Original Article

Effect of Ethanol Extract of Moringa Oleifera leaves on T-cell Proliferation

Adewale GB,[1,4] Olowoparija SF, [1,2] Olayemi JO[3]and Olayinka E[5]

[1]Department of Pharmacology & Therapeutics, College of Medicine, University of Ibadan, Ibadan, Nigeria. [2]Department of Pharmacoloy & Therapeutics, Faculty of Basic Clinical Science, University of Medical Sciences, Ondo, Ondo, Nigeria. [3]Department of Pharmacognosy, Faculty of Pharmacy, University of Ibadan, Ibadan, Nigeria. [4]Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Ibadan, Ibadan, Nigeria. [5]Department of Mathematics, Faculty of Science, Obafemi Awolowo University, Ile-Ife, Osun, Nigeria

***Correspondence**
. Olowoparija Segun Fredrick
Email: olowfed1@gmail.com

Article Metrics
Date submitted: *15/09/2020*
Date Accepted: *06/11/2020*
Date Published: *November, 2020*

https://doi.org/10.46912/wjmbs.25

ABSTRACT

Immunity boosting is an important use of Moringa oleifera (family Moringaceae) commonly called Horseradish or "Tree of Life" in ethnopharmacology. The positive health promoting actions might be due to their action on immune cells. The aim of this study was to investigate T-cell mediated immunity boosting property of the ethanol extract of the leaves of M. oleifera using Jurkat cells. Toxicity profile was evaluated using trypan blue exclusion assay, and the probable mechanism of death evaluated by staining cells with annexin V and 7AAD. The effect of extract on proliferation was measured by staining with CSFE. The pattern of homing for CD3 and CD54 or CD28 activated T-cells in presence or absence of M. oleifera was monitored by estimating expression of CCR7. The result showed a concentration dependent significant increase in death by necrosis for inactivated Jurkat cells. Pre-stimulation with CD3 and CD54 or CD28 shifted the probable mechanism of death to apoptosis in a dose dependent manner. CFSE assay showed that inhibition of proliferation by M. oleifera was concentration dependent. The extract killed more than 50% of Jurkat cells at concentration higher than 80µg/ml. Expression of CCR7 was relatively higher in CD3 and CD28 pre-stimulated Jurkat than CD3 and Cd54. The study suggests that the ethanol extract of M. oleifera leaves possesses positive immunomodulatory activity mediated via CCR7 expression. This finding justifies the traditional use of M. oleifera extract in immunity boosting

Keywords: *Moringa oleifera, Proliferation, Necrosis, Annexin V, Immunomodulation,*

INTRODUCTION

Quite a number of compounds derived from plants have been found to either suppress or enhance immune system. Sometimes these properties are the intrinsic activity indicated for beneficial use of the plants in ethnomedicine and Traditional African Medicine (TAM). But quite a few of the plants have had their

immunomodulatory effects systematically studied. Most studies have been limited to reporting immunomodulatory effect on the basis of the plethora of cytokines measured. Immunomodulation is alteration of immune response which may enhance or suppress the immune system responsiveness. Immunomodulators may include some bacterial products, lymphokines and plant derived substances. Most of the immunomodulatory agents play their role in maintaining the immune system by increasing T cell immunity, decreasing or blocking the suppressor activity, or stimulating the Natural Killer cells (NK cells) and interferon production as well as inducing specific cytokine production by activated target cells.[1,2,3] According to Ganju et al.,[4] immunomodulation using medicinal plants can provide an alternative to conventional chemotherapy for a variety of diseases particularly when host defense mechanism has to be activated under the conditions of impaired immune response or when a selective immunosuppression is desired in situation like autoimmune disorders.

Moringa oleifera or Horseradish tree is an extremely valuable medicinal plant. Different parts and preparations of *M. oleifera* are used in traditional medicine for the treatment of various conditions [5] and as nutritional supplement.[6, 7] The immune system is affected by the environmental and dietary habits and it is believed that diet rich in antioxidants and micronutrients can boost the immune system.[5] *M. oleifera* like most plants contains several secondary metabolites and quite a large number of compounds have been isolated from the leaves.[8, 9] Phytochemical studies of *M. oleifera* leaves extracts showed that it contain catechol tannins, gallic tannins, steroids and triterpenoids, flavonoids, saponins, anthraquinones, alkaloids and reducing sugars.[10] It mainly contains various glycosides of thiocarbamate and isocyanide class.[11]

