

Full Length Research Paper

***In vitro* anti-HIV and immunomodulatory potentials of *Azadirachta indica* (Meliaceae) leaf extract**

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***Azadirachta indica* (neem) is attributed to have many ethnopharmacological properties and has been postulated to have a significant effect on CD4 counts in HIV/AIDS subjects, however, the precise anti-retroviral mechanism is not yet known. In this study, a hydroacetone extract of *A. indica* leaves was investigated for its anti-retroviral and immunomodulatory potentials. The effect of the neem leaf extract on acute HIV infectivity and fusion was measured by the syncytia formation assay on HIV-1-infected C8166 CD4+ cells. The inhibitory effect of the extract on reverse transcriptase (RT) polymerase activity and HIV p24 antigen expression were evaluated in the culture supernatant. The extract was also assessed for its ability to decrease the phenotypic expression of the immune activation markers CD38 and CD69. Results from a syncytium formation assay indicated that the extract blocked HIV-1 envelope-mediated membrane fusion. The extract inhibited HIV-1 replication in C8166 CD4+ cells *in vitro* by inhibiting the biochemical activity of HIV-1 reverse transcriptase, the result being a subsequent decrease in HIV p24 antigen concentration. In the effective dose range, no cytotoxicity was detected on uninfected target cells. *Ex vivo* the extract exhibited a dose dependent reduction in the levels of the immune activation marker CD38 and CD69 on phytohemagglutinin A (PHA)-stimulated human peripheral blood mononuclear cells (PBMC). The observed anti-HIV activity and immunomodulatory potentials of the extract shows that neem could impart health benefits to HIV/AIDS patients possibly by acting as fusion or reverse transcriptase inhibitors and also down-regulate hyperimmune activation.**

Key words: Anti-HIV activity, immunomodulatory activity, *Azadirachta indica*.

INTRODUCTION

Infection by the human immunodeficiency virus type 1 (HIV-1), the etiological agent to the acquired immunodeficiency syndrome (AIDS), is a global health problem affecting more than 33.4 million people worldwide (WHO/UNAIDS, 2009). HIV-1 infects and replicates in CD4+ T lymphocytes and monocytes/macrophages using the CD4 receptor and the chemokine co-receptors CCR5 or CXCR4 to enter the target cell (Berger et al., 1999). HIV-1 persistently replicates in the lymphoid tissues, leading to a progressive deterioration of the immune system, resulting in AIDS. Treatment with highly active antiretroviral therapy (HAART) promotes a sustained decrease in viral load and a restoration of the immune response, and has reduced morbidity and mortality

of HIV-1 infection (Pallela et al., 1998). However, this treatment does not completely eradicate HIV-1 from the infected tissues (Blankson et al., 2002), and its long-term use is restricted by metabolic disorders and toxicities and emergence of drug-resistance viruses (Richman, 2001). Thus, the search for other antiretroviral agents, especially from natural sources is critical. Hyperimmune activation is one of the hallmarks in the pathogenesis of HIV/AIDS. It is thought to play a critical role in HIV disease progression and CD4+ T cell loss possibly through the induction of energy, activation-induced cell death (AICD) and apoptosis of bystander, non-infected cells (Gougeon et al., 1996; Bentwich et al., 1998).

Drivers of immune activation in chronic infection are not completely understood, but are likely influenced by a combination of host and viral factors. Increased levels of lipopolysaccharide (LPS) due to microbial translocation from the gut (Brenchley et al., 2006), soluble HIV envelope proteins, the high magnitude of host immune

