*., 2015).

Reactive oxygen intermediates and reactive nitrogen intermediates (RNI) are generated during normal cellular metabolisms, and during the intracellular degradation of engulfed pathogenic matter by the designated innate leukocytes at the site of inflammation (Punchard *et al*., 2004). Normally, the body is relatively able to regulate oxidative stress with the use of enzymes such as catalase, superoxide dismutase and various peroxidases (Saleh *et al*., 2010). However, vast production of the free radical molecules refutes the body's neutralizing mechanisms, leading to severe damages to various cellular molecules such as deoxyribonucleic acid (DNA), ribonucleic acid (RNA) templates. Free radicals also affect and destabilize proteins, lipids and polysaccharides. Ultimately, these become the predisposing factors to well-known disorders such as cancer and diabetes mellitus, neurodegenerative diseases such as Alzheimer's and Parkinson's diseases, inflammatory diseases such as atherosclerosis and rheumatoid arthritis, as well as accelerating the aging processes in general (Ďuračková 2009).

MATERIALS AND METHODS

Plant collection and verification of leaves: *Commelina benghalensis* was collected from the University of Limpopo, Mankweng, in Limpopo Province, South Africa. The plant was identified by the Larry Leach Herbarium, University of Limpopo and vouchered (UNIN 121327).

Plant extract preparation: The leaves of the plants were dried in the dark at 25oC for two weeks and grinded into fine powder (1g) which was exhaustively extracted using 10 ml of acetone. The supernatants were filtered into pre-weighed glass vials. The quantity of plant materials extracted was determined and stored in air-tight glass vials in the dark until use. The dry plant extracts were reconstituted in dimethylsulphoxide (DMSO) (Sigma Aldrich, SA) for cell-based assays.

Qualitative screening of various phytochemical constituencies from the medicinal plant

Test for tannins: After the addition of the plant extract and distilled water as well as ferric chloride, the presence of a greenish-black colour was indicative of tannins (Borokini and Omotayo, 2012).

Test for phenols: The plant leaf extract was suspended in dihydrogen oxide (H2O) and a few drops of 1 % aqueous solution plumbous acetate [Pb(C4H6O4)] added. The presence of phenols was indicated by a yellow precipitate (Qadir *et al*., 2009).

Test for saponins: The plant leaf extract was dissolved in water and heated. The sample was cooled at room temperature and vigorously agitated. The presence of saponins was indicated by persistent foam (Qadir *et al*., 2009).

Test for steroids: The plant leaf extract was mixed with chloroform (CHCl3) and sulphuric acid (H2SO4). Subsequent to agitation a reddish brown colour indicated the presence of glycosides (Borokini *et al*., 2012).

Test for carbohydrates: To the plant leaf extract, Benedict's reagent (anhydrous sodium carbonate, sodium citrate and blue vitriol) was added and boiled. The presence of carbohydrates was indicated by the appearance of a red precipitate (Borokini *et al*., 2012).

Test for flavonoids: To the plant leaf extract, a pinch of zinc (Zn) dust and hydrochloric acid (HCl) were added. The presence of flavonoids was indicated by the appearance of magenta colour after a few minutes (Borokini *et al*., 2012).

The ferric ion reducing antioxidant power analysis of the extract using spectrophotometry: Ferric reducing antioxidant power (FRAP) assay was utilized to assess the ferric ion reducing capacity of the *C. benghalensis* plant extract. Various concentrations (1.95 µg/ml to 250 µg/ml) of the plant extract in distilled water (100 µl) were prepared. A blank solution was prepared (without the extract) and ascorbic acid (C6H8O6) was used as the standard in conformity to a method described by Arulpriya (2010).

Cell culture: The Raw 264.7 adherent murine macrophage cells were cultured in FBS-supplemented RPMI-1640 medium at 37 °C in a 95% humidified atmosphere of 5% carbon dioxide (CO2).