There are many anedoctal uses and reports on the benefits of *M. oleifera* leaves as an adjuvant immune booster in HIV/AIDs in Africa. Several studies have also revealed that *M. oleifera* powder supplementation might act as an immune stimulant for patients suffering from HIV infection.[12] Ethanol extract of *Moringa oleifera* leave increased the total white blood cell count and its differential in Cadmium induced toxicity as the probable mechanism of protection.[13] Despite the popularity of the plant in immunity boosting, there are just a handful of scientific publications validating this indication in its crude form. Previous studies have shown that methanol extract of the leaves stimulate both cellular and humoral immunity *in-vivo* at almost 1g/kg.[14, 15] The present study is focused on determining immunomodulatory profile of the crude extract on T –cell immunity by assessing proliferation, necrosis, apoptosis, homing pattern of stimulated and unstimulated Jurkat cells.

MATERIALS AND METHODS

Plant Collection and Authentification

M. oleifera leaves were collected at the Botanical garden of the Obafemi Awolowo University, Ile- Ife in August, 2010. The leaves were identified and authenticated at the Forestry Research Institute of Nigeria (FRIN) Ibadan, where a voucher specimen with the number (FHI 109601) was deposited

.

Extraction

The leaves were air-dried, pulverized and 500 g was macerated in 1 L of 50% ethanol. After 72 h it was decanted and filtered several times using cotton wool and Whatman's No.1 filter paper. The filtrate was concentrated using rotary evaporator (BUCHI Rota vapor R-205) at the Central Laboratory of the University of Ibadan, Ibadan, Nigeria. The percentage yield was 15.61% representing 78.05 g extracted from the 500 g of dried pulverized leaves. On each day of experiment, the dark tan colored extract referred to as the ethanol extract of moringa leaves (EMOL) obtained was freshly dissolved in DMSO which served as a vehicle.

Abs and chemicals

Anti-CD3 (OKT3), anti-CD11a (HB202), and anti-CD54 (R6.5D6) were purchased from American Type Culture Collection (Manassas, VA) and purified from serum-free hybridoma culture medium using protein G-Sepharose. Trypan blue. CFSE was purchased from Molecular Probes (Eugene, OR). Annexin V–phycoerythrin was purchased from Pharmingen, 7AAD, CCR7

Cell culture

The leukemic T cell line Jurkat E6.1 was purchased from American Type Culture Collection (TIB-152). Culture medium for all cell was RPMI 1640 (Mediatech, Herndon, VA), containing 10% FBS (Atlanta Biologicals, Norcross, GA), 50 U/ml each of penicillin and streptomycin (Life Technologies, Grand Island, NY), and 20 mM glutamine (Life Technologies).

Trypan blue cell exclusion assay

On the day of experiment the cells were counted and plated at 100,000 cells/well in a 96 well plate. DMSO and prepared concentrations of extract in DMSO were added such that the concentration of DMSO does not exceed 0.4% of medium. The numbers of dead (dark blue stained) and viable (non-stained) cells in the grids of hemocytometer were manually counted and recorded under an optical microscope (100× magnification; Olympus, Tokyo, Japan). The death rate of Jurkat cells at the different concentrations were calculated as follows:

$$\text{Cell Death rate} = \frac{\text{Dead cell count x 100}}{\text{Dead cell count + Viable cell count}}$$

CSFE Assay

Specified amount of cells re-suspended in serum free RPMI at 2×10^6 were labeled with 2.5µM CSFE and allowed to absorb stain for about 15 minutes in the incubator. Equal volume of complete RPMI was added before stained cells were re-suspended in fresh complete RPMI and plated at 100,000 cells per well. The vehicle and extract was then added. At the various time points the cells were collected washed twice with ice cold PBS and re-suspended in 100µl 1X Annexin V buffer before running on the Accurri flow cytometer.

Annexin V

At the time points, cells were collected washed twice with ice cold PBS and stained for 15minutes with 1µl Annexin V in 99µl of 1X Annexin V buffer. The cells were re-suspended in 100µl 1X Annexin V buffer before running on Accurri flow cytometer.

AAD

The cells for the 7AAD were collected and washed twice with ice cold PBS and stained for 15 minutes with 5µl 7AAD in 95µl of 1X Annexin V buffer. The cells were re-suspended in 100µl 1X Annexin V buffer before running on Accurri flow cytometer.

CCR7 assay

The cells for CCR7 assay were collected washed twice with ice cold PBS and stained for 15minutes in the dark at room temperature with 1.5µl Annexin V in 98.5µl of 1X Annexin V buffer. The cells were re-suspended in 100µl 1X Annexin V buffer before running on Accurri flow cytometer.

