

The *in vitro* Effects of Various Immunomodulatory Nutritional Compounds on Antigen-Stimulated Whole-Blood Culture Cytokine Production

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Abstract—Immunomodulators are substances that alter immune system via dynamic regulation of messenger molecules. It can be divided into immunostimulant and immunosuppressant. It can help to increase immunity of people with a low immune system, and also can help to normalize an overactive immune system. Aim of this study is to investigate the effects of *in vitro* exposure to low and high doses of several immunomodulators which include caffeine, kaloba and quercetin on antigen-stimulated whole blood culture cytokine production. Whole blood samples were taken from 5 healthy males (age: 32 ± 12 years; weight: 75.7 ± 6.1 kg; BMI: 24.3 ± 1.5 kg/m²) following an overnight fast with no vigorous activity during the preceding 24 h. The whole blood was then stimulated with 50 μ l of 100 x diluted Pediacel vaccine and low or high dose of immunomodulators in the culture plate. After 20 h incubation (5% CO₂, 37°C), it was analysed using the Evidence Investigator to determine the production of cytokines including IL-2, IL-4, IL-10, IFN- γ , and IL-1 α . Caffeine and quercetin showed a tendency towards decrease cytokine production as the doses were increased. On the other hand, an upward trend was evident with kaloba, where a high dose of kaloba seemed to increase the cytokine production. In conclusion, we found that caffeine and quercetin have potential as immunosuppressant and kaloba as immunostimulant.

Keywords—Caffeine, cytokine, immunomodulators, kaloba, quercetin.

I. INTRODUCTION

IMMUNOMODULATION is a therapeutic approach in which we try to intervene in auto regulating processes of the host defense system. Immunomodulators can be defined as substances (drugs and nutrients) that alter the activities of the immune system via dynamic regulation of messenger molecules such as cytokines, adhesion molecules, nitric oxide, hormones, neurotransmitters, and other peptides. Immunomodulators can help to increase immunity of people with a low immune system, and also can help to normalize an overactive immune system.

Generally, these compounds can be divided into immunosuppressants and immunostimulants [1]. Numerous *in vitro* and *in vivo* studies have found that immunomodulators modulate the secretion of multiple cytokines [2].

Altering cytokine expression with immunomodulators may offer some therapeutic potential. As reviewed by Del-Rio-

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Navarro and colleagues [3], on average, immunostimulants reduce the incidence of acute respiratory tract infections in children by 40%. Several published studies have suggested that caffeine [4], [5] and quercetin [6], [7] as immunosuppressants while kaloba [8], [9] as immunostimulants.

Caffeine is present in coffee and tea and is a member of the methylxanthine family of drugs; it is the most widely consumed psychoactive substance by humans [10]. Immunomodulatory effects of caffeine, a well-known psychostimulant substance, have been extensively studied and it appears that caffeine modulates both innate and adaptive immune responses.

Kaloba is an extract derived from the root of plants that are indigenous to South Africa. It comes from the family Geraniaceae and genus *Pelargonium* and was traditionally used to treat dysentery, diarrhea, colds, wounds, fatigue, fevers, hepatic complaints, generalized malaise, and respiratory tract infections such as tuberculosis, bronchitis, and sinusitis [11], [12]. Furthermore, studies reported that it is successfully employed for the treatment of ear, nose, and throat disorders as well as respiratory tract infections [8], [13]-[15].

Quercetin is a flavonol that can be found in fruits and vegetables which is believed to reduce infection risk during intense exercise since it has anti-inflammatory and antioxidant properties, beside its psychostimulant actions and its ability to stimulate mitochondrial biogenesis [16]. This was supported by one study where it was found that quercetin ingestion significantly reduced the upper respiratory tract infection (URTI) incidence in the cyclists [17].

However, current evidence concerning their efficacy is limited. Hence, this present study intended to investigate the effects caffeine, quercetin and kaloba on antigen-stimulated whole blood culture cytokine production *in vitro*.

