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Preliminary *in vitro* inhibition of HIV-1 replication by alpha-zam, a *nigella sativa* seed formulation

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Abstract

Background: The unavailability of the highly active antiretroviral therapy (HAART) to the teeming population (about 2.4 million out of 5 million infected persons) living with human immunodeficiency virus type 1 (HIV-1) in the Western and Central Africa means that they have to seek an alternative treatment option. Therefore, traditional herbal medicine seems to be a common option. This study was aimed at investigating the efficacy of such a remedy by determining its anti-retroviral effect on HIV-1 using *in vitro* infection system.

Methods: Alpha-zam (a-zam) was examined for its anti-HIV-1 activity and cytotoxicity in acute and chronically infected cells. The anti-HIV-1 activity was determined by the inhibition of virus-induced cytopathogenicityin acutely infected MT-4/MOLT-4 cells using the MTT assay, while this was by the inhibition of p24 antigen production in chronically infected OM-10.1/U1 cells using quantitative ELISA. Also the cytotoxicity of a-zam was determined by the MTT method.

Results: A-zam did not show anti-HIV-1 activity in acutely infected cell culture.The cells displayed definitive cytopathic effect and only 23.9% - 24.9% survived at 250 to 6250 fold dilutions of the herbal formulation. Interestingly, a-zams electively inhibited the p24 antigen production in OM-10.1 cell after stimulation with tumor necrosis factor alpha (TNFα). The highest inhibition (84.6%) was achieved at the 100-fold dilution. The results appear connected with those in previous human study, where significant decrease inplasma viral load was observed in patients.

Conclusion: A-zam has a potential for anti-HIV-1 activity in chronically infected cells and may be a good candidate for treating chronic HIV-1 infections.

Keywords: *Poor antiretroviral therapy coverage, Nigella sativa, Anti-HIV-1 activity, Chronic HIV-1 infection, Western and Central Africa, Alpha-zam*

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Introduction

The HIV pandemic is currently the most socio-economic challenge that has negatively impacted populations and human capital development in many developing countries [1]. This has posed a hindrance to economic growth and severely affected development prospects; perpetuated poverty, proliferated orphanages and threatened the security and existence of many populations [1-2]. Also there has been a lowering of the life expectancy value, dramatic alteration in the age-sex structure and excessive demand on the scarce health-care facilities in many of the affected countries [2]. The AIDS prevalence has grown steadily since the beginning of the epidemic, with a total record of 74.9 million (58.3 million-98.1million) infections and 32 million (23.6 million-43.8 million) death associated with HIV/AIDS-related illnesses at the end of 2018 [3]. The advent of antiretroviral therapy (ART) in 1987 is a major factor in lowering the mortality and prevalence of HIV infections, which was observed in the global statistics of 1.7 million new infections and 770,000 deaths in 2018 [3]. Adherence to ART regimen slows down the progression of the disease, decreases the risk of death and improves the quality of life of people living with HIV (PLWHIV).

Also there is a reduced risk of horizontal and vertical transmission to sexual partners and unborn child [4]. The initiation of the first global ART target (2003) of treating 3 million people by 2005 resulted into a 20% increase in ART coverage in low and middle income countries. This was particularly dramatic in the sub-Saharan Africa (SSA) where the number of people on treatment went up from 100,000 to 810,000 at the end of 2005 [5]. Thereafter the scaling up between 2010 and 2018 resulted into 23.3 million people on treatment and a reduction in new infections and deaths from 2.1 million to 1.7 million and 770,000 from 1.2 million respectively [3]. While previous targets were for increased ART uptake and accessibility, the post 2015 declaration (90-90-90) sought to end the HIV/AIDS epidemic through improved knowledge of HIV status, increased uninterrupted drug utilization and viral suppression [6]. Despite these concerted efforts by the United Nations (UN) and World Health Organization (WHO), some regions are still lacking adequate coverage. This is true of the West and Central Africa (WCA) region, where about 280,000 new infections and 160,000 AIDS-related deaths occurred in 2018 [7].