The effect of the plant extract on cell proliferation using MTT assay: MTT [3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide] tetrazolium reduction cell proliferation assay was utilized to assess the proliferative activities of the plant extract on Raw 264.7 cells. The adherent cells (1.0x106cells/ml) were incubated (37°C, 5% carbon dioxide (CO2)) overnight with treatments of different concentrations of the dimethyl sulfoxide (DMSO) re-dissolved plant extract in a 96-well culture plate in conformity to a method described by Mosaddegh *et al*., 2012.

The effect of the extract on cellular nitric oxide synthesis: The Griess reagent system was utilized to measure nitrite (NO2–) in Raw 264.7 cells, one of two primary stable and non-volatile breakdown products of nitric oxide (NO). The (1.0x106cells/ml) were seeded to a 96-well plate in phenol-free RPMI culture media and allowed to attach overnight in conformity to a method described by Sun *et al*., 2003.

The effect of the extract on intracellular production of reactive oxygen species (ROS): Bursttest (phagoburst) assay was utilized to assess the effect of the plant extract on the oxidative burst activity of Raw 264.7 cells. Two hundred microliters of cells (1.0x106 cells/ml) were incubated overnight (37°C, 5% carbon dioxide (CO2)) on microscope coverslips in a 6-well plate in conformity to Pallavi *et al*., 2012.

Statistical analysis: Statistical analysis was performed in order to determine significant differences between samples. Differences were considered significant at, * $p<0.05$; ** $p<0.01$; *** $p<0.001$. The analysis of variants test (ANOVA), Tukey-Kramer was used to compare samples using Graph Pad InstatTM 3 software. Mean fluorescence intensities were analysed using Image J software. ChemiDoc XRS software was used to analyse western blot results.

RESULTS

Phytoconstituents analysis: A phytochemical analysis of the *C. benghalensis* plant extract was carried out and the results subsequently represented (Table 1). The screening of the plant extract revealed the presence of saponins, flavonoids, glycosides, tannins and carbohydrates, while steroids and phenols tested negative**.**

Table 1:
Determination of the presence of phytochemicals present in *C. benghalensis* plant extract.

Phytochemical	Results
Steroids	-
Saponins	+
Flavonoids	+
Glycosides	+
Tannins	+
Carbohydrates	+
Phenols	-

Keynote: (+) positive, (-) negative

The ferric ion reducing antioxidant power analysis of the extract: The ferric ion reducing antioxidant power analysis of the *C. benghalensis* plant extract was determined using spectrophotometry. Positive results were observed and represented graphically in figure 1 below. A significant increase in reducing power was observed when the plant extract was added to the reaction mixture from as low as 1.5 µg/ml. A significant increase in ferric reducing power was also observed the plant extract concentration was increased to 3.9 µg/ml. A significant increase in reducing power of the CB plant extract was also observed at a concentration of 62.5 µg/ml and 125 µg/ml. The highest reducing power of the plant extract was observed when the extract concentration was doubled to 250 µg/ml.

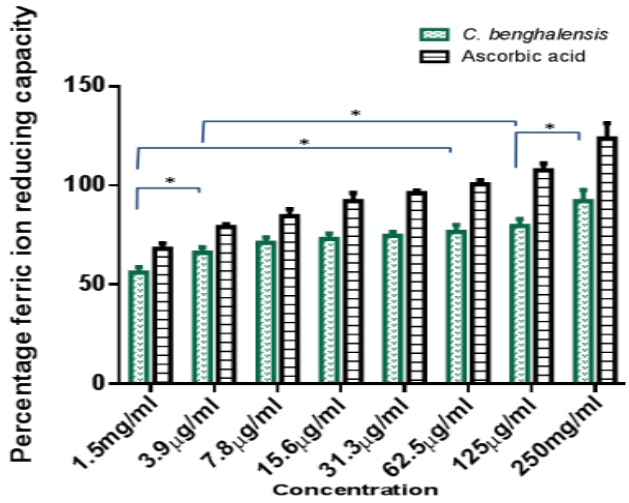

Figure 1:
The ferric-reducing antioxidant power of the *C. benghalensis* crude plant leaf extract. The percentage ferric ion reducing power of different concentrations of the *C. benghalensis* crude plant leaf extract was assayed against the control containing no extract and analysed relative to the comparative ascorbic acid (* = $p<0.05$).