II. METHODS

A. Participants

Five healthy men were recruited as participants in this study. Mean age, weight, and BMI of the participants were 32 ± 12 years, 75.7 ± 6.1 kg, and 24.3 ± 1.5 kg/m² respectively.

B. Procedures

Participants were requested to come to the laboratory between 09:00 and 10:00 following an overnight fast from 21:00. However, they were permitted to drink plain water.

They were also asked to refrain from any vigorous physical activity in the preceding 24 h. Blood samples were collected into K₃EDTA and lithium heparin collection tubes as described in the general methods. This study obtained ethics approval from the Loughborough University Research Ethics Committee.

C. Blood Analysis

Blood samples were taken from an antecubital vein by venepuncture with a 21 gauge needle into heparin and/or K₃EDTA collection tube (Starstedt, Leicester, UK) using the vacutainer method. Blood samples collected in the K₃EDTA tube were used for haematological analysis (haematocrit (Hct), haemoglobin (Hb) concentration, red blood cell (RBC) count, and total and differential leukocyte counts) using an automated haematology analyser (Ac.T.5 diff, Beckman Coulter, High Wycombe, UK). Each sample was measured twice and the average value was recorded. The intra-assay coefficient of variation for all measured variables was less than 3.0%.

Blood samples collected in heparin tube were used for determination of antigen-stimulated cytokine production; 20 h of whole blood culture incubation with immunomodulators and vaccine followed by analysis by using Evidence Investigator (Randox, UK).

D. Immunomodulatory Nutritional Compounds Preparation

Caffeine low blood dose was targeted at 9.71 mg/L [18]. Caffeine solution needed = $100 \times 9.71 \text{ mg/L} = 971 \text{ mg/L} = 97.1 \text{ mg/100 ml}$. Thus, 97.1 mg caffeine was added to 100 ml RPMI solution.

In the whole blood culture, low dose (9.71 mg/L) of caffeine was achieved by adding 10 μl of this solution into the culture wells while for high dose (97.1 mg/L), 100 μl was added.

Quercetin low blood dose was targeted at 0.02 mg/ml [19]. Quercetin solution needed = $100 \times 0.02 \text{ mg/ml} = 2 \text{ mg/ml}$. As quercetin solution has low solubility in water, the solution was made up in alcohol. Then, this solution was 10 times diluted with RPMI to make quercetin solution at 200 mg/L.

In the whole blood culture, low dose (0.02 mg/ml) of quercetin was achieved by adding 10 μl of this solution into the culture wells while for high dose (0.2 mg/ml), 100 μl was added.

Kaloba low blood dose was targeted at 3 $\mu\text{g/ml}$ [20]. Kaloba solution needed = $100 \times 3 \mu\text{g/ml} = 300 \mu\text{g/ml}$. Thus, 20 mg Kaloba was added to 66.7 ml RPMI solution.

In the whole blood culture, low dose (3 $\mu\text{g/ml}$) of Kaloba was achieved by adding 10 μl of this solution into the culture wells while for high dose (30 $\mu\text{g/ml}$), 100 μl was added.

E. Whole Blood Culture Incubation

As shown in the Table I, required volumes of RPMI-1640 medium were added into the culture wells, followed by 50 μl of 100 x diluted vaccine. Next, the appropriate amount of immunomodulators solution was added, followed by 0.25 ml of blood. After gently mixing, they were incubated for 20 h in the CO₂ incubator at 5% CO₂ and 37°C (MiniGalaxy E, Livingston, UK).

After 20 h incubation, blood culture was transferred into eppendorf tubes and spun for 4 min at 12,000 rpm in a microcentrifuge (Thermo IEC, Needham Heights, Massachusetts, USA). After that, the supernatant fluid was aspirated, placed in a labelled eppendorf tube and stored in a cryobox in a -20°C freezer until further analyses were carried out.