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responses, aberrant innate signaling, and opportunistic infections (Sodora and Silvestri, 2008) could contribute to hyperimmune activation. Drugs that can modulate hyperimmune stimulation could possibly be beneficial to HIV infected individuals. Plant products have attracted attention as possible anti-HIV drugs targeted on the different steps of the viral life cycle, such as viral attachment and entry (Luscher-Mattli, 2000; O'Keefe, 2001) and on essential enzymes that play a role during viral replication (Lednicher and Sander, 1991; Buckheit et al., 2000; Asres et al., 2001; Yu et al., 2005). Parts of the *A. indica* (neem) tree, which grows well in tropical and subtropical areas around the world have been used over the years for medicinal purposes (Verkerk and Wright 1993). *A. indica* varieties are used for the treatment of some pathological conditions related to oxidative disorders, such as inflammation and skin diseases (Clayton et al., 1996), rheumatic, arthritic disorders and treatment of fever and diabetes (Van der Nat et al., 1991). An aqueous extract of *A. indica* leaves has been reported to inhibit Dengue virus type-2 both *in vitro* and *in vivo* (Parida et al., 2002). Different parts of the neem (*A. indica*) tree are widely used traditionally throughout India, Africa and Nigeria as medicine as anti-malarial (Udeinya et al., 1993), anti-fungal (Fabry et al., 1996), anti-bacterial (Vanka et al., 2001). Clinical trials have shown that fractionated acetone-water neem leaves extract inhibited the invasion of human lymphocytes by HIV-1 *in vitro*, and caused significant improvement in CD4+ cell count in a small number of HIV/AIDS patients (Udeinya et al., 2004; Mbah et al., 2007), however, the antiretroviral activity associated with this reports is not yet known. In this study, we therefore, independently investigated the antiretroviral potential of an acetone extract of *A. indica* together with its ability to modulate hyperimmune activation in human peripheral blood mononuclear cells.

MATERIALS AND METHODS

Chemicals

The solvents acetone was purchased from EMD Biosciences (Gibbstown, NJ). Trypan blue was purchased from Fluka Chemicals, dimethyl sulfoxide (DMSO), the nucleoside reverse transcriptase inhibitor 3'-azido-3'-deoxythymidine (AZT) were all purchased from Sigma Chemical Co. (St. Louis, MO). Filtration was done with Whatman No. 1 filter paper. Phycoerythrin (PE)-conjugated annexin V, 7-amino-actinomycin (7-AAD) and the monoclonal antibodies fluorescein isothiocyanate (FITC)-conjugated anti-CD69 and phycoerythrin (PE)-conjugated anti-CD38 were obtained from BD Biosciences (Mississauga, ON). Phytohemagglutinin A (PHA) and the cell proliferation reagent WST-1 was obtained from Roche Applied Science (Laval, QC).

Crude extract preparation

The leaves of *A. indica* obtained in January 2007 from the University of Nigeria, Nsukka campus, were identified at the Herbarium, University of Nigeria, Nsukka, air-dried at room

temperature and reduced to fine powder by milling. The resulting powder was subjected to extraction with 50% acetone. The hydroacetone extract was concentrated using a rotary evaporator and further dried under reduced pressure. For the tissue culture assays, the extract was dissolved in DMSO, diluted further with RPMI 1640 complete medium (supplemented with 10 % fetal calf serum (FCS), and 1% penicillin/streptomycin) (Gibco), and filter sterilized with a 0.20µ nalgene syringe filter.

Cells and virus

Peripheral blood mononuclear cells (PBMCs) from healthy human donors were obtained by density gradient centrifugation (Hystopaque, Sigma Chem. Co, St. Louis, MO) from buffy coat preparations. Cells were suspended in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone, Logan, UT), and 1% penicillin/streptomycin (Gibco). C8166 CD4+ T-cell and HIV-Bru (N+/E+/GFP) were maintained in the supplemented RPMI 1640 medium in a humidified incubator under 5% CO₂ at 37°C. For the tissue culture, the neem extract was dissolved in DMSO, diluted further with RPMI 1640 complete medium (supplemented with 10% fetal calf serum (FCS), and 1% penicillin/streptomycin) (Gibco), and filter sterilized with a 0.20 µ nalgene syringe filter. The concentration of DMSO used was between 0.05 and 1.0% and the DMSO did not influence the cell viability.

