Report of the Internship

Abstract

From the 20th of June 2018 to the 20th of August 2018, we made an internship at the University of Hasselt in Belgium. The purpose of the study was for impregnation to some advanced immunological laboratory techniques. During the two months I acquired many knowledge and fill to have an open mind, high motivation and specific skills helpful to achieve a research career in Neuroimmunology.

Introduction

Multiple sclerosis (MS) is a neurological disease of young adults (20-40 years old), there is no treatment for this pathology and some drugs are used to reduce the clinical symptoms and are related to a wide range for side effects (Interferon beta, acetate glatiramer…). Plants are a good source for the discovery of new active principles and may present few side effects. Garcinia kola is a plant frequently used in central Africa for the treatment of a wide range of diseases; many previous works revealed its various properties but none has shown its potentialities on the in vitro models of MS. The aim of this internship was to scientifically assess the immunological effects of Gacinia kola on cell culture.

Chemicals

Alamar blue reagent, DMEM (Dulbecco modified medium), trypan blue, griess reagent, cells , standard nitric oxide provided from Sigma Aldrich.

Plant extraction

GK's seeds were bought at the Yaounde local market (mokolo), the outer coats of GK seeds were removed and sun-dried for one week. The dried seeds were milled using a local mill into a fine powder and kept in plastic bags for further use. For the in vitro experiments (cell viability assay and nitric oxide assay), we prepared a stock solution of 1000 µg/ml. In fact 100 mg of the GK's powder was weighed and mix to 1ml of KOH. From the stock solution different dilutions were made at the ratio of 1/100; 1/10; 1/10 to obtain four doses: 1000 µg/ml; 100µg/mL; 10 µg/ml and 1µg/ml.

Resazurin assay

Cell viability was confirmed by alamar blue assay, using Vero cell lines. Vero cells were seeded at a density of $1x10^5$ cells/ml and incubated with a range of concentrations of GK at

37°C in a 5% CO_2 incubator for 4hours, then the different concentrations of the plant's extract (1000; 100; 10; 1 µg/ml) were added in different lots of four wells each and incubated for 24hrs. Following incubation, $1/10^{th}$ of alamar blue reagent was added into the cell wells. Cells were incubated for further 4hrs, protected from direct light. Absorbance was read at 540 nm, using 570 nm as a reference wavelength.

Nitric oxide assay

Nitric oxide production was assayed by measuring nitrite in the supernatants of cultured cells. The assay was carried out as described previously with slight modification (Yoon et al., 2009). After pre-incubation of cells (1×10^5 cells/mL) with LPS (1 µg/mL) for 24 hours, the amount of nitrite, a stable metabolite of NO use as an indicator of NO production, in the culture medium was measured using the Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid). A volume of 50 µL of the cell culture medium was mixed with 50 µL of the Griess reagent. Subsequently, the mixture was incubated at room temperature for 15 min and the absorbance measured at 540 nm in a microplate reader. Fresh culture medium was used as a blank in every experiment. The quantity of nitrite was determined from a sodium nitrite standard curve as expressed in this equation: $Inhibition\ (\%) = Control - TestControl \times 100$.