TABLE I
VOLUME OF RPMI, 100 X DILUTED VACCINE, AND IMMUNOSTIMULANT SOLUTION ADDED TO EACH WELL FOR EACH BLOOD SAMPLE

Well	RPMI-1640 medium (μl)	Immunomodulators solution (μl)	100 x diluted Pediacel Vaccine (50 μl)
1	750	NO	NO
2	725	NO	YES
3	715	Caffeine (L) – 10 μl	YES
4	625	Caffeine (H) – 100 μl	YES
5	715	Kaloba (L) – 10 μl	YES
6	625	Kaloba (H) – 100 μl	YES
7	715	Quercetin (L) – 10 μl	YES
8	625	Quercetin (H) – 100 μl	YES

To these volumes, 0.25 ml heparinised blood was added.
L = lowdose; H = high dose

F. Evidence Investigator Cytokine Array

Supernatant fluid stored at -20°C was removed from the freezer and kept at room temperature to thaw for approximately 1 h before it was assayed for the cytokine concentrations using an Evidence InvestigatorTM cytokine and growth factors array kit (Randox, County Antrim, UK). Briefly, 200 μl of assay buffer and 100 μl of standard or sample were added to each well of the carrier. Then, for 60 min, it was incubated at 37°C at 370 rpm on the thermoshaker (Randox, County Antrim, UK). This allowed the cytokines present in the sample to bind to the immobilised antibodies bound to the base of each carriers' well. After that, the liquid from each well was decanted and washed by performing two quick washes and four 2-min soaks, followed by decanting the liquid and tapping the carrier onto lint free tissue paper.

Next, 300 μl of conjugate was added to each well and incubated at 37°C at 370 rpm on the thermoshaker for 60 min. The wells were then washed as previously described before 250 μl of signal reagent (a mixture of luminol and peroxide) were added to each well followed by 2-min incubation in the dark. Finally, it was analysed by using the Evidence Investigator machine (Randox, County Antrim, UK). The intra-assay coefficient of variation for all measured cytokines was less than 5.0%.

G. Data Analysis

Data were analysed using the Statistical Package for Social Sciences (SPSS) version 17 (SPSS Inc., USA). Descriptive analysis was used to measure mean and standard deviation (SD). Statistical significance of differences between means was analysed using paired samples t-test. All the data were expressed as mean \pm SD.

III. RESULTS

Table II presents the values of haematological parameters measured. The entire observed values fell within the normal range. This indicates that the participants recruited for the study were healthy and have no obvious haematological problem.

TABLE II
MEAN AND SD VALUES OF HAEMATOLOGICAL PARAMETERS

Parameters	Observed values	Normal range
Red blood cells ($10^{12}/L$)	4.9 ± 0.4	4.3 – 6.2
Haemoglobin (g/dL)	14.7 ± 1.4	13.2 – 16.2
Haematocrit (%)	47.3 ± 4.1	40.0 – 52.0
White blood cells ($10^9/L$)	5.1 ± 0.6	4.0 – 11.0
Neutrophils ($10^9/L$)	2.3 ± 0.5	2.0 – 8.0
Lymphocytes ($10^9/L$)	1.9 ± 0.2	0.7 – 4.4
Monocytes ($10^9/L$)	0.5 ± 0.2	0.1 – 0.8
Eosinophils ($10^9/L$)	0.2 ± 0.9	0.1 – 0.4
Basophils ($10^9/L$)	0.0 ± 0.0	0.0 – 0.1

Table III shows the concentrations of the cytokines produced in the whole blood culture after 20 h incubation with the vaccine dose 5 and immunomodulators at high or low dose. Control refers to the whole blood cultured with only vaccine without any added immunomodulators.

IV. DISCUSSION

In general, we found that caffeine and quercetin showed a tendency towards decreased cytokine production as the doses were increased (Table III). On the other hand, an upward trend was evident with kaloba, where a high dose of kaloba seemed to increase the cytokine production.

There were no significant differences (compared with control) in cytokine production in response to caffeine and quercetin with low or high dose (Table III). However, the results showed a tendency towards reduced cytokine production with high doses of both caffeine and quercetin. Previous studies have found that caffeine and quercetin act as immunosuppressants [4]-[7]. Thus, this finding is not in contradiction to those previous findings.