Cell viability assays

Flow cytometry studies by Annexin V/7-AAD staining were performed to investigate the cytotoxic/apoptotic effect of the extract. Fluorescence associated cell sorting analysis was performed to discriminate between intact and apoptotic/necrotic cells as described by Awah et al. (2010). Staining with PE-labeled Annexin V (BD), which binds to membrane phosphatidylserine and 7-amino actinomycin (7-AAD) (BD), which binds to cellular nucleic acid was performed as per the manufacturer's protocol (BD Biosciences). Briefly, freshly isolated PBMC (1 × 10⁶ cells/ml) treated with different concentrations of the neem extract for 7 days were washed and re-suspended in annexin binding buffer. The cells were then incubated with 5 µl of PE-conjugated Annexin V and 5 µl 7-AAD for 30 min in the dark and 10,000 events acquired by FACS Calibur flow cytometry. Cell Quest software (BD Biosciences) was used to analyze the acquired data. Viable cells were Annexin V⁻/7-AAD⁻, early apoptotic cells were Annexin V⁺/7-AAD⁻ while apoptotic/necrotic cells were Annexin V⁺/7-AAD⁺. Cell viability was further assessed by light microscopic analysis with the trypan blue exclusion test, based on the principle that live cells possess intact cell membranes that exclude the dye, whereas dead cells do not. Viable cells had a clear cytoplasm whereas nonviable cells had a blue cytoplasm. The percentage of viable cells was calculated by the formula:

$$\% \text{ Cell viability} = \left[\frac{\text{No. of dye negative cells}}{(\text{No. of dye positive cell} + \text{No. of dye negative cells})} \right] \times 100 \%$$

WST-1-based cytotoxicity assay

The assay is based on the reduction of WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzene disulfonate) by mitochondrial dehydrogenases in viable cells to formazan dye. Briefly, 200 µl of A2.01 cells and freshly isolated PBMC were independently seeded in 96-well, flat bottom microplates at a concentration of 5 × 10⁴ and 5 × 10⁵ cells/ml respectively. The cells

were allowed to stabilize for 24 h and were then treated with different concentrations of the plant extracts prepared in complete medium and cultured for 96 h in a humidified atmosphere of 37°C, 5% CO₂. Thereafter, 20 µl of cell proliferation reagent WST-1 was added with further incubation for 4 h at 37°C. The microplates were then shaken thoroughly for 1 min and the optical density was measured in an ELISA plate reader (Molecular Devices, Spectra Max 190 with soft max pro software) at 450 and 690 nm.

Syncytium reduction assay

The effect of the neem leaf extracts on acute HIV infectivity was measured by the syncytia formation assay as previously described by Yao et al. (1991) with slight modifications. In the presence of 100 µl of various concentrations of extracts (50 and 100 µg/ml), C8166 cells (1×10^5 ml⁻¹) were infected with HIV-Bru (N+/E+/GFP) in a 96 well ELISA plate (TCID₅₀ = 10 µl). The final volume per well was 400 µl in RPMI medium. AZT was used as a control drug. On the second day of the culture, 400 µl of media was discarded, 900 µl of media and the same concentration of extract maintained. After 5 days of culture, the cytopathic effect (CPE) was measured by counting the number of syncytia (multinucleated giant cell) in each well under an inverted microscope. Numbers of syncytia were counted using standard syncytia counting methods.

ELISA for HIV-1 p24 antigen

The effect of the neem leaf extract on HIV-1 replication was assayed by measuring p24 expression in HIV-1-infected C8166 cells as described by Yao et al. (1991) with slight modifications. C8166 cells were inoculated with viral stock at a multiplicity of infection of 5×10^{-3} and incubated at 37°C in 5% CO₂ for 1 h to allow viral absorption. Unbound virus was removed by washing and cells were plated at 1×10^5 cells/ml in the absence or presence of different concentration of extracts, harvested on Day 5 post-infection and cell-free supernatants were collected for the determination viral production levels by measurement of HIV-1 p24 antigen in each infected culture supernatant using HIV-1 p24 capture ELISA Kit (AIDS Vaccine Program of the Frederick Cancer Research and Development Center). The culture medium was incubated in microtiter wells pre-coated with anti-p24 antibody and assayed for p24 antigen by biotin-labeled anti-p24 antibody followed by streptavidin-peroxidase conjugate. The amount of captured p24 was determined by measuring the absorbance at 460 nm of tetramethylbenzidine substrate. Samples containing known amounts of p24 were used as standards.

