



Effect of Fractions, and Compounds from *Typha capensis* in LPS-Stimulated Raw 264.7 Cells. Pro and Anti-inflammatory Cytokines

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Author's contribution

The sole author designed, analyzed, interpreted and prepared the manuscript.

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ABSTRACT

Typha capensis is widely used by traditional healers to treat male fertility, venereal problems and inflammation. There are many molecular targets implicated in the inflammatory process: pro- and anti-inflammatory cytokines such as interleukin 1- β , IL-6, IL-10, IL-12p70, tumor necrosis factor alpha (TNF- α), and IL-8, and other proteins such as COX-2, and iNOS. In order to clarify the anti-inflammatory mechanism of action of compounds isolated from *T. capensis*, RAW 264.7 macrophages were activated by lipopolysaccharide and pre-treated with *T. capensis* isolated compounds. Lipopolysaccharide-stimulated RAW macrophages after treatment with *T. capensis* crude acetone extract resulted in decreasing expression of pro-inflammatory cytokines (TNF- α , IL-6,) and increased expression of immunomodulatory cytokine IL-12 P 70. Isorhamnetin-3-O- β -D-glucoside and isorhamnetin 3-O rutinoid increased the expression of pro-inflammatory cytokines TNF- α , but failed to reduce the expression of IL-1 β and TNF- α . Isorhamnetin-3-O- β -D-glucoside and isorhamnetin 3-O rutinoid increased the expression of immunomodulatory cytokine IL-12p70. Isorhamnetin-3-O- β -D-glucoside increased the expression of the anti-inflammatory cytokine IL-10 compared to quercetin and LPS-stimulated macrophages. The effect of isorhamnetin 3-O-rutinoid and isorhamnetin-3-O- β -D-glucoside on molecular targets of inflammation may provide support for the use of *T. capensis* by traditional healers against inflammation.

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1. INTRODUCTION

Typha capensis (bulrush) is one of the medicinal plants used by traditional healers in South Africa to treat male fertility problems. The rhizomes are also used during pregnancy to ensure easy delivery, diarrhea, dysentery, and to treat venereal diseases [1,2]. The anti-inflammatory properties of *T. capensis* are not well documented, although it has been said that the plant can be used against stomach pain, abdominal pain, and abscesses [3]. There is very few information about the mechanism of action involved in its anti-inflammatory activities. Inflammation is a complex process from the host to restore a tissue injury due to harmful stimuli such as stimuli exposure, trauma, and viral infection to normal physiological function. This complex process is mediated by activated immune cells such as macrophages [4].

Cytokines are soluble signaling molecules produced by a variety of cells types. They are secreted in response to specific and non-specific stimuli and involved in all stages of inflammation, and immunological responses from the body. Cytokines regulates inflammatory process, from initiation to resolution, and regulates its own synthesis and that of their cellular receptors [5,6]. There are many cytokines, divided into two main categories: Pro and anti-inflammatory cytokines. T lymphocytes are responsible for the immune system regulation via the secretion of key cytokines that can drive T-cells based on the stimuli. There are three main types of T-cells (Th-1, Th-2, and Th-17) depending on the type of cytokines they produce. Tumor necrosis factor alpha is produced by Th-1, meanwhile interleukin-6, interleukin-10 are produced by Th-2 T-cells types [7].

Lipopolysaccharide, an endotoxin is a part of the major part of Gram-negative membrane of some bacteria, plays an important role in inflammatory response via macrophage activation. The result of that macrophage activation is production of many signaling events, including various pro inflammatory cytokines, such as tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), interleukin 1- β , nitric oxide, inducible nitric oxide synthase, (iNOS), cyclooxygenase-2 (COX-2), and other inflammatory cytokines [8,9]. Inducible nitric oxide synthase expression in cells is associated

with heme oxygenase (HO-1), inhibitor of pro-inflammatory mediator expression (TNF- α , IL-6, IL-1 β) in LPS-stimulated macrophages [10]. When balanced, pro and anti-inflammatory cytokines (TNF- α and IL-10) and other inflammatory mediators play a key role on managing and restoring normal physiologic function such as inflammation imbalance and mediators in many central nervous system injuries and diseases [11,12]; However, any imbalance of inflammatory mediators such as overproduction of pro inflammatory mediators can lead to various inflammatory diseases such as rheumatoid arthritis, and inflammatory bowel diseases, but also to many other ailments like vascular diseases, asthma, vascular diseases, and even cancer ([13]).

