International Journal of Histology and Cytology ISSN 2447-9535 Vol. 3 (9), pp. 283-286, September, 2016. Available online at www.internationalscholarsjournals.org © International Scholars Journals

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Full Length Research Paper

GARCINIA KOLA extract reduced lipopolysaccharide activation of macrophages using U937 cells as a model

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Accepted 03 January, 2016

The effect of *GARCINIA KOLA* heckel seed extract on the promonocytic cell line U937 activated by lipopolysaccharide (LPS) was investigated. 200 µl of U937 cells maintained in culture at 5 x 10^5 cells per ml was delivered into wells of a culture plate according to groups. Cells were pre-treated with 20 µl of 100 ng/ml phorbol myristate acetate (PMA) for 24, 48, or 72 h in order to transform them to the macrophage form. Following the PMA treatment, some cells were incubated with 100 µg/ml LPS (group C), while others (group D) were treated with 100 µg/ml LPS and 100 µl of 100 µg/ml of the *G. KOLA* methanolic extract. Other cells (groups A, and B) were incubated with neither LPS nor extract. 24 h later, the supernatants were analysed for the production of TNF-α and IL6 as indices for the activation of macrophages. The results show that the *G. KOLA* extract reduced the tendency of LPS to activate the cells. However, highest activation of LPS was observed when the cells were incubated with PMA for 48 h prior to the addition of LPS. This suggests that stimulation of U937 cells with PMA is necessary for the production of cytokines and that *G. kola* possesses considerable immunomodulatory activity.

Key words: *Garcinia kola*, lipopolysaccharide, phorbol myristate acetate, macrophage, cytokines.

INTRODUCTION

The seeds of *Garcinia kola* heckel commonly called bitter kola in Nigeria, found in moist forest which grows as a medium sized tree up to 12 m high, belongs to the family of tropical plants known as Guttifera (Plowden, 1972). It is cultivated and distributed throughout West and Central Africa and has been shown to possess a caloric value of 35.8 kcal/g (Adegoke et al., 1998).

Before the remarkable bioactivities were explored, it was initially consumed as a stimulant (Atawodi et al., 1995). Split stems and twigs of the plant are used as chewing sticks in many parts of Africa, and have been commercialised in the major cities for years, offering natural dental care. In fact, it is probably the most important source of chewing sticks in West Africa (Agyili et al., 2006). The plant has been cultivated for various medi-cinal uses. The seeds have been used as antidote for *Strophantus gratus* infections (Holmes, 1960). The seeds are also used for the treatment of bronchitis, throat infections, antipurgative and antiparasitic (Madubunyi, 1995; Okunji and Iwu, 1991; Adefule-Ositelu et al., 2004). Other known uses include guinea worm remedy (Lewis, 1977), anti-atherogenic effects (Adaramoye et al, 2005), and antilipoperoxative effects (Emorele et al., 2005). Some other known activities of the seeds of *G. kola* H are antidiabetic and antihepatotoxic effects (Iwu, 1993; Tita et al., 2001). The plant has been shown to possess even antiviral activity as it halts the replication of the deadly Ebola virus in its tract in laboratory tests and it has been suggested that if the anti-Ebola compound proves successful in animal and clinical trials, it will be the first medicine to successfully treat the virus that causes Ebola hemorrhagic fever-an often fatal condition (BBC, 1999). All these and other remarkable bioactivities ascribed to *G. kola* H might be partly due to its great antioxidant property (Adaramola et al., 2005; Terashima et al., 2002).

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Studies have shown that *G. kola* inhibited *in-vitro* lipid peroxidation of rat liver homogenate in a dose dependent media and the active component responsible for lipid peroxidation is tentatively identified as isoflavones, a group of flavonoids (Adegoke et al., 1998). A recent study reports that extract of *G. kola* H produced duration-dependent teratogenicity in foetal rats (Akpantah et al., 2005) which suggests that continuous consumption of the seeds in pregnancy should be done with caution.

It is very obvious that the reported remarkable bio-activities of *G. kola* H may be partly due to its enhancement of some elements of the immune system. This present study is aimed at elucidating the effect of a methanolic extract of *G. kola* H on lipopolysaccharide activation of macrophages using the promonocytic cell line U937 as a model. Lipopolysaccharide is a major virulence factor of most bacteria and its ability to activate macrophages has been reported (Meng and Lowell, 1997; Rossenberger et al., 2000).