Anti-HIV-1 RT inhibitory activity

The inhibitory effect of neem leaf extract on RT polymerase activity was evaluated in the culture supernatant using recombinant HIV-1 RT enzymes, as reported by Da Matta et al., (1999) with slight modification. Briefly, after 5 days of culture, the culture supernatants were ultracentrifuged for 5 min at 360,000 × g. The viral pellets were then lysed in 20 µl of NTE (10 mM NaCl, 10 mM Tris (pH 7.8), 1 mM EDTA) containing 0.1% Triton X-100. Thereafter, 10 µl of the lysate was added to the polymerization reactions mixture containing 50 mM Tris HCl (pH 7.8), 6 mM MgCl₂, 1 mM dithiothreitol, 50 mM KCl, 20 µM dTTP, 10 µM of [³H]dTTP (47 Ci/mmol) and 150 µg/ml poly(rA) oligo(dT) template primer. One (1) U of enzyme was defined as the amount of enzyme that incorporates 1 pmol of dTTP in 30 min at 37°C under standard assay conditions. The reaction mixture was incubated at 37°C for 30 min, and stopped by adding ice cold 5% trichloroacetic acid containing 20 mM of sodium pyrophosphate. The precipitates were

collected on nitrocellulose filters, washed with sodium phosphate 0.1 M. Filters were dried for 20 min at 80°C. RT activity was quantified by measuring the level of incorporated [³H] TTP using a liquid scintillation counter (results are expressed as counts per minute per 50 µl of lysate). The RT inhibitor AZT was used as a negative control.

Cell stimulation and detection of immune activation

The ability of neem extract to inhibit the hyper-activation of lymphocytes was assessed by expression of immune activation markers CD38 and CD69 as reported by Awah et al. (2010). Briefly, freshly isolated PBMC were suspended at a concentration of 1.0×10^6 cells/ml of RPMI growth media. The cells were left unstimulated as negative control or stimulated with PHA (5 µg/ml) and incubated for 36 h in 5% CO₂ at 37°C with or without the neem extract. PHA (5 µg/ml) gave substantial stimulation after 36 h, and this time point was used throughout. After stimulation with PHA, the lymphocytes were washed twice and suspended in PBS with 2% FCS and co-stained with FITC-conjugated anti-CD69 and PE-conjugated anti-CD38. The cells were incubated for 30 min at 4°C, washed, resuspended in PBS with 2% FCS, further fixed with 1% paraformaldehyde, and analyzed by FACS Calibur flow cytometry.

RESULTS

Cell viability and cytotoxic effect of plant extract

The safety of the extract upon use in human cells was investigated by treating human peripheral blood mononuclear cells (PBMCs) with different concentrations of the extract. Cell viability was evaluated by trypan blue dye exclusion assay. After 7 days of continuous treatment, there was no significant decrease ($p > 0.05$) in the viability and membrane integrity of PBMCs compared to the control untreated cells. Viability was higher than 90% for concentrations up to 100 µg/ml, but viability declined at higher concentration (Figure 1a and 1b).

Effect of neem extract on syncytia formation

The ability of the extract to block HIV-1 entry was investigated. *A. indica* mediated a substantial anti-HIV-1 effect in infected C8166 T cells. The extract inhibited acute HIV-1 infection and cell-to-cell transmission, as shown in the syncytia formation assay. A dose-dependent inhibition of syncytia formation was observed, with the best activity at a concentration of 100 µg/ml (Figure 2).