Results

Garcinia kola's effects on the Alamar blue assay

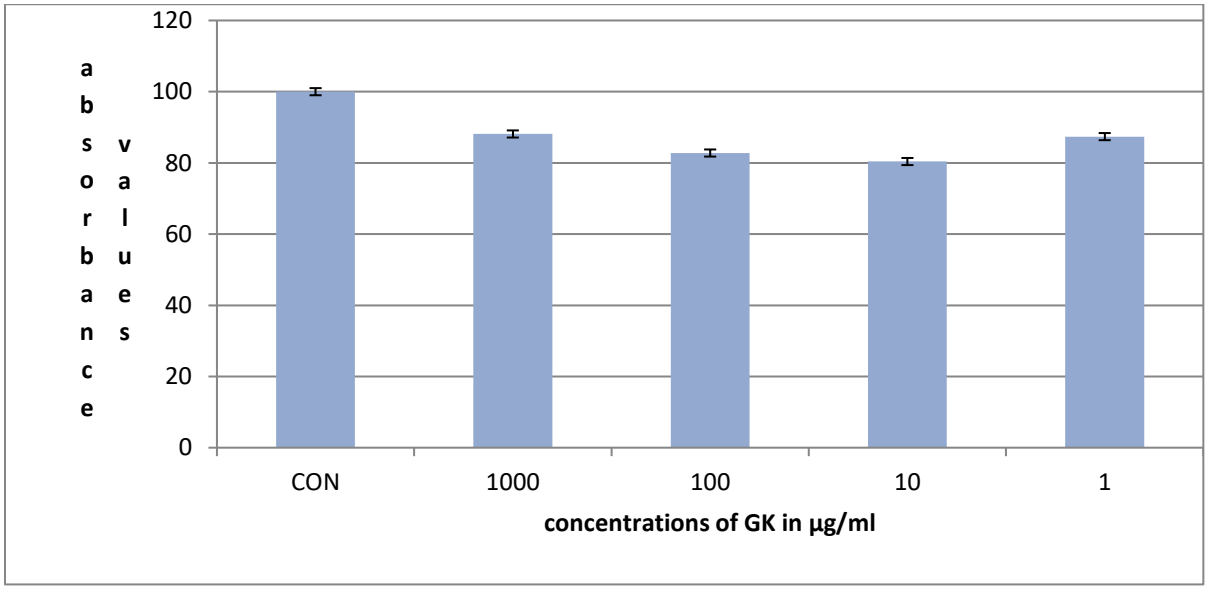


Figure 3: effects of Garcinia kola on the Alamar blue assay

Each value is expressed as mean±SEM of absorbance of Alamar blue treated cells with GK's seed extracts (1000, 100, 10 and 1µg/mL) compared to the negative control.

The seeds of GK does not have much effect on viability, reduces it to a maximum rate of about 20% (level of significance). The concentrations 1000; 100; 10 and 1 µg/ml presented respectively the absorbance values of 88±353,42; 82,77±87,09; 80,38±564,35 and 87,38±980,52, compared to the negative control group whose absorbance is 100±0, 0.

Effect GK's seeds on LPS induced NO production by …cells

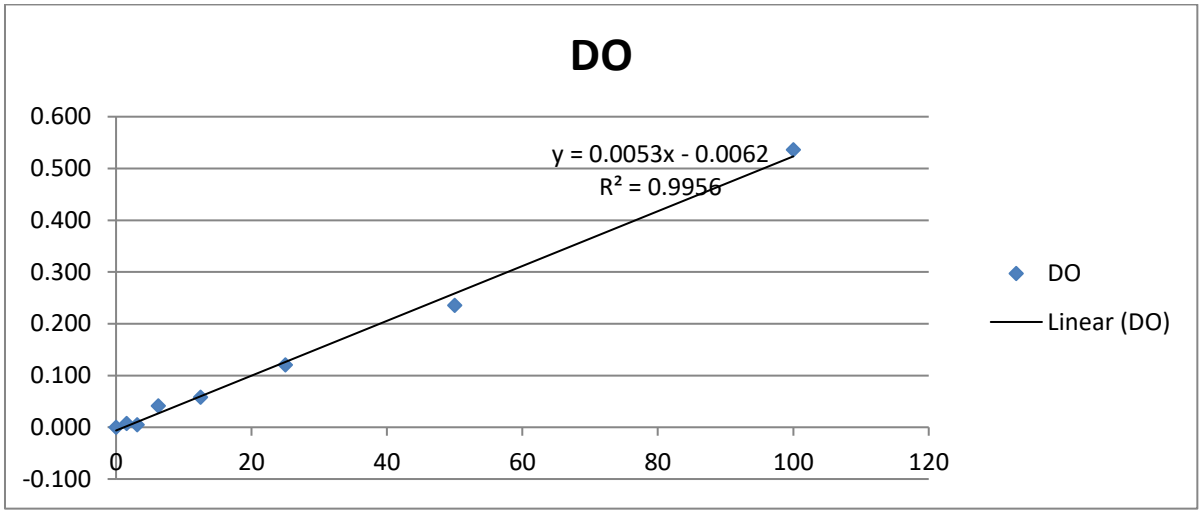


Figure 4: $NaNO_2$ standard curve used to obtain the NO concentrations

The Griess reaction, a spectrophotometric determination of nitrite level, was carried out to measure the nitrite levels in the conditioned medium … cells treated with LPS. Sodium nitrite ($NaNO_2$) was used as a standard compound for the standard curve. The NO concentrations were obtained using the standard curve equation: **Y = 0.0053 X – 0, 0062 (R^2=0, 9956)**, where Y is absorbance at 540 nm and X is the NO concentration in µM. The inhibitory activity of the extracts towards NO generated by LPS-activated macrophage was obtained from the calculated value of X.