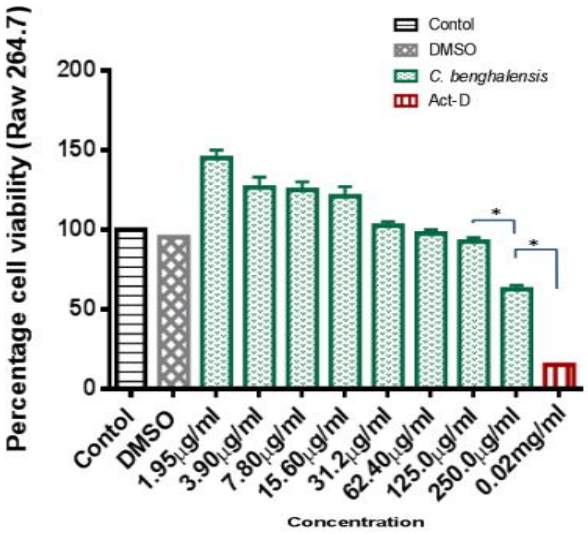

Figure 2:
The effect of *C. benghalensis* plant extract on viability of Raw 264.7 macrophages. The percentage cell viability of the Raw 264.7 cell line challenged with different concentrations of the *C. benghalensis* plant extract (*$p<0.05$).

The effect of the extract on cell proliferation: The MTT assay was employed to evaluate the effects of the test material on the viability of the Raw 264.7 cell line at different concentrations and was graphically documented. As shown in figure 2, the *C. benghalensis* crude plant leaf extract concentrations of up to 125 µg/ml had no effect on Raw 264.7 cell viability. In fact, the plant extract seemed to promote cell viability in comparison to the untreated cells. It was only when concentrations of the extracts were increased to as high as 250 µg/ml and above that significant decrease in cell viability ($p<0.05$) was observed.

The IC_{50} could be determined at 400 µg/ml, showing that the *C. benghalensis* crude plant leaf extract can be applied in the cells without induction of cell death since only concentrations lower that 65 µg/ml show reducing power activity as depicted in figure 2 above. The control cells were treated with same amount of DMSO used to solubilize the highest extract concentration of 250 µg/ml. The Actinomycin-treated positive control cells significantly lost viability to as low as 20%, showing that these cells were still sensitive to agents capable of inducing cell death. The contrasting results on the DMSO negative control cells and actinomycin-treated positive control cells could be used to demonstrate that the *C. benghalensis* crude plant leaf extract had minimal effect on cell viability.

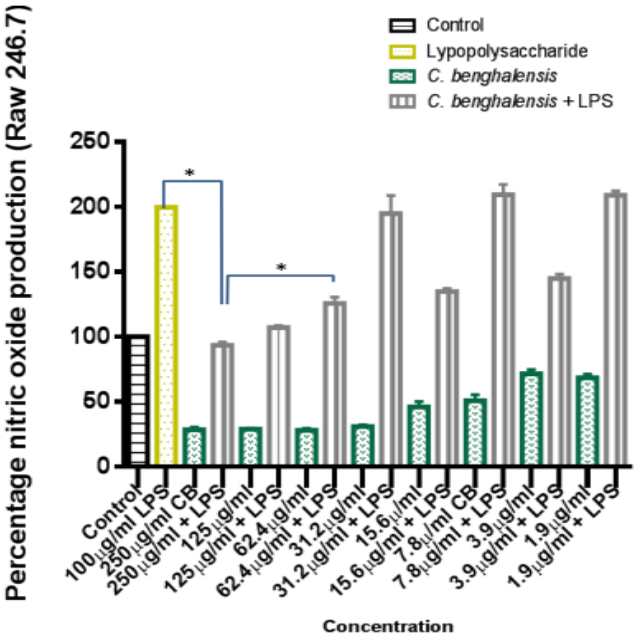

Figure 3:
The effect of *C. benghalensis* plant extract on nitric oxide production in LPS-activated Raw 264.7 macrophages. The percentage nitric oxide production by the Raw 264.7 cells following treatment with different concentrations (1.95µg/ml to 250µg/ml) of the *C. benghalensis* plant extract. Different concentrations of the plant extract supplemented with the lipopolysaccharide stimulant were also tested on the cell line.