Effect of neem extract on HIV-1 reverse transcriptase activity

The crude extract of *A. indica* exhibited strong HIV-1 RT inhibitory activity. At concentration of 50 and 100 µg/ml of the extract significantly reduced ($p < 0.05$) the polymerase activity of the recombinant HIV-1 RT with the peak inhibition of 92.4% at 100 µg/ml. Zidovudine (AZT), a

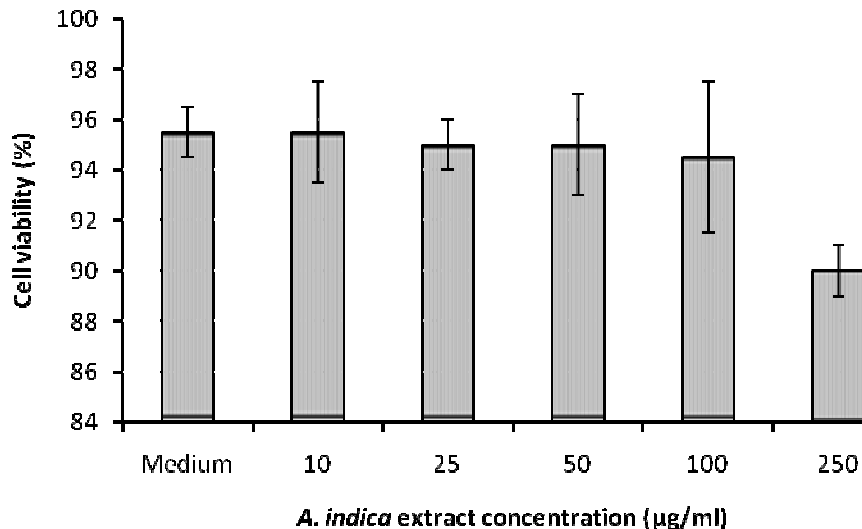


Figure 1a. Trypan blue exclusion viability (%) of PBMC after 7 days of incubation with different concentrations of extracts. Data represented as mean ± SEM.

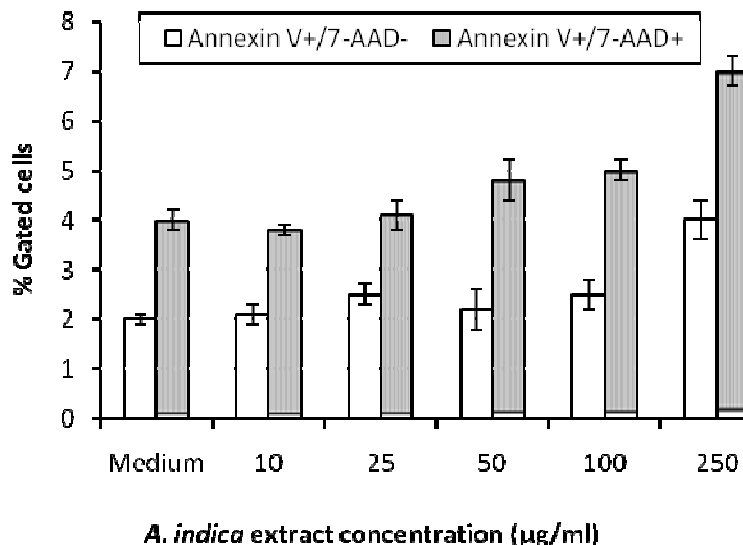


Figure 1b. Cytotoxic effect of the extract on PBMCs as shown by Annexin V/7-AAD staining. Data represented as mean ± SEM.

nucleoside analogue inhibitor of the HIV-1 enzyme reverse transcriptase, blocked viral replication as expected (Figure 3).

Effect of neem extract on HIV-1 p24 antigen expression

The extract significantly inhibited ($p < 0.05$) HIV-1 replication in C8166 cell line infected with HIV-Bru (N+/E+/GFP). In the presence of AZT and 50 and 100

µg/ml of the neem extract, there were no detectable levels of p24 antigen in the culture supernatant (Figure 4).

Inhibition of Phytohaemagglutinin A (PHA)-induced activation

Flow cytometry was used to investigate the effect of the neem extracts on PHA-induced hyperimmune activation in PBMCs. The extracts significantly ($p < 0.05$) reduced the level of expression of the immune activation markers