Although there has been an increase in the number of people on treatment (a rise from 860,000 in 2010 to about 2.6 million in 2018); the overall population coverage (53%) in the region is still very low [7]. At the end of 2018, only 14 WCA countries (Benin, Burkina Faso, Burundi, Cameroun, Cape Verde, Chad, Cote d'Ivoire, Democratic Republic of the Congo, Gabon, Niger, Nigeria, Sao Tome and Principe, Senegal, Togo) have ART coverage above 50% (51%-89%), while the remaining 11 have estimates ranging from 19% to 35% [8].The low ART coverage alongside poor economic growth prevented patient's access to other HIV/AIDS support programs available in other regions, hence the region is hardest hit by AIDS associated deaths (about 20% of annual global mortality) and rapid spread of the disease [7]. Several factors, such as the lack of will power for innovative methods in treatment delivery, poor monitoring of drug supply, financial barriers, reduced international funding, weak health systems, national/ political conflicts and competing health priorities are barriers militating against the scaling up of access to treatment [9]. This insufficient provision of ART implies that a greater proportion of PLWHIV (47% of 5 million) in the WCA will seek treatment from other sources including traditional medicine [7].

The traditional medicine practitioners are respected in communities and are engaged in the provision of personalized, holistic and culturally acceptable treatment to people living with HIV/AIDS [10]. As a result, the use of traditional medicine by PLWHIV for the management of opportunistic infections, boosting of the immune system and at times concurrent use with ART have been documented from several countries [11-17]. Also, patient's knowledge about the non-curative nature of HIV infection and the need for total adherence and dependence on ART throughout their lifetime impacted on patient's desire to seek other non-conventional treatment. Therefore, it becomes important that such non-conventional medicines are evaluated to generate evidence on effectiveness for HIV treatment. The use of herbal remedies for HIV/ AIDS is a common practice in Nigeria and in many cases these are continued after ART initiation, purposely as complementary therapy [18-19]. Many of the documented herbal remedies act on opportunistic infections, boost immunity, improves energy status of patients while *neem* leaf in particular increased CD4 count and general well-being significantly in HIV patients [19-20]. An earlier study of a-zam found that it poses no risk to user, when used alone or in combination with ART. The study showed that it significantly reduced the viral load (HIV-RNA), and increased the CD4 count of patients

[21]. Also, sero-reversion was observed in a 46 year old patient following a 6 month treatment course with a-zam [22]. In addition, a pregnant HIV patient who sero-reversed at 1 year of treatment had 4 HIV negative children and had maintained the seronegative status for 9 years when last tested [23]. These experiences therefore prompted the need to investigate and demonstrate the effectiveness of a-zam for the treatment of HIV-1 infection through an *in vitro* study.

Methods

Ethical Approval

The approval for this study (UI/EC/14/0277) was obtained from the University of Ibadan/University College Hospital Ethics Committee.

Sample Preparation: The herbal remedy, a *Nigella sativa* seed formulation was prepared for analysis by diluting in warm water as described previously by Onifade*et al*. 2011 [21]. The suspension was then filtered with 0.22µm Millipore filter attached to vacuum pump, to remove microbiological contaminants and other extraneous matter.

*Sample Screening for Anti-retroviral Compounds***:** To be able to ascertain that any anti-retroviral effect observed in the study is produced by the herbal formulation, a sample was submitted to the National Agency for Food and Drug Administration and Control (NAFDAC)Yaba, Lagos for analysis.

Cell viability and cytotoxicity assay: A colorimetric test utilizing MTT (3-(4, 5-dimethythiazol-2-yl)-2-5-diphenyltetrazolium bromide) was used to assess cytotoxicity of the formulation on cultured cells. The MTT assay is particularly useful for drug screening and it is based on the activity of mitochondrial dehydrogenase in living cells to reduce yellow tetrazolium salt to insoluble purple formazan which is then measured by light absorbance at specific wavelength. The method previously described by Pauwels *et al*. (1988) was modified for the assay [24]. Briefly, the test agent (a-zam) was diluted with RPMI 1640 (medium developed at the Roswell Park Memorial Institute for culturing mammalian cells in suspension and monolayer) in triplicates using two-fold method in 96-well plates. One hundred microliters (100µl) of the cell ($1x10^6$cells/mL) was added to each well and plates incubated at 37^0C for 4 days. Plates were observed daily, and cell viability assessed on day 4 by the MTT method. The formazan crystals were dissolved in isopropanol prior to reading at 570/690nm in a multi-well spectrophotometer. The experiments were done 3 times.