Statistical Analysis

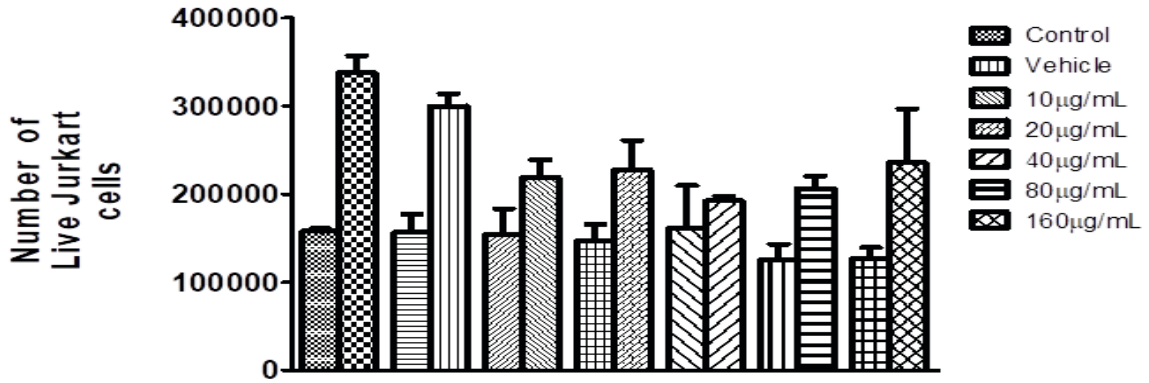
Results of the experiments and observations were expressed as mean ± standard error of mean (SEM). The significance of differences between groups was determined using 2-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. A level of significance ($P < 0.05$ or 0.01) was considered for each test. GraphPad Prism version 5 (GraphPad Software Inc., La Jolla, CA) was used for the statistical analysis.

RESULTS

EMOL was toxic on Jurkat cells at 160µg/mL

EMOL reduced the number of live cells to lower than 100,000 after 6 hours of treatment. After 24 hours post treatment all the concentrations has doubled almost twice except 40, 80 and 160µg/mL (Figure 1).

Figure 1: Crude extract *Moringa oleifera* inhibited cell division in Jurkat cells. Jurkat cells were plated at 100000



cells/well and total number of cells counted using trypan blue cell exclusion assay at time points (1,2,4,6 and 24 hours). Data are mean ± (SEM) total number of live cells within entire population. Representative of n= 4 experiments

EMOL reduced proliferation of Jurkat cells

The number of rapidly dividing Jurkat cells reduced with increasing concentration of EMOL. Jurkat cells are highly proliferating cells showing a bulk shift in histogram to the right from day 1 to 4 (Figure 2A). MFI of Jurkat cells in live gate show that at 80µg/mL, cells were not proliferating hence the high fluorescence value (Figure 2B). A plot of fold change in MFI for day 4 and 7 revealed that more inhibition was before day 4 (Figure 2C). EMOL inhibit number of proliferating cells in a concentration dependent manner (Figure 2D).