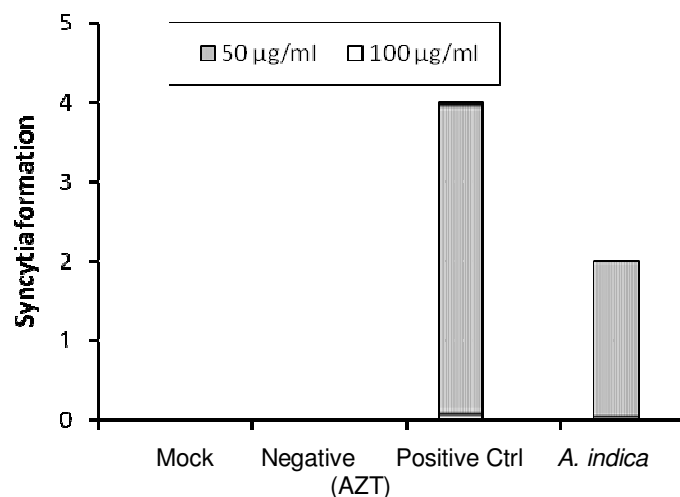


Figure 2. Effects of neem extract on envelope-mediated fusion. Various concentrations of extract were added to the fusion assay and formation of syncytia was measured after 5 days of culturing C8166 CD4+ cells with HIV-1.

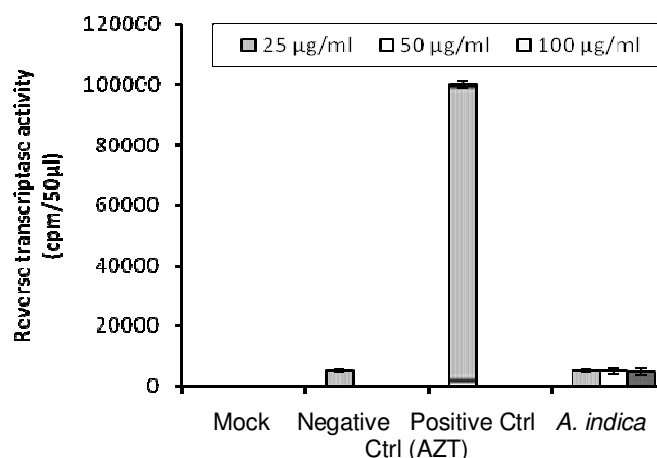


Figure 3. Effect the extract on HIV-1 RT activity. Recombinant HIV-1 RT was incubated with the indicated concentrations of the extract or AZT and the enzyme polymerase activity was evaluated as described in Materials and methods. Data represent means \pm SEM of two independent experiments. AZT was used as a positive control for RT inhibition at 1 μ M.

CD38 and CD69 in PHA-activated lymphocytes, thereby suggesting suppression of hyperimmune activation of human lymphocytes (Figure 5).

DISCUSSION

Over the years, parts of the *Azadirachta indica* tree have been used for medicine including claims of its anti-

retroviral potential. Anti-HIV agent which could possibly inhibit the early stages of the HIV replicative cycle would be very useful in treating HIV-infection. Our results demonstrate that compared to the standard anti-HIV drug AZT, a hydroacetone extract of *A. indica* significantly ($p < 0.05$) inhibited acute HIV-1 infection and cell-to-cell transmission, as assayed by syncytia formation (Figure 2). At 100 μ g/ml the extract completely inhibited syncytium formation but the anti-fusion activity of the extract was slightly lower when 50 μ g/ml of extract were used (Figure 2). Formation of syncytium is mediated by interaction of viral glycoprotein gp120 with specific regions of the host cell CD4 receptor (Tang and Levy, 1990). Neem extract almost completely inhibited the fusion interaction between normal C8166 and HIV-1 chronically infected C8166 cells in tissue culture. The active principles in the extract possibly inhibited syncytia formation by binding to the viral gp120, thereby blocking the gp120-CD4 interaction, thereby inhibiting not only HIV binding to the CD4+ cells but also preventing HIV-induced syncytium (giant cell) formation. The anti-HIV effect of neem extract could not have been due to the toxic effect of the extracts on the C8166 cell line since cell viability studies showed that the tested concentrations were relatively non-toxic to the cells in tissue culture (Figure 1a and 1b).