There are many studies exploring the role of cytokines and other molecules related to inflammatory process networks not only in inflammation, but also in some specific diseases such as atherosclerosis, cancer, and neuro inflammation [14,15]. In the present study, we investigated the mechanism of *T. capensis* crude extract, fractions and isolated compounds on lipopolysaccharide (LPS)-mediated inflammation in macrophages. The biological effects of *T. capensis* on the production of inflammatory mediators, pro and anti-inflammatory cytokines, in LPS-stimulated macrophages to elucidate the potential anti-inflammatory mechanism through iNOS and COX-2 pathways. In addition, we determined whether the induction of HO-1 expression explain the anti-inflammatory mechanism of *T. capensis*.

2. PLANT MATERIAL

Typha capensis leaves were collected in February 2016 in the vicinity of Midrand, Gauteng. One kilogram of leaves were collected and air-dried for five days and ground to fine powder using KMF-10 Basic grinder (IKA-WERKE-GMBH & CO.KG), then extracted in acetone for 48H. After extraction, the solution was filtered using whatmann filter paper and the filtrate was concentrated using rotary evaporator under reduced pressure (Büchi Rotavapor, CH 9230, Flawil, Switzerland). Fifty g were collected and left for another week on bench in honey jar to complete dryness before use.

2.1 Reagents

The mouse macrophage cell line RAW 264.7 was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). RPMI 1640 medium, foetal bovine serum (FBS) and antibiotics were purchased from (Basel, Switzerland). LPS, bovine serum albumin (BSA), was obtained from Sigma-Aldrich (St. Louis, MO, USA). Anti-iNOS antibody ab15323, abcam USA, Anti-COX-2/Cyclooxygenase 2 antibody ab52237, abcam USA, Goat Anti-rabbit IgG H&L (HRP) ab6789, abcam USA. Nitrocellulose (NC) membranes were acquired from Millipore (Bedford, MA, USA).

2.2 Instruments Used

SH 30L Reciprocating Shaker FINEPCR, Korea; UVITEC Cambridge CB4 QB, UK; Bio-Rad Mini Protean tetrasystem, China; Labcon waterbath; AccuBlock digital drybath Labnet International, NJ07095, USA; Sonicator JENCONS, model VC50 Danbury City, USA; Bio-Rad 041BR125388, Singapore; Ependorf centrifuge 5417R Merck, Germany.

2.3 Raw 264.7 Macrophage Cultivation

The RAW 264.7 macrophages cell lines were obtained from the American Type Culture Collection (ATCC) and they were grown at 37°C with 5% CO₂ in Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5g/L of glucose and 4mM of L-glutamine and supplemented with 10% foetal calf serum (FCS) and 1% penicillin/streptomycin/fungizone (PSF). Raw 264.7 macrophages cell lines were cultured at a density of two milliliters of (6 x 10⁵cells/mL) in 6-wells plate overnight at 37°C with 5% CO₂ to allow attachment. The RAW 264.7 cells were activated by incubation in a medium containing 1 µg/mL of lipopolysaccharide (LPS), except for blank (cells only plus DMSO) and incubated one hour before adding three concentrations of crude extract (*Typha capensis*), butanol fractions (*Typha capensis*,) and isolated compounds from *Typha capensis*. The cells were treated with extracts or compounds dissolved in DMSO and further diluted in culture medium. The concentration of DMSO in the experiment did not exceed 1%.

2.4 Pre-treatment of Raw 264.7 Macrophages for Cytokines Using Flow Cytometry Method

RAW 264.7 macrophages obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA) were grown at 37°C with 5% CO₂ in Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5 g/L of glucose and 4 mM of L-glutamine (Hyclone™) supplemented with 10% foetal calf serum (FCS) (Capricorn Scientific GmbH, South America) and 1% penicillin/streptomycin/fungizone (PSF). The cells were sub-cultured three times a week. One hundred micro litres of a cell suspension (2 x 10⁶ cells/mL) of RAW 264.7 cells at the logarithmic phase of growth (about 80% confluence) were seeded in 96-well micro litre plate and incubated overnight at 37°C with 5% CO₂ to allow attachment.