The effect of the extract on cellular nitric oxide synthesis: The effect of the plant extract on nitric oxide production by the Raw 264.7 cells was examined using nitrite assay (Griess reagent) and the results graphically documented in Figure 3. The plant extract by itself was observed to inhibit the production of nitric oxide in comparison to the untreated cells. The pharmaceutically acknowledged stimulant, lipopolysaccharide, was observed to double the amount of nitric oxide relative to the untreated cells. This inhibition in nitric oxide production by the plant extract proved to have an inverse relationship with extract concentration. Figure 3 clearly depicts a marked decrease in nitric oxide production because of the increase in the extract concentration. When coupled with lipopolysaccharide however, the plant extract was observed to induce nitric oxide production in the cells (in comparison to the untreated cell). The inverse relationship between the nitric oxide production and extract concentration (coupled with LPS) was still observed, with the decrease in extract concentration having a causal effect of an increased nitric oxide production (see figure 3). It was in the concentration of 7.8µg/ml of the LPS-coupled extract; however, that the plant extract tremendously induced nitric oxide production, more pronounced even to that of the lipopolysaccharide stimulant.

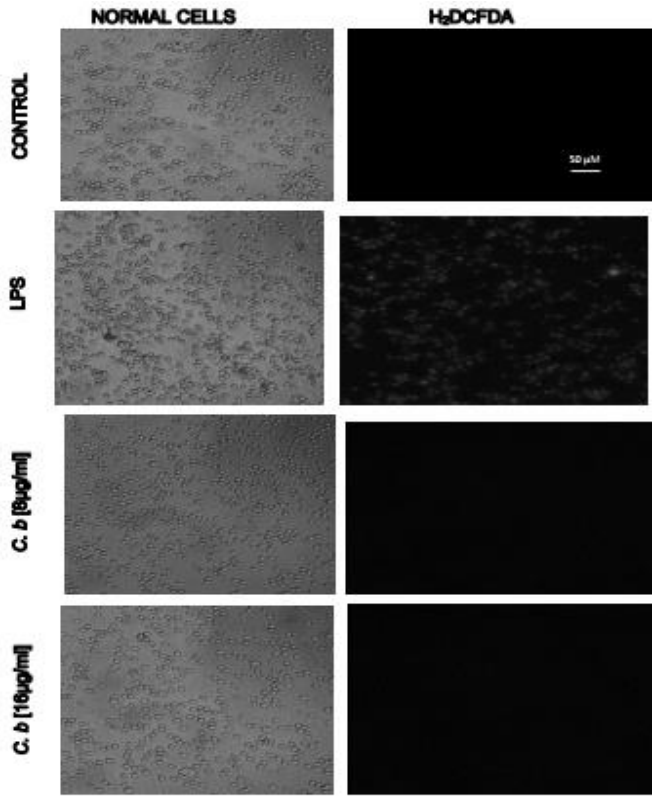

Figure 4:
The effect of *C. benghalensis* crude plant leaf extract on ROS production in LPS-activated Raw 264.7 cells. Fluorescence microscopic (40X magnification) images displaying Raw 264.7 macrophage cells treated with different concentrations (8µg/ml and 16µg/ml) of the *C. benghalensis* crude plant leaf extract stained with the H2DCFDA oxidative stress indicator were captured. Fluorescence brightness represents the intracellular oxidation of H2DCFDA. The brightness of the fluorochrome (2,7-dichlorofluorescein) reflects the relative amount of the reactive oxygen species.

DISCUSSION

Commelina benghalensis , has been observed in the treatment of a variety of inflammatory infections by traditional healers. In this study, owing to its traditional use in treatment of these infections-related ailments, the *C. benghalensis* crude plant leaf extract was shown to possess maximum potency against a variety of infectious bacteria even though the compounds responsible for the plant's medicinal properties have not yet been identified. It is therefore evident that its ability to stimulate the immune system is apparent (Jemilat *et al*., 2010; Khan *et al*., 2011).