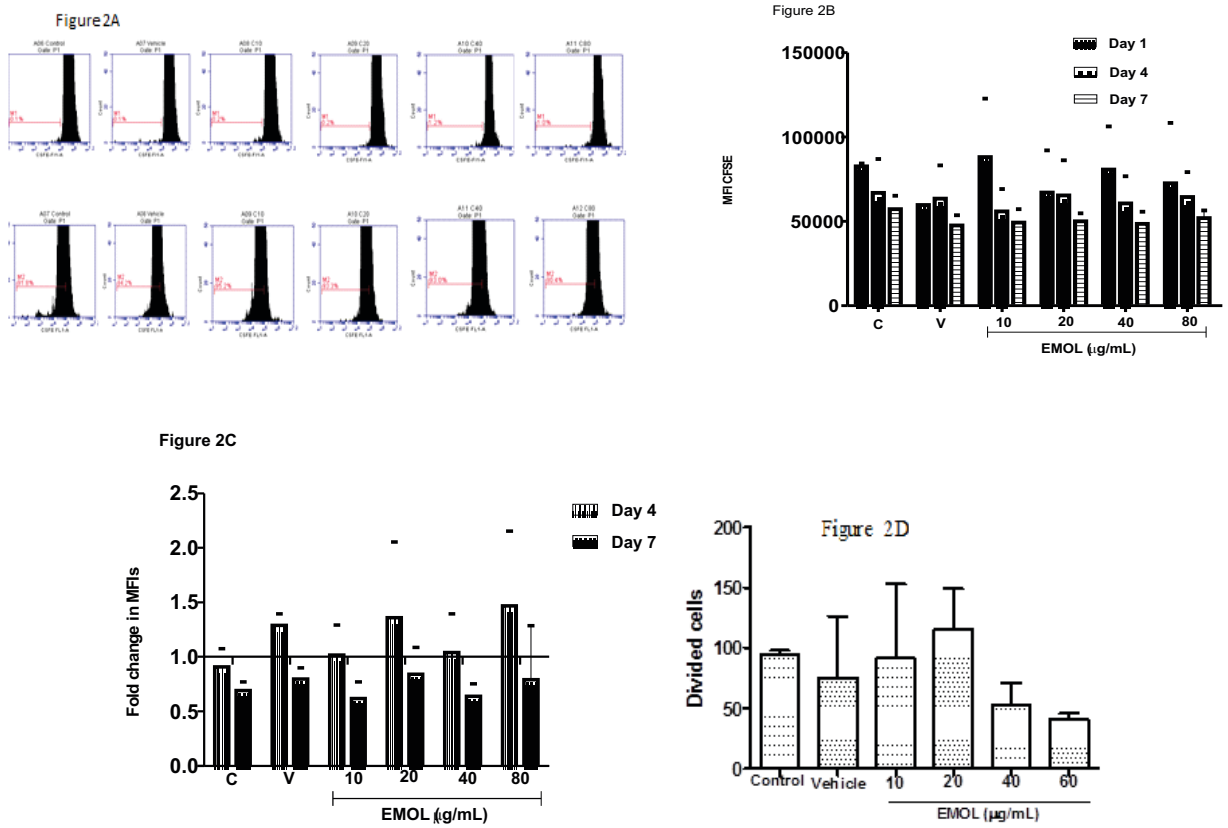


Figure 2: Crude *Moringa oleifera* inhibit proliferation of Jurkat cells.
Jurkat cells sub-cultured at 500000 cells/mL 24 hours before staining with CFSE and treated with or without crude extract. Gating was performed on rapidly dividing cells and counted at time points. (A) Representative histogram plots of CFSE at days 1 and 4. (B) Plot of CFSE mean florescence intensity (MFI) in presence or absence of extract at time points. (C) Fold change in MFIs at day 4 greater than 1. (D) Dose dependent reduction in number of dividing cells on day 4.

EMOL showed a concentration dependent induction of cell death by necrosis

Plot of 7AAD and annexin V showed that Jurkat cells become more $7AAD^+$ with increase in concentration of EMOL (Figure 3A). Untreated Jurkat cells were both $7AAD^-$ and annexin V^-, while at 80µg/mL most of the cells were $7AAD^+$ and few were annexin V. Result also showed a concentration driven effect for necrotic Jurkat cells on days 1 and 4.

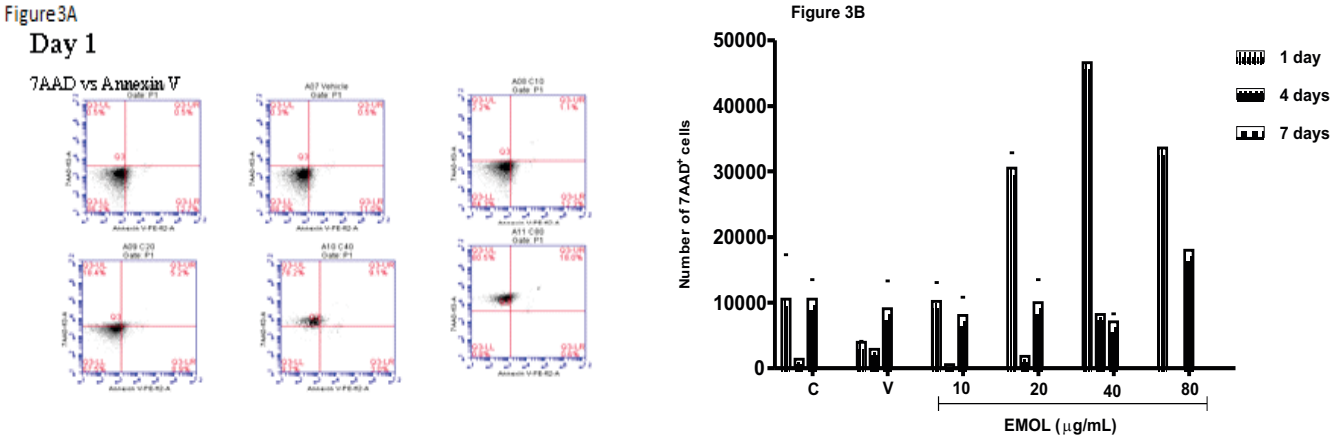
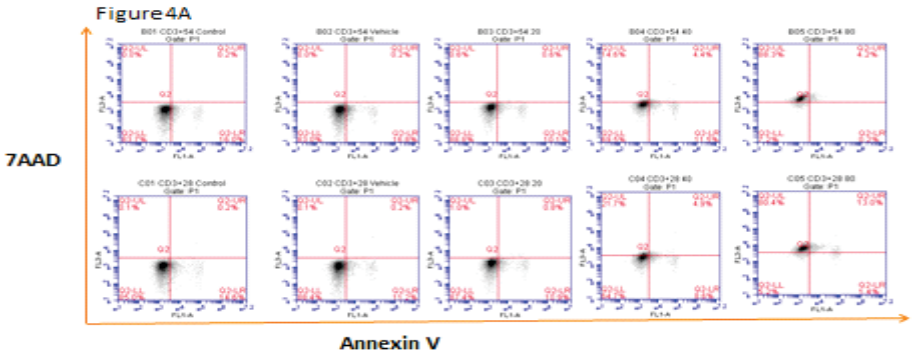