*Anti-HIV-1 assay in acutely infected cells***:** The antiretroviral activity of a-zam against acute HIV-1 infection was based on the inhibition of virus-induced cytopathic effect (CPE) as described previously by Baba *et al*. 1991 [25]. Briefly, one hundred microliters of various dilutions of a-zam was added to each well of a 96 well plate and thereafter 100 µl of HIV-1 (III_B strain) infected and mock-infected MT-4 and MOLT-4 cells (1×10^5 cells/mL) were added. For the infected cells, a multiplicity of infection (MOI) of 0.1 was applied and cells were incubated for 4 days at 37˚C. While MT-4 cell was examined for viability by the MTT method at the end of incubation, the MOLT-4 cell was sub-cultured on day 4 at a ratio of 1:5 with fresh culture medium containing appropriate concentrations of the test agent. The cell viability test was assessed on day 8, because the initial incubation period (4 days) was not sufficient for HIV-1 replication. The antiretroviral activity of a-zam was evaluated based on the viability of mock-infected cells, as determined by the MTT method. The experiments done in triplicate were repeated three times for accuracy and reproducibility.

*Anti-HIV-1 assay in chronically infected cells***:** The anti-HIV-1 activity in chronic infection was based on inhibition of p24 antigen production in OM-10.1 and U1 cells as described previously [26]. Briefly, one hundred microliter (100µl) of the latent HIV-1-infected cells OM-10.1 and U1 (1×10^5cells/mL) was cultured in 96-well plates in the presence of varying dilutions of a-zam. After incubation for 2 h at 37°C, 10 µl of 0.1ng/mL of tumor necrosis factor-α (TNF-α) was added to each well and plates were further incubated for 3 days. After incubation, culture supernatants were collected and stored at -20˚C until analyzed for p24 antigen levels. The p24 antigen level in culture supernatant was measured using a quantitative enzyme-linked immunosorbent assay (ELISA) kit (Zepto-Metrix Corporation, Buffalo NY 14202, USA). The assay was conducted according to the manufacturer's instructions. The MTT assay was also performed to determine cell viability. All experiments were carried out in triplicate and repeated three times for determination of anti-HIV-1 activity.

Results

Sample Screening for Anti-Retroviral Compounds: The result of the analysis done by NAFDAC on the herbal sample revealed that Lamivudine, Zidovudine, Abacavir and Nevirapine were not present in the formulation.

Antiviral activity in acute infection

A-zam was tested for its inhibitory effect on the replication of HIV-1 (IIIB strain) in acutely infected cells. The formulation did not show the capacity to inhibit HIV-1 replication in the acutely infected cells (MT-4 and MOLT-4). The infected MT-4 cell showed the characteristic virus-induced cytopathogenicity at low concentrations (6250x-250x), with only 23.2% to 24.9% cell survival. However, at high concentrations (10x-50x) both infected and mock-infected cells died due to cytotoxicity (Fig. 1). Similar result was obtained in infected MOLT-4 cell except for a slight increase in cell viability at 200-fold dilution, however this was found to be toxic to mock-infected cell which has only 24% cell survival compared to the 72% to 100% survival at other dilutions (Fig. 2).

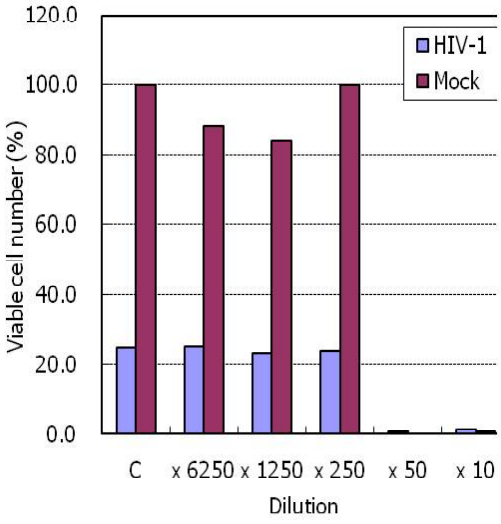

Fig.1: Anti-HIV-1 activity of A-zam in acutely infected MT-4 cells. Thecells were infected or mock-infected with HIV-1 (III_B strain) at a MOI of 0.1 and incubated in the presence of various dilution of A-zam. After 4 days, the number of viable cells was determined by the MTT method.