Some of the biologically active compounds detected in the phytochemical analysis of the plant extract (Table 1) support not only the suggestion that the plant is of medicinal importance but also supports the basis for some of its ethno-uses. The presence of flavonoids suggests that the plant has some antioxidant, anti-allergic, anti-inflammatory, anti-microbial and anti-cancer activities. Other secondary metabolites do have antiviral and antibacterial activities and can aid in the healing of wounds and burns (Jamilet *et al*., 2010). Saponins and glycosides are cardio-active and used in the treatment of heart conditions (Evans, 2002).

In the evaluation of the *C. benghalensis* plant extract's ferric reducing antioxidant activity, positive results were obtained. This was visualized in the assay, where $K_4[Fe(CN)_6$ (potassium ferro-cyanide) (which forms the Prussian blue ferric-ferrous complex after reacting with ferric chloride) could only be obtained from the successful ferric-reduction-antioxidation of the K3[Fe(CN)6] (potassium ferri-cynide). The study provided by Kunle and Egharevba (2009) on the phytochemicals present in the *C. benghalensis* plant leaf extract also reports on the presence of flavonoids which suggests that the plant extract contains some degree of antioxidant activity, and thus, the eliminant anti-allergic activity. Although the antioxidant activity of the plant extract (in the form of ferric reducing antioxidant power) seemed relatively low to that of the ascorbic acid (figure 1). This effect might be attributed to the fact that ascorbic acid is a pure compound while the plant extract contains an assortment of biologically active compounds (which may possess effects which are antagonistic in nature) (Kunle and Egharevba, 2009).

The plant extract was observed to have inhibitory effect on nitric oxide production by the Raw 264.7. Nitric oxide production is mostly due to an intracellular up-regulation of the inducible nitrogen oxide synthase (iNOS) in phagocytes (during inflammation), a reduction in which can reduce the detrimental effects of an inflammatory response which is chronic in nature (Mills *et al*., 2015). The inhibition of nitric oxide production corresponds with the report that, the crude plant leaf extracts of C. benghlensis are medicinally used in tropical Asia and Africa, as a treatment for inflammations of the skin as well as leprosy (Khan *et al*., 2011). Thus, the study should be furthered to ascertain the extract's mode of action. However, when coupled with lipopolysaccharide, the plant extract proved to be super stimulatory to nitrogen oxide production (figure 3). This effect would prove highly useful (clinically) in engineering a rapid bust of the inflammatory response (along with its accomplice mediators) in cases where an individual is immune-compromised and is unable to mount a strong enough immunological attack on invading pathogens, or rather the rate at which the individual's immune system responds, is inadequate to the elimination of said pathogens (to induce an immunological scenario similar in nature to that of passive immunization and avoid imminent catastrophe) (Mark and Saunders., 2011).

Upon the assaying of the respiratory burst activity of the cell line by evaluating the oxidation of 2'7'-dichlorofluorescin-diacetate (H2DCFDA), the plant extract showed inhibitory effects on the production of the reactive oxygen molecules (figure 4). This is highly important, as it is the oxidative stress which is implicated in the pathogenesis of lipo-toxicity in both human and animal studies. Moreover, it is the chronic deregulated oxidative stress which is linked to insulin resistance in multiple tissues (Rosenkranz *et al*., 1992).

In conclusion, the plant extract shown to contain various phytochemical constituents though had minimal effects on cell proliferation or induction of apoptosis while production of Nitric Oxide (NO) was inhibited in a concentration-dependent manner. The compounds contained in *C. benghalensis* can be used in the treatment of oxidative stress-related disorders with the potential development of novel immunomodulatory drugs. Further studies will be ongoing to determine the effect of the extract on the expression underlying mechanisms of Immunomodulatory and Anti-inflammatory with the aim of identifying therapeutic principles contained in the extract.

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