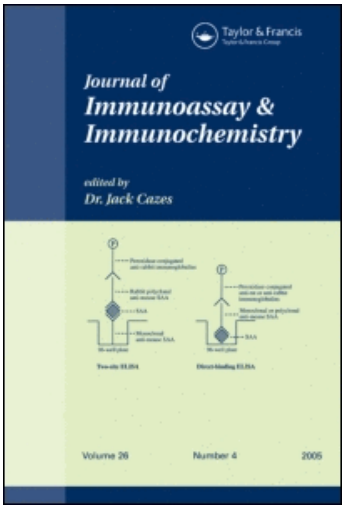

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THE *IN VITRO* EFFECTS OF ROOIBOS AND BLACK TEA ON IMMUNE PATHWAYS

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□ *The* in vitro *effects of Aspalathus linearis (Rooibos tea) and Camellia sinensis (Black tea) on biomarkers of specific immune pathways were determined using whole blood culture assays. Stimulated and unstimulated whole blood cultures were incubated with tea extracts. Enzyme linked immunosorbent assays were used to screen spent culture medium for Interleukin-6, Interleukin-10 and Interferon gamma as biomarkers for inflammation, humoral immunity, and cell mediated immunity, respectively. Rooibos and Black tea addition to unstimulated whole blood cultures induced higher Interleukin-6, Interleukin-10, and Interferon gamma secretion. Addition of Rooibos tea to stimulated whole blood cultures induced higher Interleukin-6, lower Interleukin-10, and had no effect on Interferon gamma secretion. Black tea addition to stimulated whole blood cultures inhibited Interleukin-6, Interleukin-10, and Interferon gamma production. The data indicates that Rooibos and Black tea modulates immune function in vitro.*

Keywords Black tea, immune pathway biomarkers, immunomodulatory, Rooibos tea, whole blood cultures

INTRODUCTION

The immune system of vertebrates functions to eliminate foreign micro-organisms. The immune system is made up of several organs and cell types. The immune defences can be subdivided into two types, namely the innate and adaptive immune reactions.[1] The adaptive and innate immunity differ in function; however, they do work in parallel. The innate immunity can also be defined as natural immunity or the part of the immune system that people are born with. It defends against bacteria, fungi, secreted molecules, waste, and transformed cells. Innate immunity is regulated by pattern recognition receptors (PRR) which recognize a wide variety of conserved and molecular regions of micro-organisms.[2] Adaptive

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immunity is specific for antigens and produces immunological memory. This results in a more pronounced response upon re-exposure to the antigen.[3] Adaptive immunity relies on cellular and molecular adaptive mechanisms such as B and T-lymphocytes.[2] This type of immunity may generate cellular and humoral immunity. The cellular immunity is regulated by immune cells such as T-lymphocytes, macrophages, and natural killer cells (NK cells).

On the other hand, humoral immunity is characterized by antibody production by B-lymphocytes.[4] The T-lymphocytes can be divided into CD4 (helper) and CD8 (cytotoxic) T cells. Helper T-cells facilitate the cytotoxic T-cells to kill target cells and allow B cells to produce antibodies. Functions of cytotoxic T cells include killing cells that are infected with intracellular pathogens. B-cells secrete antibodies against pathogens and, thereby, destroy them.[5]

B and T lymphocytes directly recognize and bind antigens. B cells have antigen receptors called immunoglobulins (Ig) while T cells have T cell receptors (TCR). Ig binds to free antigens; however, TCR bind to processed antigens that are presented on major histocompatability complex (MHC) molecules. MHC-2 molecules are found on antigen presenting cells.[6–8]

The innate and adaptive immunity effector pathways coincide. The molecular mediators of these two pathways are protein molecules called cytokines.[9] These cytokines are secreted by various cells and their functions differ. The function of the cytokines depends on the cell they are secreted from. They may act as autocrine, paracrine, or endocrine messengers. The cytokines that are secreted by lymphocytes are known as interleukins.[10]