TABLE III
ANTIGEN-STIMULATED WHOLE BLOOD CULTURE CYTOKINE PRODUCTION (PG/ML) IN RESPONSE TO CAFFEINE, QUERCETIN AND KALOBA AT LOW AND HIGH DOSES

	Dose					
		IL-2	IL-4	IL-10	IFN- γ	IL-1 α
CONTROL		2.3 \pm	5.7 \pm	27.5 \pm	792 \pm	175 \pm
		1.3	2.3	11.5	600	105
CAFFEINE	Low dose	2.5 \pm	5.6 \pm	48.2 \pm	579 \pm	293 \pm
	(9.71 mg/L)	1.3	3.5	35.5	553	172
	High dose	1.5 \pm	3.9 \pm	47.4 \pm	84 \pm	234 \pm
	(97.1 mg/L)	0.6	1.3	29.3	96	184
QUERCETIN	Low dose	3.2 \pm	3.1 \pm	25.9 \pm	196 \pm	193 \pm
	(20 μ g/ml)	2.2	0.2	22.2	107	208
	High dose	2.5 \pm	2.6 \pm	44.7 \pm	52 \pm	177 \pm
	(200 μ g/ml)	1.9	0.8	54.2	48	219
KALOBA	Low dose	3.6 \pm	3.7 \pm	25.5 \pm	768 \pm	237 \pm
	(3 μ g/ml)	3.1	2.3	11.7	558	167
	High dose	2.4 \pm	4.7 \pm	51.9 \pm	860 \pm	299 \pm
	(30 μ g/ml)	1.8	2.9	39.9	532	190

The mechanism of action of caffeine has been debated. However, it is well established that caffeine inhibits cyclic adenosine monophosphate (cAMP)-phosphodiesterase. But, this only occurs with high concentration of caffeine, higher than average human consumption [10]. Yet, growing evidence suggests that the mechanism of action of caffeine is mediated by antagonism of adenosine receptors [10], [21]. Generally, activated adenosine receptors will lead to suppression of pro-inflammatory cytokines (especially TNF- α) [22], [23], thus if caffeine act to antagonise adenosine receptors (by blocking the action of endogenous adenosine), this will result in increased TNF- α production. However, one study found that caffeine decreased TNF- α production [4], thus they hypothesised that the suppressive effect of caffeine on TNF- α production observed in their study may be due to the inhibition of cAMP-phosphodiesterase, a resultant increase in intracellular cAMP concentrations, and activation of protein kinase A.

The potential mechanism of action for the anti-inflammatory action of quercetin (blocked TNF- α mediated inflammation or insulin resistance in adipocytes) are proposed by interfering with TNF- α receptor (TNFR) binding, suppressing TNF- α -TNFR signalling, and altering the activity of proteins involved in inflammation or glucose and lipid metabolism [7]. Quercetin also was proposed to exert its effects in inflammation prevention and insulin resistance via inhibition of TNF- α mediated reactive oxygen species (ROS - increase inflammatory gene expression) production [24]. We found no significant differences in cytokine production in response to kaloba in this study. Nevertheless, there was an evident upward trend of kaloba on cytokine production (Table III) where a high dose of kaloba seemed to increase the cytokine production. This is in agreement with previous findings which suggested kaloba as an immunostimulant [8], [9].

Kaloba was reported to have multifactorial mode of action; antiviral and cytoprotective properties, antibacterial properties, secretomotor properties and inhibition of 'sickness behaviour'. Several *in vitro* studies found that kaloba triggers stimulation of IFN synthesis [9], [25] and improves phagocyte function [20]. It also increased the ciliary beat frequency, thus increasing the transportation of mucous and pathogen from the respiratory tracts. This in turn reduced pathogen count, removed the nutrient medium for their subsequent proliferation and substantially improved respiration [26].