Garcinia kola's effects on NO production

Figure 3: Effects of Garcinia kola on the NO production

These results present the values of the nitric oxide obtain in each treated cell wells. It shows that, GK's extract does not have a dose-dependent effect of the NO production. The concentrations 1000; 100; 10 and 1µg/ml presented respectively 54, 54±5,74; 48,74±5,21; 44,89±2,41 and 52,60±6,39. The cells treated by LPS and not treated with LPS presented respectively the NO values of 51,19±4,43 and 3,42±1,05.

Discussion:

The AlamarBlue Cell Viability Assay Reagent is used to quantify cellular metabolic activity and in turn determine the concentration of viable cells in a given sample (O'Brien et al.,2000).. This result may reveals that GK's extract is less harmful to cells, the concentration of 10µg/ml may be much harmful for cells, because at this rate, about 20% of cells died, implying that this concentration would be the most toxic. The weak cytotoxic effects revealed by this plant can explain its current consumption by the local population (Adjanohoun et al., 1991) and thus shows that it could have low side-effects in the treatment of multiple sclerosis, and might suggest that its constituents may not have a major negative impact on the cells that make up the blood brain barrier.

Nitric oxide is an important signaling molecule involved in diverse physiological and pathophysiological mechanisms in cardiovascular, nervous and immunological systems. Inhibition of iNOS (inducible nitric oxidesynthase) may be beneficial for the treatment of inflammatory disease (Aktan et al.,2003). Nitric oxide is a reactive molecule that is synthesized by macrophages and microglia during inflammation (Hendriks et al., 2005). Concerning GK's seed extract effect on the NO production, we can say that GK does not have a big impact on the reduction of the nitric oxide production; it implies that it does not have a great in vitro anti-inflammatory activity. Thus the plant extract cannot be a good candidate for the discovery of a new active principle which can be effective in the treatment of multiple sclerosis (MS). In fact it has been shown that a good MS drug should greatly reduce the NO production by macrophages. For example, a cytokine like interferon beta has antiinflammatory properties which permit to inhibit the T cell proliferation and reduce migration of inflammatory cells across the BBB, (Lopez De padilla et al., 2015) or corticosteroids reduce the inflammatory response by inhibiting lymphocyte proliferation and cell-mediated immune response, down regulate cytokine gene expression and have independent effects on the bloodbrain barrier permeability (Sapolsky et al., 2000). The weak inhibitory effect of GK on the NO production may not be helpful in this process.

Conclusion

This study revealed that the seed extract of Garcinia kola is less cytotoxic, this is explain by its current consumption by population but it does not have a great and dose-dependent effect on the nitric oxide production thus cannot be a good candidate for the treatment of the

inflammatory process occurring during the multiple sclerosis genesis. Further studies will be done to assess its effect on the clinical symptoms of the disease.

Aknowledgement

I will like to thank the International Brain Research Organization (IBRO) with all my heart for having sponsored this Internship, it helps me to learn an important thing ‘ Reseach in Europe is done with high rigor to ameliorate the well-being of the humanity’ and this is not the case in my country where it is just a formality; by coming back home I will give all my best and used the acquired knowledge for a better research.

Reference:

Adjanohun, E. *et al.*, 1991. Traditional medicine and pharmacopoeia: contribution to ethnobotanical and floristic studies in western Nigeria. 0AU/STRC
Akpantah, A.O., Oremosu, A.A., Noronha, C.C., Ekanem, T.B., and Okanlawon, A.O. (2005).

Aktan, F., Henness, S., Roufogalis, B. D. and Ammit, A. J. (2003). Gypenosides derived from *Gynostemma pentaphyllum* suppress NO synthesis in murine macrophages by inhibiting iNOS enzymatic activity and attenuating NF-B-mediated iNOS protein expression. Nitric Oxide: *Biology and Chemistry*, 8(4): 235 – 242.
Braide VB. Antispasmodic extracts from seeds of *Garcinia kola. Fitoterapia* 1989; ix: 123

Dalziel, JM (1937). The Useful Plants of West Tropical Africa Crown Agents for Colonies. Westminster, London. pp. 89-91.

Etkin NL (1981). A Housa herbal pharmacopoeia: Biomedical evaluation of commonly used plant medicines. J. Ethnopharmcol., 4: 75-98.