MATERIALS AND METHOD

Plant material

G. kola seeds were purchased from a local market. They were sun dried after removal of the seed coats and ground to a fine powder using a blender. It could not be guaranteed whether all the seeds were from the same source. The resulting powder was transferred to an 80% methanol solution in a round-bottomed flask, and kept airtight for three days. It was filtered and the extract concentrated using a rotary evaporator at 40^{o}C. The resulting residue was further air-dried. A 1 mg/ml solution of the extract was prepared by dissolving 10 mg of the dry extract in 10 ml of distilled water and stored under -4^{o}C until when required for analysis.

Propagation of the cell line U937

All procedures were carried out in a cell culture facility unless otherwise stated. One vial of the cell line U937 was grown in RPMI-1640 medium (Roswell Park Memorial Institute 1640) (Obtained from invitrogen life technologies) supplemented with 50 ml heat inactivated foetal calf serum, 5 ml of 1% glutamine, and 5 ml of penicillin-streptomycin solution (thus known as the complete RPMI medium). The vial of the cell line U937 from the liquid nitrogen was allowed to thaw and delivered to 5 ml of complete RPMI. 5 ml of RPMI medium was added to the stock and centrifuged at 2500 rpm for 10 min. They were washed three times in RPMI and re-suspended in 10 ml complete RPMI between 2 x 10^5 – 1 x 10^6 cells per ml. The cells were then kept in a humidified incubator maintained at 37^{o}C and gassing up to 5% CO_2.

Generation of macrophages

The cells were re-suspended at 5 x 10^5 cells per ml in complete RPMI medium. 200 µl of this suspension was added into each well of columns 2 - 9 of a 96 well culture plate. 20 µl of 100 ng/ml Phorbol 12-Myristate 13-Acetate (prepared in RF10 complete medium) was added to the cells of columns 4 - 9 of the culture plate and kept in the humidified incubator for 24 h.

Incubation of macrophages with test solutions

After 24 h the supernatants were removed from the culture plate (bearing the stimulated U937 cells). 100 µl of 1µg/ml lipopolysaccharide was added to the cells in each well of columns 6, 7, 8, 9. 1 µg/ml of the *G. kola* extract was prepared by dissolving 10 µl of the 1 mg/ml extract in 10 ml RPMI medium. 100 µl of this solution was added to the cells in each of the wells in columns 8, and 9. The treatment regimen could be summarised as follows:

Columns 2 and 3: No PMA, no LPS, no extract (group A);
Columns 4 and 5: PMA only (Group B);
Columns 6 and 7: PMA and LPS (Group C);
Columns 8 and 9: PMA and LPS and extract (Group D).

The culture plate was kept in the humidified incubator for 24 h. U937 cells were also added to two other culture plates and subjected to the same treatment as above but one of the plates was kept for 48 h after adding PMA, while the other for 72 h after adding PMA. The supernatants of the cell culture were later analysed for the activation of macrophages.

IN VITRO assay for macrophage activation

The activation of the macrophages was assayed by cytokine ELISA and the products of the cytokines TNF-α and IL6 were used as the indices of the activation of macrophages. The antibodies, purified rat anti- human TNF-α, purified rat anti- human IL6, biotin mouse anti-human TNF-α, and biotin mouse anti-human IL6 were obtained from Pharmagen (Becton Dickinson). Extravidin-HRP conjugate was obtained from Sigma Chemicals Ltd. Washes in all cases was carried out in phosphate buffered saline/tween (PBS/Tween) and blocking done in PBS/BSA/tween.