Furthermore, the effect of the extract on HIV-1 replication in C8166 CD4+ cells was investigated *in vitro* by determining HIV-1 reverse transcriptase (RT) activity and p24 antigen expression levels. The extract significantly inhibited ($p < 0.05$) the biochemical activity of HIV reverse transcriptase in the culture supernatant (Figure 3). The potency was not significantly different ($p > 0.05$) from that of the standard drug AZT. Since, the p24 antigen is one of the major capsid proteins expressed by matured HIV, decreased concentrations of the antigen suggests low levels of HIV particles and hence, anti-retroviral activity. The level of expression of the p24 antigen was also significantly decreased ($p < 0.05$) in the culture supernatant of cells treated with neem extract as shown in Figure 4. The reverse transcriptase inhibitory activities of the active principles present in the extract possible contributed to the decrease in HIV p24 antigen concentration. The inhibitory potential of the extract on HIV RT activity and p24 antigen expression were apparently not significantly different ($p > 0.05$) from that of AZT the standard anti-HIV drug, suggesting a very potent anti-HIV activity of the plant extract. The antiretroviral mechanism of this extract is however, not completely understood. The extract completely inhibited syncytia formation and the biochemical activity of HIV reverse transcriptase, resulting in a decreased p24 antigen expression with its activity not significantly different ($p > 0.05$) from that of AZT the standard anti-HIV drug. These inhibitory effects of the plant extracts were all dose-dependent and no cytotoxicity on uninfected target cells was detected over a 1,000-fold concentration from 0.1 to 100 μ g/ml of the extracts (Figure 1a and 1b).

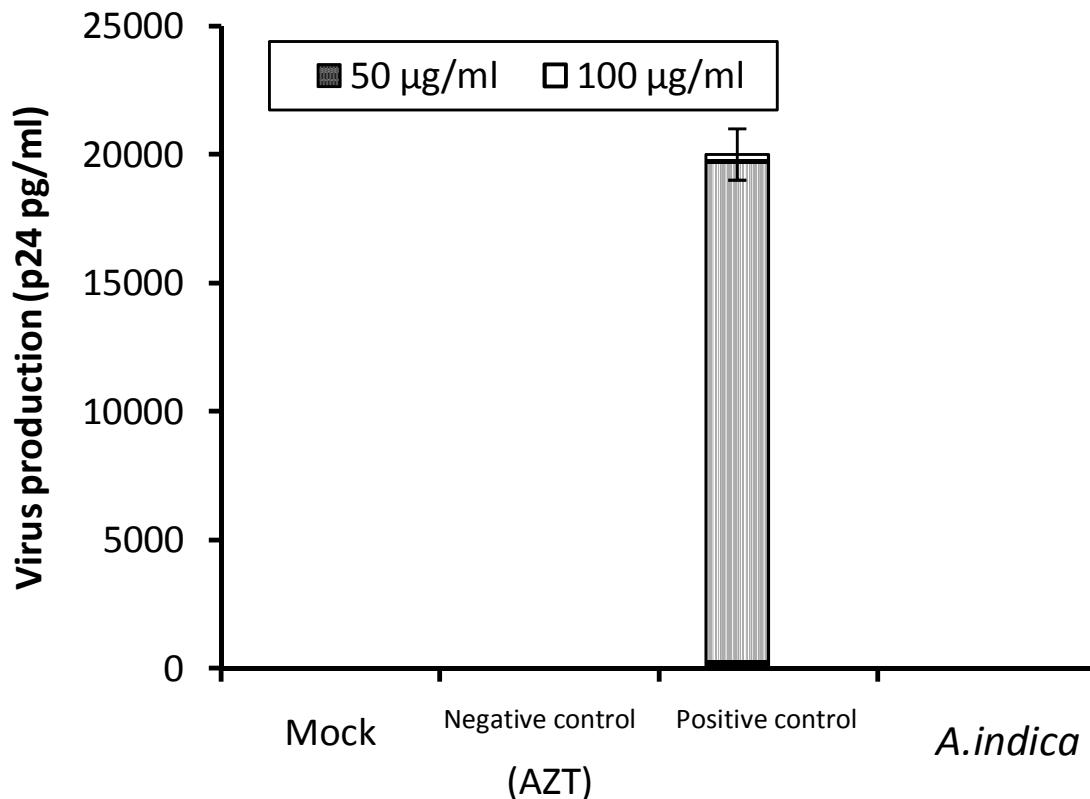


Figure 4. Effect of extract on supernatant p24 antigen expression. C8166 cells were infected with X4-tropic HIV-1 and exposed to neem extract and AZT, the viral replication was measured in the culture supernatants. Supernatant p24 values are the mean of two independent determinations; error bars represent the standard error of the mean.