Interleukin-6 (IL-6) plays an important role in innate immunity. It is both a pro-inflammatory and anti-inflammatory cytokine. Immune cells such as monocytes, macrophages, lymphocytes, and mast cells secrete IL-6.[11] IL-6 functions to promote inflammation by stimulating expansion and activation of T cells. Moreover, IL-6 causes B-cells to differentiate and causes the stimulation of acute phase reactants by hepatocytes.[12]

Interleukin-10 (IL-10) is a cytokine that is produced by T-helper 2 cells (Th2). This cytokine causes the inhibition of IFNγ synthesis in Th1 cells.[13] Several cell types may synthesise IL-10 such as Th2 cells, monocytes, macrophages, and B-cells.[14–16] IL-10 functions in stimulating B-cells to proliferate and to differentiate. It plays an important role in protecting against intestinal parasites, neutralisation of bacterial toxins, and in local mucosa defence. IL-10 suppresses cellular immunity by inhibiting Interferon gamma (IFNγ) production of T-lymphocytes.[15]

IFNγ is a cytokine that regulates various cellular programs. IFNγ stimulates direct anti-microbial and anti-tumour mechanisms as well as stimulating antigen processing. This cytokine also attracts leukocytes to the site of

infection and regulates the growth, maturation, and differentiation of various cell types.[17]

Drugs and food can modulate the immune system resulting in reactions such as immune system activation, immune system sensitization, or immune system impairment. Activation of the immune system entails development of inflammation or autoagressive reactions. Drugs or compounds may also induce naive lymphocytes to differentiate to effector or memory cells, resulting in sensitization of the immune system.[18] In addition, impairment of the immune system, such as immunosuppression, may result due to consumption of drugs or other compounds.[18]

Second to water, tea is the most widely consumed beverage in the world.[19] This study focused on two teas, namely *Aspalathus linearis (A. linearis)* or Rooibos tea and *Camellia sinensis (C. sinensis)* or Black tea, respectively. These teas are rich in flavonoids and antioxidants and have been reported to have many beneficial effects to humans.

A. linearis or Rooibos tea is a plant that is indigenous to South Africa.[20] It has been used as a health beverage and is very popular due to its unique taste and its versatility.[21] Characteristics of this tea include high levels of flavonoids, which result in potent antioxidant activity.[20] Rooibos tea has several other physiological effects, such as anti-microbial activity in cells,[22] an inhibitory effect on oxidative stress in diabetic rats,[23] and hepatoprotective effects in rats.[24]

C. sinensis or Black tea is a beverage that is consumed worldwide by approximately 73–78% of the world's population.[19] One of the main flavonoids found in Black tea was identified as epigallocatechin gallate.[25] Black tea has many physiological effects, such as anti-mutagenic and anti-cancer effects.[26]

Very few studies have been done on the immunomodulatory activity of Rooibos and Black tea. The aim of this study was to screen Rooibos tea and Black tea extracts for their immune modulating effects on biomarkers of specific immune pathways using an *in vitro* whole blood culture (WBC) assay.

EXPERIMENTAL

Sample Preparation for Whole Blood Cultures (WBC)

Samples were prepared by seeping 10 teabags (25 g) of Rooibos (Batch no: P 22.11.01 05:03 E19.02.09) and 10 teabags (25 g) of Black tea (Batch no: P 28.11.07 13:11 E 26.11.08) in 1 litre of boiling water, respectively. The samples were allowed to cool to room temperature. Aliquots of the extracts were stored at −80°C. For assay, aliquots were thawed and then the teas were sterilised by filtration using a 0.22 μM sterile filter (Lasec, SA). Two-fold dilutions of sterile samples were made with sterilized distilled water.

Preparation of WBC

Blood was collected from healthy male volunteers (24–28 years of age) after informed consent was obtained in line with the South African Ethical Advisory Council. Volunteers were not on medication for at least 1 month before blood collection. Blood samples were collected using endotoxin-free evacuated blood collection tubes (Greiner Bio One GmBH, Austria) containing sodium citrate (3.2%).