Procedure

Each of the antibodies, Purified rat anti –human TNF-α (in case of TNF-α determination), or purified rat anti human IL6 (in case of IL6 determination) were diluted (1:1000) in sodium phosphate buffer and 100 µl was delivered into each well of Nuncimmuno Maxisorp 96 well plates and incubated overnight at 4^{o}C. The contents of the wells were discarded and washed thrice. 125 µl of blocking solution was then added to each well and incubated for 1 h under room temperature. The plates were washed again and 50 µl of each supernatant of the cell culture and 50 µl of the blocking solution were added to the wells and incubated for 90 min under room temperature. The contents of the plates were discarded and washed thrice as usual. Either 100 µl of biotin mouse anti-human TNF-α (1:1000 in blocking solution) (in the case of TNF-α) or 100 µl biotin mouse anti-human IL6 (1:1000 in blocking solution) (in the case of IL6) was added to each well and incubated for 1 h under room temperature. The plates were washed again and 100 µl of ExtrAvidin conjugate (Sigma Chemicals) (1:1000) was added to each well and incubated for 1 h under room temperature. The plates were washed and 100 µl of a chromogen was added to the wells and incubated for 30 min. The chromogen substrate was a tablet of o-phenylenediammine dihydrochloride (OPD) dissolved in 25 ml citrate phosphate buffer pH 5, and 25 µl hydrogen peroxide. 100 µl of 10% concentrated sulphuric acid was added to each well in order to stop the reaction and the absorbance of each well read at 490 nm using an Automated Ultra Microplate Reader. Quantification was done by the use of TNF-α and IL6 standards obtained from Pharmagen (Becton Dickinson).

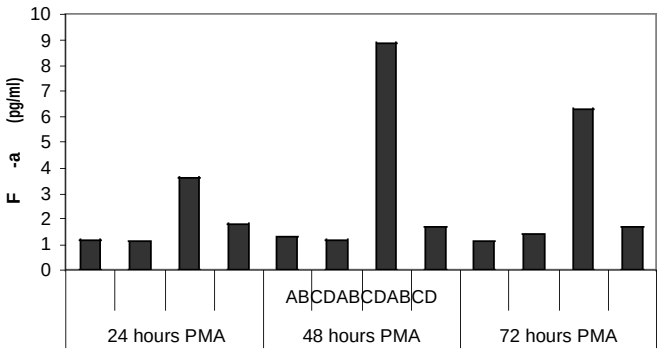

Figure 1. TNF-α in cells incubated with PMA, LPS and *G. kola* extract at different times. **Group A:** no PMA, No LPS, No extract; **Group B:** PMA only; **Group C:** PMA + LPS; **Group D:** PMA + LPS + extract.

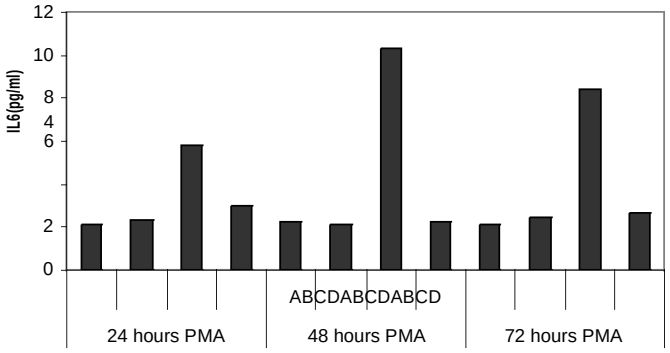

Figure 2. IL6 in cells incubated with PMA, LPS and *G. kola* extract at different times. **Group A:** no PMA, No LPS, No extract; **Group B:** PMA only; **Group C:** PMA + LPS; **Group D:** PMA + LPS + extract.

Statistical analysis

Comparison between more than two means was made using a one way ANOVA, while the data were subjected to a two-tailed student's t-test where comparison between two means arises. In all cases, a confidence level exhibited at $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

TNF-α result of U937 cells incubated at different times with PMA and/ or LPS are shown in Figures 1 and 2. U937 cells that did not have pre-treatment with PMA did not produce TNF-α, and IL6 in significant amounts. When cells were pre-treated with PMA alone, once again there was no significant production of TNF-α, and IL6. Once cells were treated with PMA followed by LPS, the proinflammatory cytokines TNF-α, and IL6 were produced significantly. In both cases, maximum concentrations of the cytokines were produced after 48 h pre-treatment with PMA and with LPS for 24 h. From Figures 1 and 2, it was obvious that the cytokine levels produced by group D were significantly lower than cells in group C. This shows that the extract reduced the tendency of lipopolysaccharide to activate the cells.

The seeds of *G. kola* H have been subjected to various studies and the remarkable bioactivities have been reported and of utmost importance is the possession of great antioxidant potentials (Adegoke et al., 1998; Adaramola et al., 2005; Terashina et al., 2002). The antioxidant may be tentatively taken as a major factor that enhances the immune response and that is the main reason for the current study.