In addition, it should be noted that *in vitro* and *in vivo* studies might give different result outcomes due to some limitations. These include poor bioavailability of the nutritional compounds in the body fluids and tissues due to poor absorption and distribution of the compounds following oral ingestion in the *in vivo* situation. Furthermore, *in vivo* some nutrients may be metabolised and inactivated by gut epithelial cells and the liver or rapidly cleared from the circulation by the kidneys.

V. CONCLUSION

There were no significant differences in the production of IL-2, IL-4, IL-10, IFN- γ , and IL-1 α in antigen-stimulated

whole blood culture in the presence of the selected immunomodulators. Nevertheless, we found that caffeine and quercetin have potential as immunosuppressants and Kaloba as immunostimulant.

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REFERENCES

- [1] Patil, U. S., Jaydeokar, A. V., and Bandawane, D. D. (2012). Immunomodulators: A pharmacological review. *Int J Pharm PharmSci*, 4(1), 30-36.
- [2] Spelman, K., Burns, J., Nichols, D., Winters, N., Ottersberg, S. and Tenborg, M. (2006). Modulation of cytokine expression by traditional medicines: a review of herbal immunomodulators. *Altern Med Rev*, 11(2), 128-150.
- [3] Del-Rio-Navarro, B. E., Espinosa Rosales, F., Flenady, V. and Sienra-Monge, J. J. (2006). Immunostimulants for preventing respiratory tract infection in children. *Cochrane Database Syst Rev*, 18(4), CD004974.
- [4] Horrigan, L. A., Kelly, J. P. and Connor, T. J. (2004). Caffeine suppresses TNF-alpha production via activation of the cyclic AMP/protein kinase A pathway. *Int Immunopharmacol*, 4(10-11), 1409-1417.
- [5] Horrigan, L. A., Kelly, J. P. and Connor, T. J. (2006). Immunomodulatory effects of caffeine: friend or foe? *Pharmacol Ther*, 111(3), 877-892.
- [6] Chen, C. C., Kong, M. S., Lai, M. W., Chao, H. C., Chang, K. W., Chen, S. Y., Huang, Y. C., Chiu, C. H., Li, W. C., Lin, P. Y., Chen, C. J. and Li, T. Y. (2010). Probiotics have clinical, microbiological, and immunologic efficacy in acute infectious diarrhea. *Pediatr Infect Dis J*, 29(2), 135-138.
- [7] Chuang, C. C., Martinez, K., Xie, G., Kennedy, A., Bumrungrert, A., Overman, A., Jia, W. and McIntosh, M. K. (2010). Quercetin is equally or more effective than resveratrol in attenuating tumor necrosis factor- α -mediated inflammation and insulin resistance in primary human adipocytes. *Am J Clin Nutr*, 92(6), 1511-1521.
- [8] Kolodziej, H. and Kiderlen, A. F. (2007). In vitro evaluation of antibacterial and immunomodulatory activities of Pelargonium reniforme, Pelargonium sidoides and the related herbal drug preparation EPs 7630. *Phytomedicine*, 14(Suppl 6), 18-26.
- [9] Trun, W. et al., (2006). Nitric oxide synthase and cytokine gene expression analyses in Leishmania-infected RAW 264.7 cells treated with an extract of Pelargonium sidoides (EPs7630). *Phytomedicine*, 13, 570-575.
- [10] Fredholm, B. B., Bättig, K., Holmén, J., Nehlig, A. and Zvartau, E. E. (1999). Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. *Pharmacol Rev*, 51(1), 83-133.
- [11] Kolodziej, H. (2000). Traditionally used Pelargonium species: chemistry and biological activity of umckaloabo extracts and their constituents. *Curr Top Phytochem*, 3, 77-93.
- [12] Lewu, F. B., Grierson, D. S. and Afolayan, A. J. (2006). Clonal propagation of Pelargonium sidoides: a threatened medicinal plant of South Africa. *Afr J Biotechnol*, 5, 123-125.