Figure 3: Crude *Moringa oleifera* extract dose dependently increased necrotic death in Jurkat cells. Jurkat cells sub-cultured at 500000 cells/mL 24 hours before treatment with or without crude extract. To directly measure apoptosis and necrosis, Jurkat cells were labelled with annexin V and 7AAD at various time points. Gating was performed on live cells and the total number of viable cells (mean ± SEM) was measured by flow cytometry (A) Representative plots of 7AAD and annexin V. (B) Total number of $7AAD^+$ cells at time points.

MOL inhibited apoptosis in CD3, ICAM and CD28 activated Jurkat cells

CD3 and either CD54 or CD28 co-stimulation of Jurkat (Figure 4A). EMOL caused a concentration dependent inhibition of apoptotic cell death in stimulated Jurkat cells (Figure 4B). EMOL as expected showed a concentration dependent increase in necrotic death (Figure 4C).



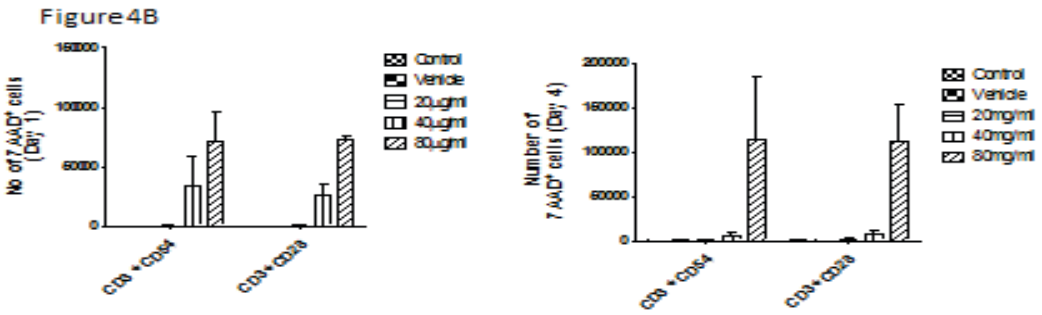


Figure 4: Sustained and efficient activation of Jurkat cells following co-stimulation through ICAM-1 and CD28. Jurkat cells were stimulated with anti-CD3 and anti-CD54 or anti-CD28. Cells were harvested on the days indicated and the total number of viable cells (mean, ± SEM) was measured by flow cytometry. Data (mean ± SEM) are presented as the number of cells relative to the entire population. Representative of three experiments. (A) Representative plots of 7AAD and annexin V. (B) Number of $7AAD^{+}$ cell on day 1 and 4.

EMOL showed slight increase expression of CCR7 in CD3 + CD28 treated Jurkat cellsEMOL caused a slight shift in CCR7 expression MFI of EMOL treated Jurkat cells when compared to untreated (Fig 5). CD3 + CD54 activated Jurkat did not affect CCR7 expression.