Macrophages are major players of the immune system which are at peak when various micro organisms enter the body. The remarkable function in phargocytosis makes macrophages a suitable model for study. Neutrophils would have been better for the experiment but obtaining neutrophils in large amounts from patients every week is hard. However macrophages also possess the same Fcγ receptors that neutrophils possess, hence a worthy substitute.

The antioxidant factor in *G. kola* has been reported to be flavonoids which have shown to possess various bioactivities which include antimicrobial, antiviral and anti-inflammatory properties (Madubunyi, 1995; Braide, 1990; BBC, 1999). The anti-inflammatory effect of flavonoids is believed to be the inhibition of cyclooxygenase (Epsey, 1983).

Lipopolysaccharide is a major virulence factor of most bacteria and its ability to enhance the activation of macrophages has been reported (Meng and Lowell, 1997; Rossenberger et al., 2000). In fact Meng and Lowell (1997) reported further that lipopolysaccharide triggers the abundant release of TNF-α, IL6 and IL1. Phorbol myristate acetate was used to enhance the transition of the monocyte form of U937 to the macrophage form because phorbol esters induce the differentiation of U937 to macrophage morphology and acquire considerable phargocytic activity (Pagliara et al., 2005; Liu and Wu, 1992). This was further confirmed in this current work, as the measured cytokines were at the background level when the cells were not pre-treated with phorbol myristate acetate in all cases (Figures 1 and 2). It was also found that the differentiation of U937 cells peaked at 48 h pro-treatment with phorbol myristate acetate. This supports the findings of Joyce and Steer (1992) who reported that treatment of U937 with phorbol myristate acetate resulted in the cells having characteristics of macrophages after two days.

When the cells were pre-treated with phorbol myristate acetate for various periods, the subsequent incubation of the *G. kola* extract for 24 h caused marked activation but the activation recorded after pre-treatment of phorbol myristate acetate for 48 h was significantly higher than 24 h PMA ($p = 0.002$) and also higher than 72 h PMA ($p = 0.013$) (Figures 1 and 2). Of course, treatment with the extract alone produced no activation as the recorded values for the measured indices were even smaller than the negative control values. This further confirms that the

pre-treatment with phorbol myristate acetate is necessary for the production of the proinflammatory cytokines.

Several reports have credited the antioxidant property of *G. kola* to the presence of flavonoids (Braide and Vitrotio, 1989; Cotterih et al., 1978) but some prooxidant effects of flavonoids have also been reported hence arbitrarily ascribing antioxidant effects to flavonoids should be revisited (Awad et al., 2001; Galati and O'Brein, 2004). Terpenes have also been reported to possess some remarkable bioactivities (Zhou et al, 2006) which are a group of important phytochemicals which may be present in *G. kola* extracts. In conclusion, the extract of *G kola* H seed possesses considerable immunomodulatory effects as judged, reducing lipopoly-saccharide activation of macrophages. Progress in this area will surely extend the frontiers of the search of immunomodulatory agents.

REFERENCES

Adaramoye OA, Nwaneri VO, Anyanwu KC, Farombi EO, Emerole GO (2005). Possible anti-atherogenic effect of kolaviron (a *Garcinia kola* seed extract) in hypercholesterolaemic rats. Clin. Exp. Pharm. Physiol. 32(1-2): 40-46.

Adefule-Ositelu AO, Adefule AK, Oosa BO, Onyenefa PC (2004). Antifungal activity of *Garcinia kola* nut extract as an ocular bacterial isolates in Lagos. Niger. Qualt. J. Hosp. Med 14: 112-114.

Adegoke GO, Kumar MV, Sambaiah K, Lokesh BR (1998). Inhibitory effect of *Garcinia kola* on lipid peroxidation in rat liver homogenate. Indian J. Exp. Biol. 36(9): 907-910.

Agyili J, Sacande M, Kouame C (2006). *Garcinia kola* Heckel. Seed leaflet No. 113. Millenium seed bank project, Ghana.

Akpantah AO, Oremosu AA, Noronha CC, Ekanem TB, Okanlawon AO (2005) Effects of *Garcinia kola* seed extract on ovulation, oestrous cycle and foetal development in cyclic female sprague - dawley rats. Niger. J. Physiol. Sci. 20(1-2): 58-62.