The Effects of Rooibos Tea and Black Tea on Endotoxin Stimulated Blood

Stimulated WBC were prepared by mixing blood, Roswell Park Memorial Institute 1640 (RPMI-1640) (Sigma- Aldrich, St. Louis, MO, U.S.A.) medium, and 10 μg/mL endotoxin (lipopolysaccharide, LPS) (Sigma-Aldrich, U.S.A.) in dimethyl sulfoxide (DMSO) in the ratio 10:89:1. Unstimulated WBC was prepared by mixing blood, RPMI-1640 medium and DMSO in the ratio 10:89:1. Rooibos tea or Black tea samples (25 μL/well) at different concentrations (250, 125, 62.5, 31.25, 15.625, 7.812 μg/mL) with controls (sterilized distilled water) were dispensed in a 96 well microtiter plate (Nalge Nunc International, Thermo Fisher Scientific, NY, U.S.A.). Endotoxin stimulated or unstimulated diluted blood was added to all samples and controls (225 μL/well). The WBC were incubated at 37°C for 18 hours. After the incubation period, supernatants were collected and assayed for LDH and IL-6.

The Effects of Rooibos Tea and Black Tea on Phytohemagglutinin (PHA) Stimulated Blood

Stimulated WBC were prepared by mixing blood with RPMI-1640 medium and 1.6 mg/ml PHA in DMSO in the ratio 10:89:1. Unstimulated WBC were prepared by mixing blood and RPMI-1640 medium in DMSO in the ratio 10:89:1. Rooibos tea or Black tea samples (25 μL/well) at different concentrations (250, 125, 62.5, 31.25, 15.625, 7.812 μg/mL) with controls (sterilized distilled water) were dispensed in a 96 well microtiter plate (Nalge Nunc International, Thermo Fisher Scientific, NY, U.S.A.). PHA stimulated or unstimulated diluted blood was added to all samples and controls (225 μL/well). The WBC were incubated at 37°C for 48 hours. After the incubation period, supernatants were collected and assayed for LDH, IFNγ, and IL-10.

Cytotoxicity Assay (Lactate Dehydrogenase Assay)

Lactate Dehydrogenase was used as a biomarker for cytotoxicity. LDH assays were performed on all culture supernatants using a commercially

available kit (Biovision Research Products, CA, U.S.A.). Assays were done according to the instructions supplied in the kit manual.

IL-6, IL-10 and IFNγ Enzyme Linked Immunosorbent Assays (ELISAs)

Cytokine kits were purchased from eBioscience (Human IL-6, IL-10, and IFNγ ELISA Ready-Set-Go, eBioscience, Inc, San Diego, U.S.A.) and used to quantify cytokine production from the supernatants of whole blood cultures. Nunc maxisorp microtiter plates (Nalge Nunc International, Thermo Fisher Scientific, NY, U.S.A.) were used for the assays. All the reagents and diluents required for the ELISA were supplied with the kit. The assay was performed according to the protocol provided in the kits.

Statistical Analysis

All data is presented as mean $\pm$ standard deviation (SD). One way analysis of variance (ANOVA) was used to compare results with $P < 0.001$ considered as significant.

RESULTS

Effect of Rooibos Tea and Black Tea Extracts on Cellular Toxicity

Rooibos tea and Black tea were tested for cellular cytotoxicty using an LDH assay. Results showed that neither of the tea extracts was cytotoxic at the concentrations tested (data not shown).

Effect of Rooibos Tea and Black Tea Extracts on IL-6 Production

IL-6 was used as a biomarker to determine the inflammatory response induced by Rooibos and Black tea. Results for IL-6 production by unstimulated and stimulated WBC exposed to Rooibos tea extracts are shown in Figure 1. Addition of Rooibos tea to unstimulated WBC induced higher IL-6 secretion across all concentrations (7.8125 µg/mL–250 µg/mL) compared to the control ($P < 0.001$). Addition of Rooibos tea extracts at a concentration of 62.5 µg/mL to stimulated WBC resulted in a significant increase of IL-6 production compared to the control ($P < 0.001$).