2.5 Pro and Anti-inflammatory Quantification Using Multiplex Cytokines Kit

The RAW 264.7 cells were activated by incubation in a medium containing 5 µg/mL of lipopolysaccharide (LPS) alone (control) and treated simultaneously with two different concentrations of *T. capensis* crude extract, fraction, and isolated compounds dissolved in DMSO and further diluted in culture medium (final concentration (100µg/mL and 50µg/mL). The concentration of DMSO in the experiment did not exceed 0.5%. Each sample was further incubated eight hours then spin 3000rpm for 10 min then the supernatant was transferred in new labeled 96-well plate. The pellets were discarded. The samples were analysed using Cytometric Bead Array (CBA) Human Inflammatory Cytokines Kit assay according to the manufacturer instructions.

2.6 Fractionation of Acetone Leaf Extracts of *Typha capensis*

The acetone leaf extract of *Typha capensis* was selected for further solvent-solvent fractionation. *T. capensis* fine powder (1000 g) was extracted with 10 L acetone to yield 44 g of dry acetone extract. This crude extract was dissolved in an equal mixture volume of chloroform/water and fractionated by solvent/solvent extraction to yield hexane (9.62 g), chloroform (5.34 g), butanol (7.7 g), ethyl acetate (3.88 g), methanol/water (8 g), and water (4 g) fractions [16]. The hexane

fraction was kept aside and fractionation was discontinued because of its oily appearance which was difficult to work with. For the purification of the compound, the ethyl acetate and butanol fractions were mixed and further fractionated using silica gel column chromatography. The column was eluted with chloroform: methanol (gradient 0-100% methanol) to afford nine subfractions. Subfraction nine was further purified using Sephadex LH20 and preparatory thin layer chromatography until two compounds were obtained in their pure forms.

2.7 Structure Elucidation of Isolated Compounds

Two compounds were identified by means of 1D and 2D NMR spectroscopic and mass spectrometry (MS) analyses. ¹H NMR and 2D NMR experiments data were acquired on a 400 MHz NMR spectrometer (Bruker Avance III 400 MHz). The molecular weight of each compound was determined by using a Waters Acquity Ultra Performance Liquid Chromatography (UPLC®) system hyphenated to a quadrupole-time-of-flight (QTOF) instrument. The analysis of NMR data and the acquisition and processing of NMR spectra were performed by using Bruker's TopSpin™ software package. Chemical shifts were reported with reference to respective deuterated solvent peaks. The structures were identified after analysing various NMR spectra and comparison with established literature in the Dictionary of Natural Products and the Chemical Abstracts Services (Scifinder) [17,18].

3. RESULTS AND DISCUSSION

3.1 Multiplex Flow Cytometry Bead Assay

The multiplex flow cytometry bead assay enables simultaneous quantification of six pro and anti-inflammatory cytokines in cell culture supernatants using a standard cytometer. The aim was aimed to compare the activity of *T. capensis* crude extracts, fraction and compounds on pro inflammatory cytokines (TNF- α , IL-6, IL-1 β), anti-inflammatory cytokines (IL-8, IL-10), and cytokines implicated in immunomodulatory (IL-12 P70). The multiplex flow cytometry assay enables a large number of cytokines to be measured in supernatant in a short time and the results can be compared to the standard uniplex ELISA assay. Previous studies showed a good

correlations between standard ELISA and CBA assays on cytokines [19,20]. Multiplex flow cytometry assay can be a useful tool to measure a large number of pro and anti-inflammatory cytokines concentration in LPS-stimulated RAW 267.4 macrophages in a short period of time [21].