Farombi, E.O., Adepoju, B.F., Ola-Davies, O.E. & Emerole, G.O. (2005): Chemoprevention of aflatoxin B1-induced genotoxicity and hepatic oxidative damage in rats by kolaviron, a natural biflavonoid of *Garcinia kola* seeds. -European Journal of Cancer Prevention 14(3): 207-214.

Hendriks, J.J., Teunissen, C.E., de Vries, H.E., Dijkstra, C.D., 2005. Macrophages and neurodegeneration. Brain Res. Brain Res. Rev. 48, 185–195.

Ibironke G.F., Olaleye S.B., Balogun O. and Aremu D.A. (1997). Antiulcerogenic effects of diets containing seeds of Garcinia kola (Heckel) Phytotherapy Research 11, 312 - 313.

Iwu M. Biflavanones of garcinia: Pharmacological and biological activities. Progression in Clinical Biological Research. 1986;213:485.

Lopez De Padilla, C. M., C. S. Crowson, et al. (2015). "Interferon-regulate chemokine score associated with improvement in disease activity in refractory myositis patients treated with rituximab." Clin Exp Rheumatol **33**(5): 655-663.

O’Brien J, Wilson I, Orton T, et al (2000). Investigation of the Alamar Blue (resazurin), fluorescent dye for the assessment of mammalian cell cytotoxicity. *Eur J Biochem*, **267**, 5421-6.

Sapolsky, R.M., Romero, L.M., Munck, A.U., 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr. Rev. 21, 55–89.

Yoon, W. J., Kim, S. S., Oh, T. H., Lee, N. H. and Hyun, C. G. (2009). *Abies koreana* essential oil inhibits drug-resistant skin pathogen growth and LPS-induced inflammatory effects of murine macrophage. *Lipids*, 44: 471 – 476.