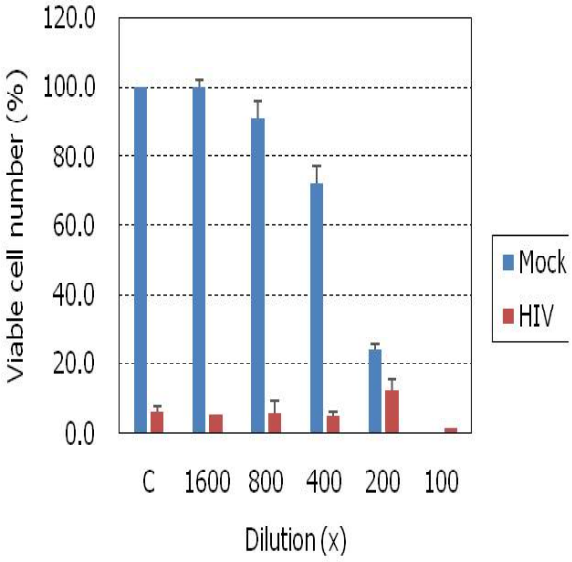

Fig.2. Anti-HIV-1 activity of A-zam in acutely infected MOLT-4 cells. The cells were infected with HIV-1 (III_B strain) at a MOI of 0.1 and incubated in the presence of various dilutions of A-zam. After 4 days, the cells were subcultured at a ratio of 1:5 and further incubated in the presence of its appropriate concentrations. After 4 days (on day 8), the number of viable cells was determined by the MTT method.

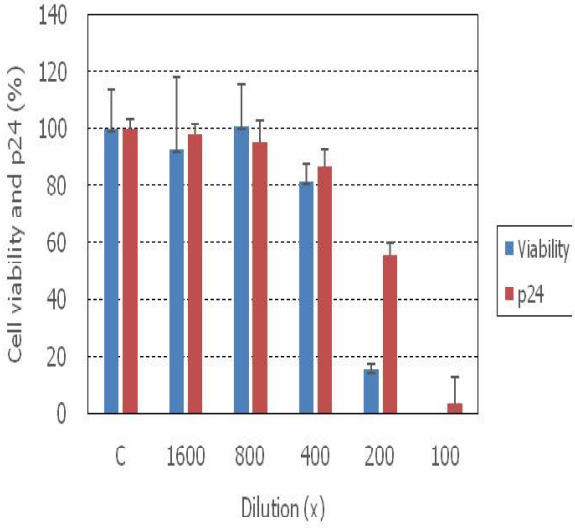

Fig.3: Anti-HIV-1 activity of A-zam in U1 cells. The cells were incubated in the presence of various dilutions of A-zam for 2 h. The cells were stimulated with 0.1 ng/mL TNF-α and further incubated. After 3 days, the number of viable cells and p24 antigen levels ofculture supernatants were determined by the MTT and p24 ELISA methods, respectively.

Antiviral activity in chronic infection

Both U1 and OM-10.1 cells were latently infected with HIV-1, producing little amount of viral particles and antigens under basal culture conditions. However, there was a rapid increase in virus replication following stimulation with TNFα. At low concentrations (1600 to 400-fold dilutions), a-zam did not affect HIV-1 production as well as cell viability in U1 cells (Fig. 3). However, there was a slight reduction in the p24 antigen at 200-fold

dilution (56% compared to 86.8% to 100% at higher dilutions) but this occurred at lower cell viability of 15.5%. This showed that a-zam is not a selective inhibitor of HIV-1 replication. The observation in OM-10.1 cell was quite different and a-zam was found to be non-cytotoxic (100% cell survival) at higher dilutions (200x-1600x), but showed moderate cytotoxicity (45.3% cell survival) at 100-fold (Fig.4). Interestingly, a dose-dependent reduction of p24 antigen was observed at dilutions 100-400 fold and a reduction of 84.6% was achieved at 100 fold. This result suggested that there was a selective inhibition of HIV-1 replication, and a-zam had a potential for anti-retroviral activity in chronically infected cell. Plans are underway to do a more comprehensive study using fractionated components, to identify the active component as well as reduce cytotoxicity on cultured cells.