Human cytokine quantification on LPS-stimulated RAW 264.7 macrophage supernatant was higher than cells only for IL-12 p 70, IL-1 β , IL-6, TNF- α , and IL-10, but lower than cells only (IL-8). LPS-mediated activation of macrophages initiates the production of TNF- α , IL-6, IL-10, IL-1 β , nitric oxide, and interferon gamma [8,22]. Lipopolysaccharides-stimulated RAW macrophages after treatment with *T. capensis* crude acetone extract resulted on decreasing expression of pro-inflammatory cytokines (TNF- α , IL-6, IL-1 β), and increased expression of immunomodulatory cytokine IL-12 p70. Isorhamnetin-3-O- β -D-glucoside (compound 1), and Isorhamnetin 3-O-rutinoside (compound 2), two compounds isolated from *T. capensis* reduced the expression of pro inflammatory cytokines (TNF- α , IL-6, IL-1 β) compared to quercetin used as positive control. Compounds 1 and 2 increased the expression of immunomodulatory cytokine IL-12 p70 and anti-inflammatory cytokine IL-8 compared to quercetin used as positive control (See Figs. 1, 2, 3, 4, 5, 6). Isorhamnetin 3-O-rutinoside increased the expression of anti-inflammatory cytokine IL-10 compared to quercetin and LPS-stimulated macrophages. Compounds 1 and 2 were isolated from *T. capensis* butanol fraction, which shows results similar to the *T. capensis* crude extracts, and isolated compounds.

Cytokines are target proteins released from immune cells (monocytes, macrophages, and lymphocytes) and activated in response to inflammatory-related infection to repair damage tissues and restore homeostasis [23]. Anti-inflammatory cytokine (IL-8, IL-10) plays the essential role in preventing inflammation in the gut, regulates the intestinal inflammation, and prevent neurodegenerative disorders [24,25]. Interleukin-1 β and TNF- α are potent pro inflammatory cytokines while IL-6 can play both pro- and anti-inflammatory roles [26]. The mechanism of action of pro-inflammatory cytokines (IL-1 β , TNF- α , and IL-6) is enhanced via the activation of NF- κ B and MAPK pathways. Recent studies have shown that secondary metabolites from plants such as phenolic and flavonoid compounds have protective effect on inflammation via inhibition of inflammasome-

mediated secretion of IL-1 β from ex-vivo mouse macrophages and LPS-induced human macrophages [27]; Zhang et al., 2014; [28]). Several flavonoids and phenolic compounds

have been isolated from the *Typha capensis* (typhasterols, afzelekin, epiafzelekin, (+)-catechin, and (-)-epicatechin [29].

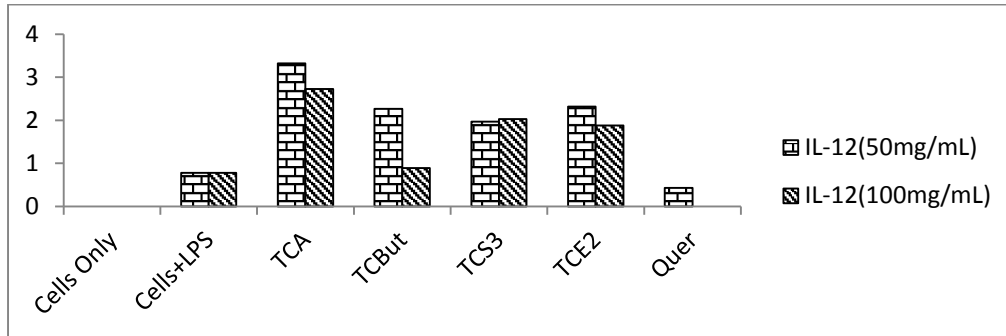


Fig. 1. Concentrations (pg/mL) of IL-12 in the LPS-activated RAW 264.7 macrophages cell lines supernatant at 50 mg/mL and 100 mg/mL. TCA= T. capensis acetone crude extract, TCbut= T. capensis butanol fraction. TCE2= Isorhamnetin-3-O- β -D-glucoside, and TCS3 = Isorhamnetin 3-O-rutinoside. Quer= quercetin used as positive control