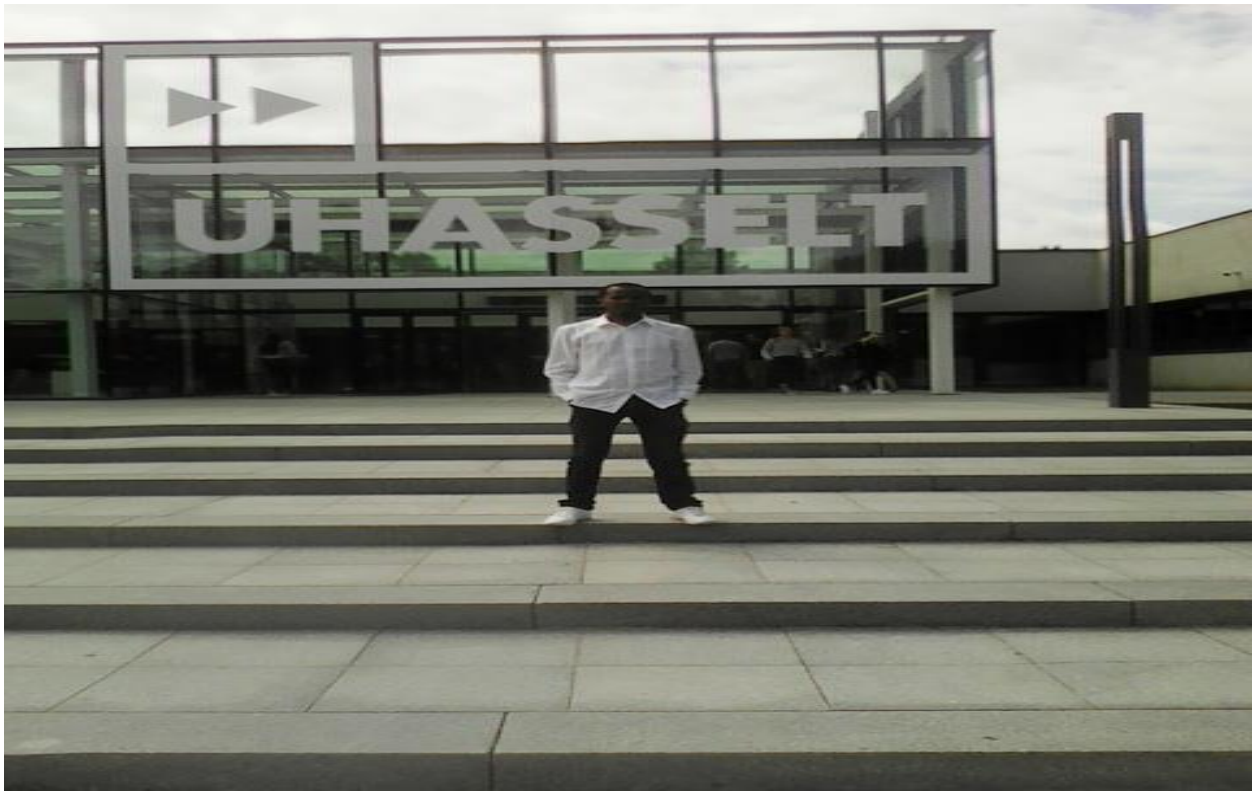
Outcome and benefit of the award

From this internship I can say that I have learned how to do in vitro cell culture, and how to achieve some experiments like RTPCR and Immunohistochemistry. The results obtained reveals that Garcinia kola is less harmful to cell, revealing its less toxic character this may explain its current consumption in Cameroon, and that it has no significant effect on the reduction of the nitric oxide production.

Financial report:

Expense made	Prize
Ticket transport (go and return)	1000 Euros
Accommodation:	1100 Euros for two months
Passport and Insurance	400 Euros
Total	2500 Euros

Photographs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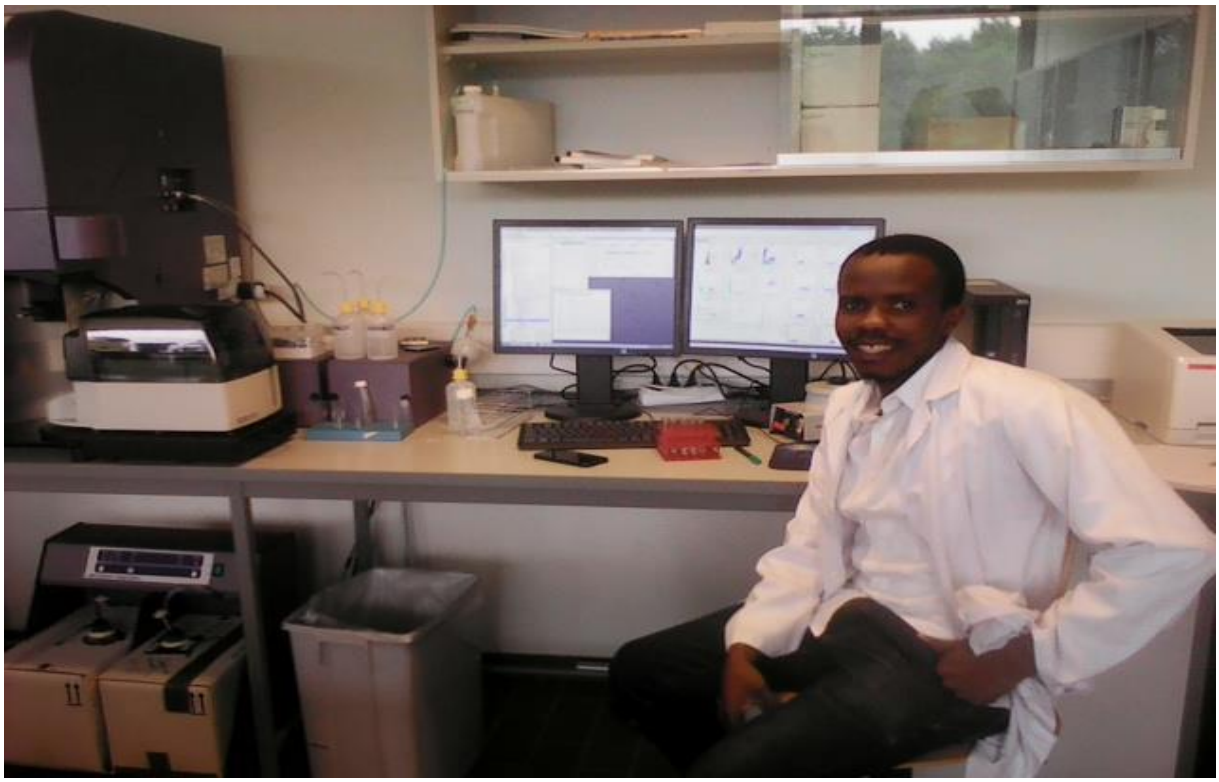


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