Figure 2 shows a graphical illustration of results obtained for IL-6 production for unstimulated and stimulated WBC exposed to Black tea extracts. Addition of Black tea extracts at concentrations from 7.8125 µg/mL–125 µg/mL

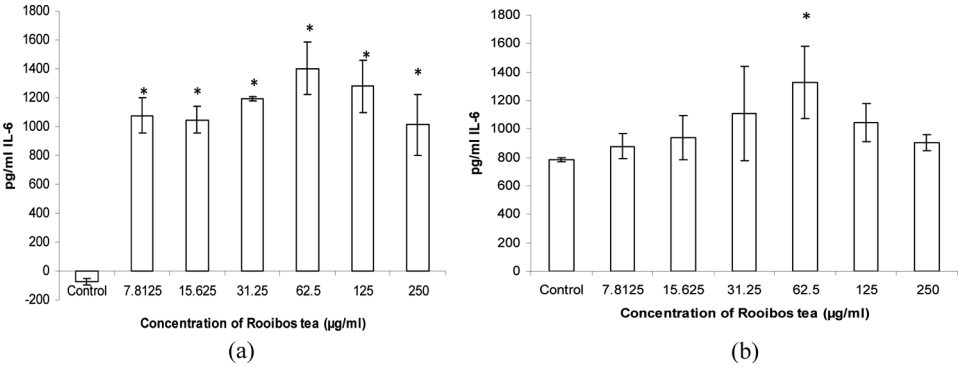

FIGURE 1 IL-6 production (pg/ml) of human whole blood cultures exposed to Rooibos tea. (a) In the absence of a stimulus. (b) In the presence of a stimulus (LPS). *Statistically significance ($P < 0.001$) compared to the control. Bars = Standard deviation.

induced a higher IL-6 cytokine secretion from unstimulated WBC compared to the control ($P < 0.001$). Addition of 250 µg/mL of Black tea extracts to stimulated WBC resulted in a decrease of IL-6 production compared to the control ($P < 0.001$).

Effects of Rooibos Tea and Black Tea Extracts on IL-10 Production

IL-10 was used as a biomarker for humoral immunity. Results for IL-10 production by human WBC exposed to Rooibos tea are shown in Figure 3. It can be seen that addition of Rooibos tea (7.125 µg/mL–250 µg/mL) to unstimulated WBC resulted in a significant induction of IL-10 secretion compared to the control ($P < 0.001$). Addition of Rooibos tea extracts

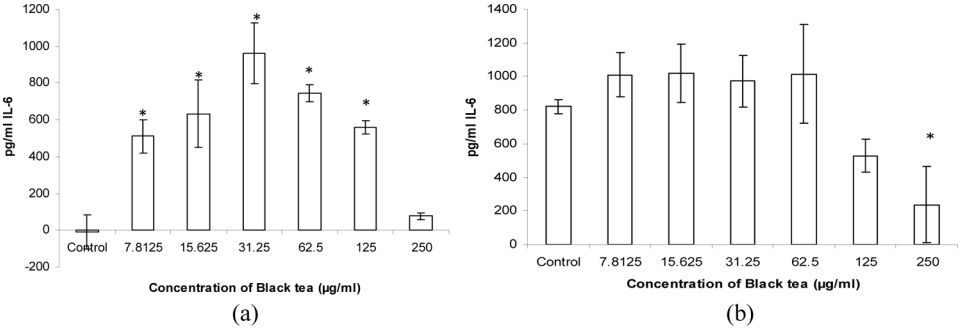

FIGURE 2 IL-6 production (pg/ml) of human whole blood cultures exposed to Black tea. (a) In the absence of a stimulus. (b) In the presence of a stimulus (LPS). *Statistically significance ($P < 0.001$) compared to the control. Bars = Standard deviation.