- [13] Matthys, H. and Funk, P. (2008). EPs7630 improves acute bronchitis symptoms and shortens time to remission. Results of a randomised, double-blind, placebo-controlled, multicentre trial. *Planta Med*, 74(6), 686-692.
- [14] Thäle, C., Kiderlen, A. and Kolodziej, H. (2008). Anti-infective mode of action of EPs 7630 at the molecular level. *Planta Med*, 74(6), 675-681.
- [15] Matthys, H., Lizogub, V. G., Malek, F. A. and Kieser, M. (2010). Efficacy and tolerability of EPs 7630 tablets in patients with acute bronchitis: a randomised, double-blind, placebo-controlled dose-finding study with a herbal drug preparation from Pelargonium sidoides. *Curr Med Res Opin*, 26(6), 1413-1422.
- [16] Davis, J. M., Murphy, E. A. and Carmichael, M. D. (2009). Effects of the dietary flavonoid Quercetin upon performance and health. *Curr Sports Med Rep*, 8(4), 206-213.
- [17] Nieman, D. C., Henson, D. A., Gross, S. J., Jenkins, D. P., Davis, J. M., Murphy, E. A., Carmichael, M. D., Dumke, C. L., Utter, A. C., McAnulty, S. R., McAnulty, L. S., and Mayer, E. P. (2007b). Quercetin reduces illness but not immune perturbations after intensive exercise. *Med Sci Sports Exerc*, 39(9), 1561-1569.
- [18] Fletcher, D. K. and Bishop, N. C. (2011). Effect of a high and low dose of caffeine on antigen-stimulated activation of human natural killer cells after prolonged cycling. *Int J Sport Nutr Exerc Metab*, 21(2), 155-165.
- [19] Nieman, D. C., Henson, D. A., Davis, J. M., Dumke, C. L., Gross, S. J., Jenkins, D. P., Murphy, E. A., Carmichael, M. D., Quindry, J. C., McAnulty, S. R. and McAnulty, L. S., Utter, A. C. and Mayer, E. P. (2007a). Quercetin ingestion does not alter cytokine changes in athletes competing in the Western States Endurance Run. *J Interferon Cytokine Res*, 27(12), 1003-1011.
- [20] Conrad, A. et al., (2007). Extract of Pelargonium sidoides (EPs7630) improves phagocytosis, oxidative bursts, and intracellular killing of human peripheral blood phagocytes in-vitro. *Phytomedicine*, 14(Suppl. VI), 46-51.
- [21] Mandel, H. G. (2002). Update on caffeine consumption, disposition and action. *Food Chem Toxicol*, 40(9), 1231-1234.
- [22] Haskó, G., Kuhel, D. G., Chen, J. F., Schwarzschild, M. A., Deitch, E. A., Mabley, J. G., Marton, A. and Szabó, C. (2000). Adenosine inhibits IL-12 and TNF- α production via adenosine A2a receptor-dependent and independent mechanisms. *FASEB J*, 14(13), 2065-2074.
- [23] Link, A. A., Kino, T., Worth, J. A., McGuire, J. L., Crane, M. L., Chrousos, G. P., Wilder, R. L. and Elenkov, I. J. (2000). Ligand-activation of the adenosine A2a receptors inhibits IL-12 production by human monocytes. *J Immunol*, 164(1), 436-442.
- [24] Goossens, V., Stangé, G., Moens, K., Pipeleers, D. and Grooten, J. (1999). Regulation of tumor necrosis factor-induced, mitochondria- and reactive oxygen species-dependent cell death by the electron flux through the electron transport chain complex I. *Antioxid Redox Signal*, 1(3), 285-295.
- [25] Kayser, O. et al., (2007). Immunomodulatory principles of Pelargonium sidoides. *Phyter Res*, 15, 122-126.
- [26] Neugebauer, P. et al. (2005). A new approach to pharmacological effects on ciliary beat frequency in cell cultures – exemplary measurements under Pelargonium sidoides extract. *Phytomedicine*, 12, 47-52.