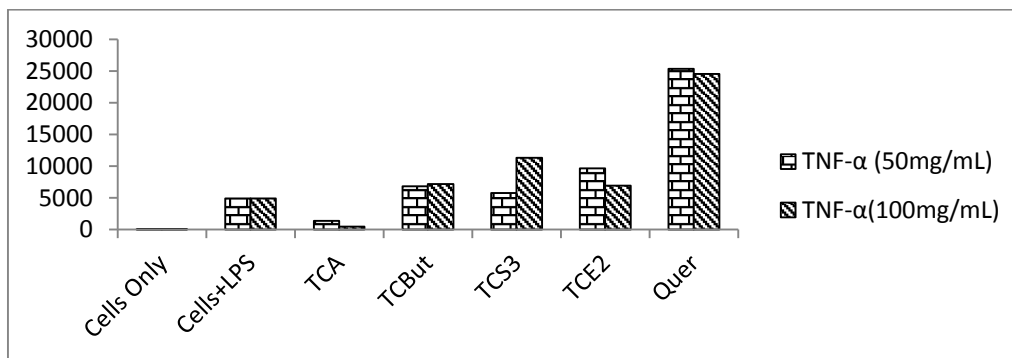


Fig. 2. Concentrations (pg/mL) of TNF- α in the LPS-activated RAW 264.7 macrophages cell lines supernatant at 50 mg/mL and 100 mg/mL. TCA= T. capensis acetone crude extract, TCbut= T. capensis butanol fraction. TCE2= Isorhamnetin-3-O- β -D-glucoside, and TCS3 = Isorhamnetin 3-O-rutinoside. Quer= quercetin used as positive control

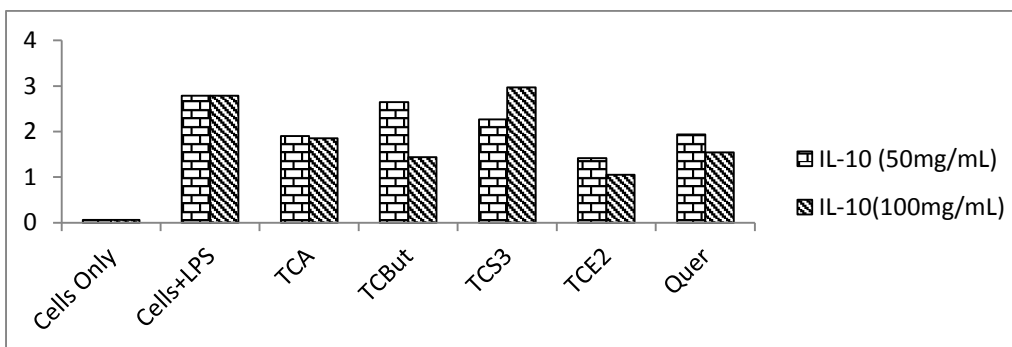


Fig. 3. Concentrations (pg/mL) of IL-10 in the LPS-activated RAW 264.7 macrophages cell lines supernatant at 50 mg/mL and 100 mg/mL. TCA= T. capensis acetone crude extract, TCbut= T. capensis butanol fraction. TCE2= Isorhamnetin-3-O- β -D-glucoside, and TCS3 = Isorhamnetin 3-O-rutinoside. Quer= quercetin used as positive control

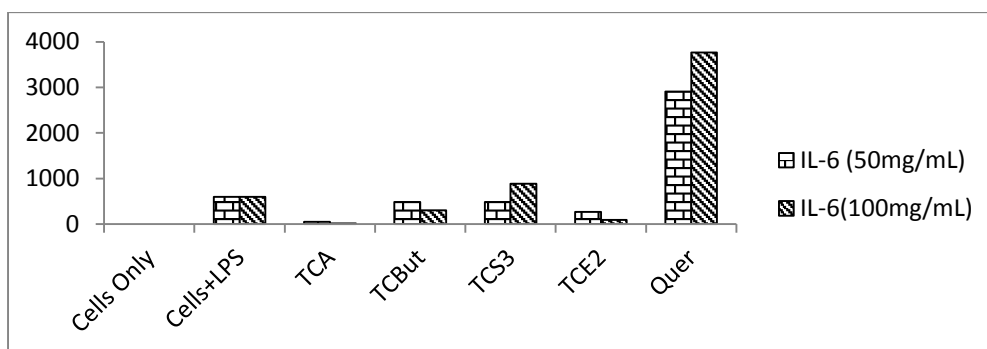


Fig. 4. Concentrations (pg/mL) of IL-6 in the LPS-activated RAW 264.7 macrophages cell lines supernatant at 50 mg/mL and 100 mg/mL. TCA= T. capensis acetone crude extract, TCbut= T. capensis butanol fraction. TCE2= Isorhamnetin-3-O-β-D-glucoside, and TCS3 = Isorhamnetin 3-O-rutinoside. Quer= quercetin used as positive control