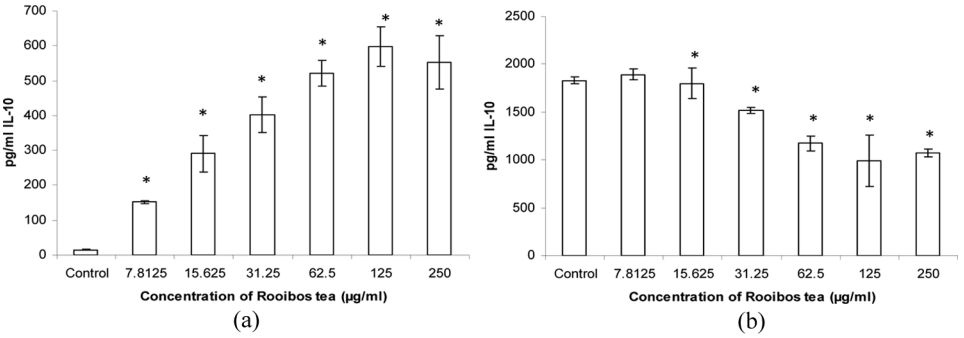

FIGURE 3 IL-10 production (pg/ml) of human whole blood cultures exposed to Rooibos tea. (a) In the absence of a stimulus. (b) In the presence of a stimulus (PHA). *Statistical significance ($P < 0.001$) compared to the control. Bars = Standard deviation.

(15.625, 62.5, 250 µg/mL) to stimulated WBC resulted in a significant decrease in IL-10 secretion compared to the control ($P < 0.001$).

Results for IL-10 production by human WBC exposed to Black tea extracts are shown in Figure 4. Addition of Black tea extracts (7.8125 µg/mL–62.5 µg/mL) to unstimulated WBC induced a higher IL-10 secretion compared to the control ($P < 0.001$). Addition of Black tea extracts (7.8125 µg/mL −250 µg/mL) to stimulated WBC resulted in a statistically significant decrease in IL-10 production compared to the control ($P < 0.001$).

Effects of Rooibos Tea and Black Tea Extracts on IFNγ Production

IFNγ was used as a biomarker for cell mediated immunity. Figure 5 shows a graphical representation for IFNγ production of human unstimulated and

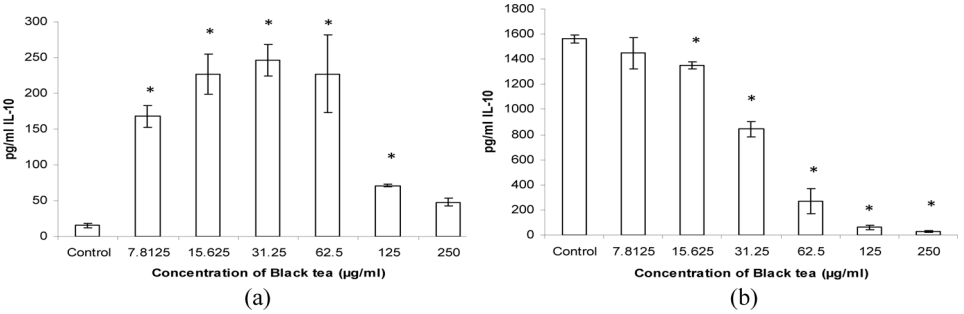

FIGURE 4 IL-10 production (pg/ml) for human whole blood cultures exposed to Black tea. (a) In the absence of a stimulus. (b) In the presence of a stimulus (LPS). *Statistical significance ($P < 0.001$) compared to the control. Bars = Standard deviation.

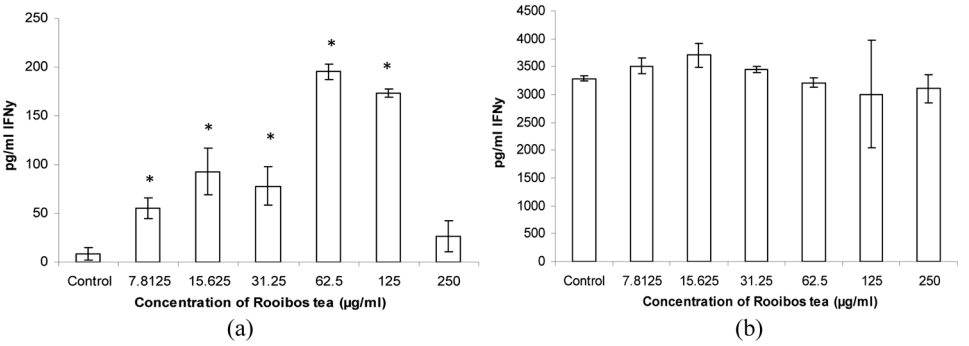

FIGURE 5 IFNγ production (pg/ml) for human whole blood cultures exposed to Rooibos tea. (a) In the absence of a stimulus. (b) In the presence of a stimulus (LPS). *Statistical significance ($P < 0.001$) compared to the control. Bars = Standard deviation.