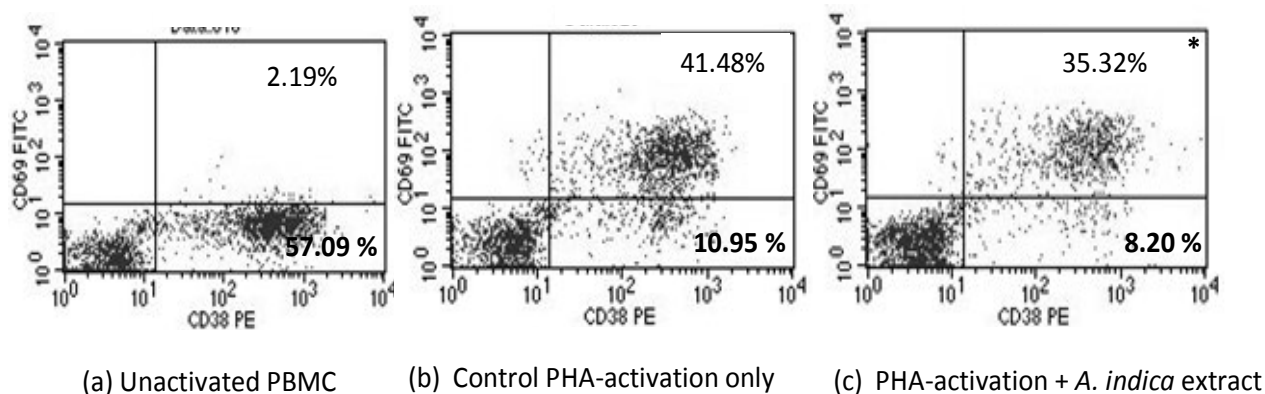


Figure 5. Influence of extract on the phenotypic expression of immune activation markers (CD38 and CD69) on PHA-stimulated lymphocytes. (a) Unactivated cells (b) PHA activation only (c) PHA activation (5 μ g/ml) in the presence of 10 μ g/ml *A.indica* extract. The result is a representative of three independent experiments. * $p < 0.05$, statistical significance of difference from control.

Mbah et al. (2007) postulated that neem extract was beneficial to HIV/AIDS patients though the mechanism of action was not known. This result therefore suggests a possible anti-retroviral mechanism of action of the active

principle in neem extract using well-established bioassays. The extract also significantly reduced ($p < 0.05$) the level of expression of the hyperimmune activation markers CD38 and CD69 in PHA-activated lymphocytes (Figure 5), thereby

indicating inhibitory effects. Prior to stimulation with PHA, only about 2.19% of the PBMCs expressed CD69 but after stimulation 41.18% of the PBMC expressed CD69 in the absence of the extract and in the presence of the extract only 35.32% of the PBMC expressed CD69 (Figure 5). The capability of the neem extract to down-regulate the expression of these immune activation markers suggests immunomodulatory potential of the extract. The mechanism of action of the inhibitory effect of the extract is however not known. Neem extract may be blocking the PHA binding site or possibly inhibiting enzymes involved in hyperimmune activation such as the mitogen activated proteins kinases. The immunomodulatory potential observed in this study is consonance with previous reports on the immunomodulatory potentials of neem extract (Upadhyay et al., 1993). These findings are of particular interest given that neem appears to have anti-retroviral and immunomodulatory activities, raising the possibility of the extract modulating the hyperimmune activation observed in HIV/AIDS as well as reducing the viral load. Our present results warrant further investigation to ascertain the mechanism by which *A. indica* extract decreases HIV-1 replication *in vitro* and *in vivo*. Furthermore, studies need to be conducted in order to identify the active compound responsible for the anti-HIV activity.

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