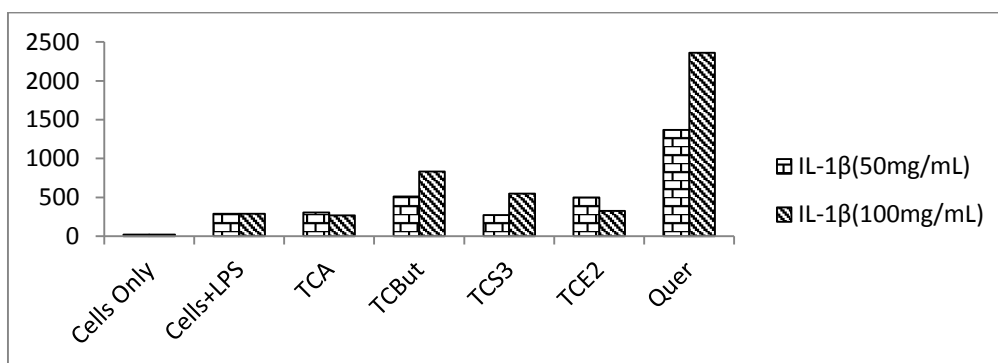


Fig. 5. Concentrations (pg/mL) of IL-12 in the LPS-activated RAW 264.7 macrophages cell lines supernatant at 50 mg/mL and 100 mg/mL. TCA= T. capensis acetone crude extract, TCbut= T. capensis butanol fraction. TCE2= Isorhamnetin-3-O-β-D-glucoside, and TCS3 = Isorhamnetin 3-O-rutinoside. Quer= quercetin used as positive control

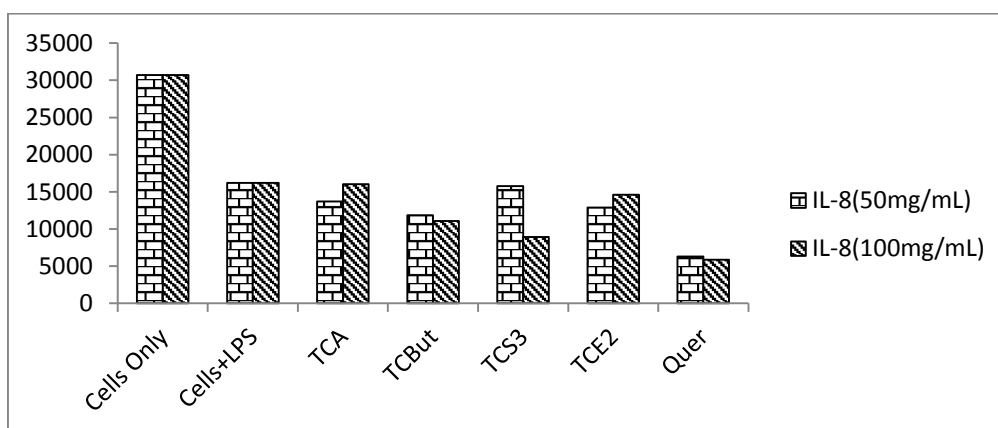


Fig. 6. Concentrations (pg/mL) of IL-12 in the LPS-activated RAW 264.7 macrophages cell lines supernatant at 50 mg/mL and 100 mg/mL. TCA= T. capensis acetone crude extract, TCbut= T. capensis butanol fraction. TCE2= Isorhamnetin-3-O-β-D-glucoside, and TCS3 = Isorhamnetin 3-O-rutinoside. Quer= quercetin used as positive control

4. CONCLUSION

Isorhamnetin-3-O-β-D-glucoside, and Isorhamnetin 3-O-rutinoside, two compounds isolated from *T. capensis* reduced the expression of pro inflammatory cytokines and increased the expression of anti-inflammatory cytokines. The mechanism of action of *T. capensis* crude extract, fraction and isolated compound in pro and anti-inflammatory expression of cytokines might be due to the presence of phenolic and flavonoid compounds present in the plant.

DISCLAIMER

The products used for this research are commonly and predominantly used products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors

COMPETING INTERESTS

Author has declared that no competing interests exist.

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