stimulated WBC exposed to Rooibos tea. Addition of Rooibos tea extracts (7.8125 µg/mL–125 µg/mL) to unstimulated WBC showed a stimulatory effect on IFNγ secretion compared to the control ($P < 0.001$). Addition of Rooibos tea extracts (7.8125 µg/mL–250 µg/mL) to stimulated WBC showed no significant difference of IFNγ production compared to the control.

IFNγ production for WBC exposed to Black tea extracts are shown in Figure 6. Addition of 15.625 µg/mL Black tea extracts to unstimulated WBC showed a significant increase in induction of IFNγ secretion compared to the control ($P < 0.001$). Addition of Black tea extracts (15.625 µg/mL–250 µg/mL) to stimulated WBC showed a statistically significant decrease in IFNγ production compared to the control ($P < 0.001$). This inhibition is proportional to the concentration of Black tea extracts.

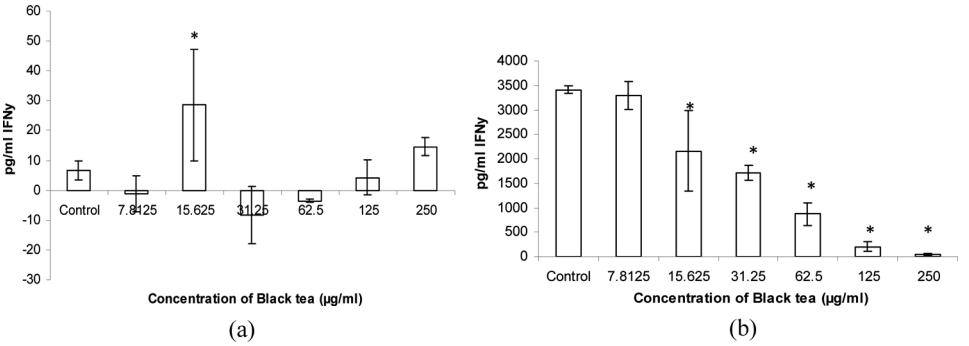

FIGURE 6 IFNγ production (pg/ml) for human whole blood cultures exposed to Black tea. (a) In the absence of a stimulus. (b) In the presence of a stimulus (LPS). *Statistical significance ($P < 0.001$) compared to the control. Bars = Standard deviation.

DISCUSSION

Rooibos tea and Black tea are beverages that claim to have wide physiological and pharmocological effects.[22,23,25,26] However, very little is known about the immunomodulating activity of these teas. Tea consists of many compounds that could play a role in immunomodulation. Antioxidant activity of teas, which is very high, may have an effect on immune status.[27]

Rooibos and Black tea showed no cytotoxic effects on WBC (data not shown). The LDH assay is a sensitive assay that determines cytotoxicity. Absence of cytotoxicity of samples does not necessarily indicate that the samples have no effect on cell physiological systems.[28] Therefore, Rooibos tea and Black tea were further analysed for their effects on the immune system using biomarkers of specific immune pathways.

IL-6 is a cytokine, that is synthesized and secreted from T-lymphocytes, monocytes, and macrophages activated by antigen or mitogen.[11] In this study IL-6 was used as a biomarker for inflammation. IL-6 secretion from unstimulated WBC and stimulated WBC was markedly increased by Rooibos tea extract. This study shows that Rooibos tea is capable of inducing inflammatory cytokines *in vitro*. The immunostimulatory effect of Rooibos tea on IL-6 may result in activation of the immune system. This may lead to the activation of hepatocytes to produce acute phase proteins which in turn activates complement and allows for the phagocytosis of pathogens.[12] The consumption of Rooibos tea may thus lead to an antimicrobial response.