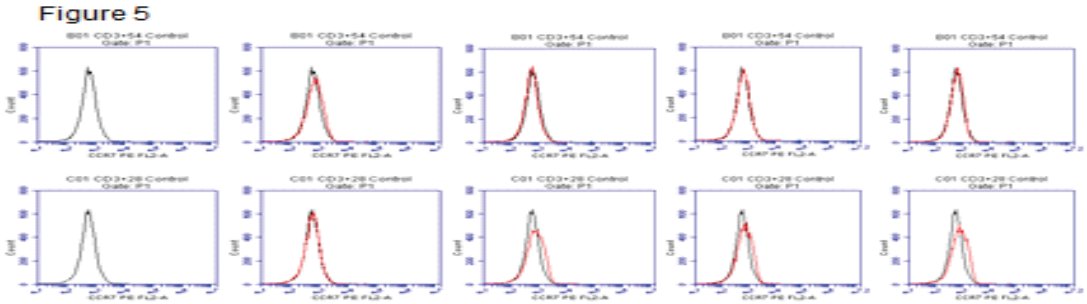


Figure 5: *Moringa oleifera* extract relatively increased expression of CCR7 in Jurkat cells following co-stimulation through ICAM-1 and CD28. Jurkat cells were stimulated with anti-CD3 and anti-CD54 or anti-CD28. Cells were harvested on the days indicated and the total number of viable cells (mean, ± SEM) was measured by flow cytometry. Data (mean ± SEM) are presented as the number of cells relative to the entire population. Representative of three experiments. Representative histogram plot of CCR7.

DISCUSSION

The present study suggests that the crude extract of *M. oleifera* possesses immunomodulatory action in vitro. These features could also be translated to control of CCR7 expression *in-vivo* and therefore be the likely reason for its use as immunity boosting. Though, *M. oleifera* is commonly used for this purpose, there is paucity of information on its effects on classical immune cell target as T-cell.

Previous study on acute toxicity of methanol extract of the root by Kasolo et al., [16] gave an LD_{50} as high as 15 g/kg, while another study by Ferreira et al., [17] gave LD_{50} lower than 700 mg/kg. In the present work, we provide the evidence to demonstrate that leaf extract of *M. oleifera* causes toxicity by necrosis and therefore advocates its cautious use in ethnomedicine.

The trypan blue exclusion test exclusion assay determines the amount of cells that are viable, but cannot differentiate between necrotic and apoptotic death as the cause of death. The extract killed more than 50% of Jurkat cells at concentration higher than 80µg/ml; surprisingly the cells were able to recuperate after their doubling time of twelve hours. Though the extract cause cell death, its nutritive potential was brought to bear by promoting doubling at the concentrations higher than number of death. The higher concentration still has a total number of live cells similar to the control and lower concentrations despite the high number of dead cells. *M. oleifera* like other natural products contains several numbers of chemical constituents used as hormones and secondary constituents.[11] Leaf extract of *M. oleifera* has been reported to contain a high number of nutrients and has been used in folkloric medicine to boost immunity in patients on antiretroviral therapy.[7] The difference in toxicity *in-vivo* and *in-vitro* might be explained by the pharmacokinetics of the extract. Some of the phytoallexins in plants are tolerable *in-vivo* because same able and efficient enzymatic pathway and transport system used in metabolism of dietary

constituents are also used in metabolism of these crude drugs.