Atawodi S, Mende P, Pfundstein B, Preussmann R, Spiegelhalder B (1995). Nitrosatable amines and nitrosamide formationformation in natural stimulants, *Cola acuminata*, *C. nitida* and *Garcinia cola*. Food Chem Toxicol 33(8): 625-630.

Awad HM, Boersma MG, Boeren S, van Bladeren PJ, Vervoort J, Reitjens IM (2001). Structure-activity study of the quinone/quinone methide chemistry of flavonoids. Chem. Res. Toxicol. 14: 398-408.

BBC (1999). Garcinia-Antioxidant and more. News bulletin on Thursday August 5, 1999. Published at 14: 37 GMT.

Braide VD (1990). Pharmacology Effects of Chronic Ingestion of *Garcinia kola* Seeds in the Rats. Phytother. Res. 4: 39-41.

Braide VD, Vittrotio G (1989). Histological Alterations by a Diet Containing Seeds of *Garcinia kola*: Effects on liver, Kidney and intestine in the Rat. In: Gedenbaurs Morphol. Jahrb, Leipzig. 1334 I.S. 95-101.

Cotterih P, Scheinmenn F, Stenhuise I (1978). Composition of *G. kola* seeds. J. Chem. Soc. Perkin. Trans. 1: 532-533.

Emorele GO, Farombi EO, Adaramoye OA, Adeyemi EO (2005). Comparative study on the antioxidant properties of flavonoids of *Garcinia kola* seeds. Pak. J. Med. Sci. 21(3): 331-339.

Epsey LL (1983). Comparison of the effect of non steroidal and steroidal antiinflammatoryagents on prostaglandin production during ovulation in the rabbits. Prostaglandin, 6: 71 -77.

Galati G, O'Brien PJ (2004). Potential toxicity of flavonoids and other dietary phenolics: Significance for their chemoprotective and anticancer properties. Free Radical Biol. Med. 37: 287-303.

Holmes EH (1960). Notes on the Medicinal Plants of Liberia. Pharm. J.

rd Iwu M (1993). Handbook of African medicinal plants. CRC Press, Boca Raton, FL.

Joyce DA, Steer JH (1992). Differentiation of the U-937 promonocytic cell line induced by phorbol myristate acetate or retinoic acid: effect of aurothiomalate. Agents Actions 37(3-4): 305-310.

Lewis WH (1977). Medic Botany: Plants Effecting Mainsheath. New York: John Wiley-Int. Pub. pp. 231-232.

Liu MY, Wu MC (1992). Induction of human monocyte cell line U937 differentiation and CSF-1 production by phorbol ester. Exp. Haematol 20(8): 974-979.

Madubunyi II (1995). Antimicrobial activities of the constituents of *Garcinia kola* seeds. Int. J. Pharmacog. 33: 232-237.

Meng F, Lowell CA (1997). Lipopolysaccharide (LPS) –induced macro-phage activation and signal transduction in the presence of Src-family kinases Hck, Fgr and Lyn. J. Exp. Med. 185(9): 1661-1670.

Okunji CO, Iwu MM (1991). Molluscidal activity of *Garcinia kola* biflavanones. Fitoterapia, 62: 74 -76.

Pagliara P, Lanubile R, Dwikat M, Abbro L, Dini L (2005). Differentiation of monocytic U937 cells under static magnetic field exposure. Eur. J. Histochem. 49(1): 75-86.

Plowden CC (1972). A manual of plants names. 3" ed. London.

Rossenberger CM, Scott MG, Gold PP, Hancock EE, Finlay BB (2000). Salmonella typhirium infection and lipopolysaccharide stimulation induce changes in macrophage gene expression. J. Immunol 164(11): 5894-904.

Terashima K, Takaya Y, Niwa M (2002). Powerful antioxidative agents based on garcinoic acid from *Garcinia kola*, Bioorg. Med Chem 10(5): 1619-1625.

Tita RK, Odeigah PG, Agomo PU, Bassey E (2001). Some properties of Medicinal Plants used by the Igbos of Nigeria. In Triats, tracts and traces. (Germany). Edited by Wolfgang Kreis, pp. 209-210.

Zhou Q, Xie H, Zhang L, Stewart JK, Gu X, Ryan JJ (2006). *cis*-Terpenones as an effective chemopreventive agent against aflatoxin B1-induced cytotoxicity and TCDD-induced P4501A/B activity in HepG2 cells. Chem. Res. Toxicol. 19: 1415-1419.