IL-10 is a cytokine that is produced by T-cells, B-cells, and macrophages[14–16] and was used in this study as a biomarker for humoral immunity. IL-10 stimulates the proliferation and differentiation of B-cells and also regulates Ig synthesis of B cells.[15] In this study, an increase in IL-10 production by unstimulated WBC exposed to Rooibos tea extracts were seen. This result suggests that the induction of IL-10 by the Rooibos tea extract may contribute to stimulation of B-cells and synthesis of Ig. This confirms data results published in which a Rooibos fraction resulted in an increase in IL-10 and Immunoglobulin M (IgM) production by murine splenocytes.[29] Rooibos tea extracts resulted in a suppression of IL-10 synthesis from stimulated WBC ($P < 0.001$). These results indicate that, in the presence of a pathogen, Rooibos tea may have an immunosuppressive effect on the differentiation of T-helper cells to Th2 cells. Consequently, having an impact on cytokines that are needed to mount an effective immune response.

Exposure of unstimulated WBC to Rooibos tea resulted in an increase in IFNγ production. No significant effect on IFNγ production was observed for stimulated WBC exposed to Rooibos tea. These results are contrary to a study in which a Rooibos fraction decreased IFNγ secretion by murine

splenocytes.[29] Rooibos tea has many constituents such as saccharides and polyphenols that could play a role in this pleiotropic effect seen in this study. WBC contain all the cells present in the circulation and the activation of one cell type may have an influence on the functioning of another.[30] Consequently, IL-10 could have inhibited T-cells to synthesise IFNγ; however, other cells present in the WBC may have synthesised it.

Low concentrations of Black tea induced unstimulated WBC to increase IL-6 production. Several components in Black tea such as the polyphenols are shown to possess antioxidant activity. This antioxidant activity may play a role in anti-inflammatory processes. However, a study showed that tea consumption in smoking subjects that have increased oxidant stress does not decrease the plasma levels of IL-6.[31] Consequently, these authors disproved the hypothesis that increased antioxidant activity may decrease oxidants and, thereby, inflammatory response.[31] At high concentrations, Black tea caused a decrease in IL-6 secretion of stimulated WBC. This result confirms previous studies where stimulated monocytes exposed to Black tea extracts resulted in a decrease in IL-6 production.[32]

Addition of Black tea extracts to unstimulated WBC resulted in an increase in IL-10 production compared to the control. IL-10 production is important in protecting against intestinal parasites, neutralisation of toxins and in local mucosa defense. The augmentation of IL-10 by Black tea extracts makes it an ideal dietary component to result in activation and sensitisation of the immune system and, thereby, possibly provide protection against infection. The Black tea extracts in this study were found to decrease IL-10 production in stimulated WBC. In contrast, no effect was seen on LPS-stimulated WBC exposed to Black tea extracts containing approximately 80% theaflavins.[33]

Unstimulated WBC exposed to Black tea extracts at a concentration of 15.625 μg/mL causes an increase in IFNγ production ($P < 0.001$). Increased IFNγ production could play a role in anti-tumour mechanisms. In this study, stimulated WBC exposed to Black tea extracts resulted in a decrease in IFNγ production ($P < 0.001$). In contrast, one *in vitro* study showed that $\gamma\delta$ T cells results in the production of IFNγ in response to alkylamine antigen found in brewed tea.[34] Taken together, the results of this study suggest that, in the presence of an intracellular pathogen or a tumour, Black tea extracts may inhibit cell mediated immunity and, thus, increase susceptibility to the host.

CONCLUSIONS

The results of this study show that Rooibos tea and Black tea play a role in modulating the immune system. Specifically, Rooibos tea and Black tea

may have an effect on cytokine secretion by human WBC. It is thus possible to use Rooibos tea and Black tea as a dietary component to either stimulate or suppress immunity. Limitations to this study include only examining *in vitro* effects of Rooibos tea and Black tea. These *in vitro* studies do not fully elucidate the *in vivo* immune mechanisms of action of these teas. Further work should be done to elucidate the immune effects of these teas *in vivo*. These findings contribute to our understanding of the effects of Rooibos tea and Black tea on specific immune pathways.

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