With interest in the probable mechanism of cell death, further experiment was performed at optimum concentrations (concentrations showing death lower than 50%). The experiment showed that the mechanism of cell death might be necrosis. There are three major morphologies of cell death; apoptosis, cell death associated with autophagy and necrosis.[18] Apoptosis is an active process of self destruction associated with profound structural changes including morphological alteration, increased membrane permeability and nuclear collapse characterized by chromatin condensation and DNA fragmentation.[19] Two standard cytofluorometric methods of apoptosis quantification, each assay detecting distinct cellular alterations of the apoptotic process (7AAD and annexin V) were used. 7AAD evaluates the alteration in plasma membrane integrity and annexin V measures the translocation of phosphatidylserine from the inner to the outer layer of the plasma membrane. 7AAD staining has since been used to replace propidium iodide (PI) stain because of its ability to simultaneously identify cells in the various stages of apoptosis and death.[20] Apart from these, it is also able to stain necrotic cells and it is not radioactive like PI. All the foregoing is dependent on the followed protocol. Staining with 7AAD at 15µg/100µl in Annexin V buffer for 15 minutes in the dark at room temperature indicated cell death by necrosis. $7AAD^+$ cells increased in a dose dependent manner for day 1 (Figure 3B). The preponderance of $7AAD^+$/Annexin V^+ cells cannot be overemphasized. The number of $7AAD^+$/Annexin V^+ cells increased in a dose dependent manner . Measuring of these multiple parameters (7AAD and Annexin V) permit precise quantification of apoptosis. The forward and side scatter plot easily detects cell shrinkages.[21] This is reflected in the decrease in average number of cells in the live gate across concentration. The death might be a result of progressive loss of membrane permeability and flipping out or translocation of phosphatidylserine.[22] In the absence of phagocytosis, apoptotic cells proceed to a stage of secondary necrosis, which shares many features with primary necrosis.[18] The ability of *M. oleifera* to increase $7AAD^+$/Annexin V^+ cells might be an intrinsic pro-apoptotic property which could be beneficial in neoplastic diseases. Antiproliferative activity of *M. oleifera* leaves might thus, be related to its intrinsic apoptotic property which have been shown to cause emerging of apoptotic bodies, chromatin condensation, cell shrinkage, DNA fragmentation and induction generation of ROS in carcinoma KB cells.[23, 24] Antiproliferative effect of the extract was measured using CFSE. Although the assay has its nuances, the protocol was strictly followed to rule out interference of concentration of cells/CFSE and duration of labeling on toxicity by CFSE. The number of cells dividing reduced in group treated with the extract. Though this seems very small compared to the proliferating cells, but this can be attributed to the intrinsic ability of Jurkat cells to proliferation. Cell proliferation is controlled by growth factors which activates transcription factors that binds DNA to either turn on or turn off production of the proteins which results in cell division. This pathology is very important in neoplastic diseases and the ability of *M. oleifera* to negatively regulate this mechanism, might probably portray some kind of futuristic purpose for which it could be pursued.

CCR7 is an important surface marker broadly used to distinguish dedicated memory T cells. Along with CD44 and CD62L which indicate TCR stimulation and tendency for a T cell to take residency in secondary lymphoid organs, CCR7 expression is used to characterize types of memory T cells, but is also expressed on immature naïve T cells. The relative increase in the expression of CCR7 by CD3 and CD28 stimulated T cells in comparison with the CD3 and CD54 stimulated cells might be indicative of *M. oleifera* ability to boost immunity. Nominal use of the extract has been reported in TAM as immunity boosting. This might be explained by its ability to increase CCR7 expression, making it already tending to memory T cell take residency in the secondary lymphoid organ as a central memory T cell (T_{CM}). Effector memory T cells (T_{EM}) like T_{CM} expresses CD62L and CD44 but no CCR7.

CONCLUSION

In summary, this study suggests that the ethanol extract of *M. oleifera* leaf modulates immunity by increasing the trafficking of immune cells to the secondary lymphoid thereby enhancing immune-surveillance probably via CCR7 expression. Findings in this study justify the traditional use of *M. oleifera* extract in immunity boosting.

Acknowledgements

AGB is immensely grateful to the US government for the Fulbright JSD award. He is grateful to Dr. Stephen Benedict, University of Kansas for providing space and facilities in his laboratory for conduct of this work and to Amanda Dunbar and Anuja Bhatta for instruction on some of the techniques.

REFERENCES

1.Gabius HJ. Probing the cons and pros of lectin-induced immunomodulation: case studies for the mistletoe lectin and galectin-1. Biochimie. 2001 Jul 1;83 (7):659-66.

2. Stanilove, S. A.; Dobreva, Z. G.; Slavov, E. S.; Mitera, L. D. C3 binding glycoprotein from *Cuscuta Europea* induced different cytokine profiles from human PBMC compared to other plant and bacterial immunomodulators. Int. Immunopharmacol. 2005, 5:723-734.

3. Lam, H. Y.; Yeap, S. K.; Alitheen, N. B.; Ho, W. Y.; Omar, A. R. Understand the role of natural killer. Am. J. Immunol. 2010, 6:54-61.

4. Ganju, L.; Karan, D.; Chanda, S.; Srivastava, K. K.; Sawhney, R. C.; Selvamurthy, W. Immunomodulatory effects of agents of plant origin. Biomedicine & pharmacotherapy. 2003, 57, 296-300.

5. Fahey, J.W. *Moringa oleifera*: A review of the medical evidence for its nutritional, therapeutic, and prophylactic properties. Part 1. Trees for life Journal. 2005, 1, 21205-21285.

6. Andoh, A. K. 2008. *Moringa oleifera*: Research and Scientific Publications. Retrieved Nov 23, 2010, from www.abibitumikasa.com/forum

7. Maponga, C.C.; Monera, T.G. *Moringa oleifera* supplementation by patients on antiretroviral therapy. Journal of the International AIDS Society. 2010, 13:188.

8. Manguro, L. O.; Lemmen, P. Phenolics of *Moringa oleifera* leaves. Natural Product Research. 2007, 21, 56-68.

9. Mishra, G.; Singh, P.; Verma, R.; Kumar, S.; Srivastav, S.; Jha, K.; Khosa, R. L. Traditional uses, phytochemistry and pharmacological properties of *Moringa oleifera* plant: An overview. Der Pharmacia Lettre. 2011, 3, 141-164.

10. Kasolo, J. N.; Bimenya, G. S.; Ojok, L.; Ochieng, J.; Ogwal-Okeng, JW. Phytochemicals and uses of *Moringa oleifera* leaves in Ugandan rural communities. Journal of Medicinal Plants Research 2010, 4, 753-757

11. Ganatra, T. H.; Joshi, U. H.; Bhalodi, P. N.; Desai, T. R.; Tirgar, P. R. A panoramic view on pharmacognostic, pharmacological, nutritional, therapeutic and prophylactic values of *Moringa oleifera* lam. IRJP. 2012, 3:6

12. Nworu, C. S.; Okoye, E. L.; Ezeifeka, G. O.; Esimone, C.O. Extracts of *Moringa oleifera* Lam. showing inhibitory activity against early steps in the infectivity of HIV-1 lentiviral particles in a viral vector-based screening. African Journal of Biotechnology. 2013, 12.

13. Chatterjee, P. K.; Anantharaya, V. N.; Singhal, A.; Chatterjee, P.; Shiva, R. K.; Mallya, R. *Moringa oleifera* aqueous leaf extract: role on total leucocyte count and its differentials in cadmium toxicity in adult Wistar Albino rat model. National Journal of Physiology, Pharmacy and Pharmacology. 2016, 6, 119.

14. Gaikwad, A.; Sura, H.; Shaphrang, B.; Shalini, K.; Moutrisha, R.; Krishnan, K. Prediction of interaction between antitumor compounds and target proteins of different cancers by in silico molecular docking studies. Pharmacologyonline. 2011, 3, 692-699.

15. Sudha, P.; Asdaq, S. M.; Dhamingi, S .S.; Chandrakala, G. K. Immunomodulatory activity of methanolic leaf extract of *Moringa oleifera* in animals. *I*ndian J Physiol Pharmacol. 2010, 54(2), 133-40.

16. Kasolo, J. N.; Bimenya, G. S.; Ojok, L.; Ogwal-Okeng, J. W. Phytochemicals and acute toxicity of *Moringa oleifera* roots in mice. Journal of Pharmacognosy and Phytotherapy. 2011, 3(3), 38-42.

17. Ferreira, P. M.; Carvalho, A. F.; Farias, D. F.; Cariolano, N. G.; Melo, V. M.; Queiroz, M. G.; Martins, A.; Machado-Neto, J. G. Larvicidal activity of the water extract of *Moringa oleifera* seeds against *Aedes aegypti* and its toxicity upon laboratory animals. Anais da Academia Brasileira de Ciências. 2009, 81, 207-16.

18. Krysko, D. V.; Berghea, T. V.; Herde, K. D.; Vandenabeele, P. Apoptosis and necrosis: Detection, discrimination and phagocytosis. Methods. 2008, 44, 205–221.

19. Wyllie, A. H.; Kerr, J. F. R.; Currie, A. R. Cell death: The significance of apoptosis. In: International Review of Cytology, Bourne, G. H., Danielli, F. J., Jeon, K. W. Eds.; Academic Press: New York, 1980, 251-306.

20. Zembruski N. C.; Stache, V.; Haefeli, W. E.; Weiss, J. 7-Aminoactinomycin D for apoptosis staining in flow cytometry. Analytical biochemistry. 2012, 429, 79-81.

21. Petit, J. M.; Ratinaud, M. H.; Cordelli, E.; Spano, M.; Julien, R. Mouse testis cell sorting according to DNA and mitochondrial changes during spermatogenesis. Cytometry. 1995, 19, 304–312.

22. Van Engeland, M.; Kuijpers, H. J.; Ramaekers, F. C.; Reutelingsperger, C. P.; Schutte, B. Plasma membrane alterations and cytoskeletal changes in apoptosis. Experimental cell research. 1997, 235, 421-30.

23. Weinberg, R. A. The Biology of Cancer. Bethesda, USA: Garland Science; 2007.

24. Sreelatha, S.; Padma, P. R. Antioxidant activity and total phenolic content of *Moringa oleifera* leaves in two stages of maturity. Plant Foods for Human Nutrition 2009, 64, 303-311.