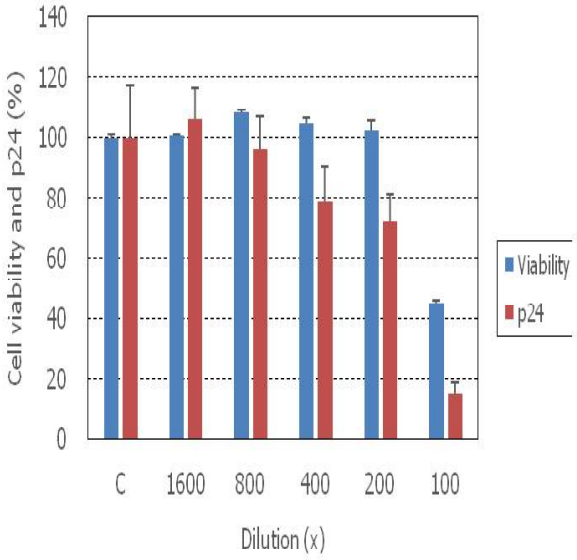

Fig.4: Anti-HIV-1 activity of A-zam in OM-10.1 cells. The cells were incubated in the presence of various dilutions of A-zam for 2 h. The cells were stimulated with 0.1 ng/mL TNF-α and further incubated. After 3 days, the number of viable cells and p24 antigen levels ofculture supernatants were determined by the MTT and p24 ELISA methods, respectively.

Discussion

The therapeutic effect of NS has been utilized by man for ages and across several cultures. These included treatment for different kinds of ailments, like jaundice, fever, respiratory and digestive disorders, rheumatoid arthritis, cardiovascular complications, numerous cancers and a whole lot of others [27]. In fact, the prophetic medicine according to the Islamic heritage is a remedy for all diseases except death [27]. The medicinal, pharmacological, and therapeutic properties have been traced to various bioactive components of the miracle seed. These included nigellicine, nigellicimine, thymoquinone, thymol, nigellicimine N-oxide and carvone. Although the importance of NS for alleviating the signs and symptoms of infectious diseases has been reported by several investigators, not much is known about its antiviral activity. Hence, only few reports are available on its potential use for ameliorating viral symptoms in both *in vivo* and *in vitro* systems. These include studies by Aqil *et al*. (2018), Salem and Hossain (2000) and Khan *et al*. (2018) which reported potent and selective inhibition of virus replication in test systems infected with Pestes des Petits Ruminants virus (PPRV), Murine cytomegalovirus (MCMV) and Newcastle disease virus (NDV) [28-30].

The virus inhibition and consequent antiviral activities of NS were observed as reduction in CPE, lowered viral load, milder symptoms and reduced mortality in *in-vitro*, *in-vivo* and *in-ovo* systems for PPRV, MCMV and NDV respectively. As a result, NS was suggested as a potential agent for controlling viral infections in animals. Also, the capacity of NS to selectively inhibit Hepatitis C virus (HCV) RNA replication was reported by authors in a previous study, using genotype 1b sub-genomic and full genome HCV replicon cells (LucNeo#2, NNC#2). The anti-HCV activity determined by luciferase expression assay and HCV RNA synthesis inhibition was further confirmed by a real-time reverse transcriptase polymerase chain reaction [31]. The direct acting antiviral activity of NS on HCV may have been responsible for the success of the Egyptian study; where a significant decrease of viral load and sero-reversion of 16.7% of patients was observed. Also there was improvement in the prognosis of the disease among patients with cirrhosis and chronic liver disease [32]. Taken together, NS is shown to possess antiviral properties and could be a novel therapy for viral diseases of man and animal in the nearest future.

In spite of the rich antimicrobial property of the NS seed, details are still unknown about its antiretroviral activity. The scarcity of literature therefore calls for intense research to explore the possibility of having an effective anti-HIV agent from the miracle seed. Therefore, the present study may be the first to demonstrate the *in vitro* anti-HIV-1 effect of NS. The result of the present study showed a dose dependent inhibition of HIV-1 replication in OM-10.1 chronically infected cell. The inhibition of viral replication and consequently lower p24

antigen measurement observed in the present study is in accordance with results from previous antiviral studies. The studies which clearly demonstrated virus inhibition reported reduced CPE, decrease in viral load, viral RNA replication inhibition, improved clinical conditions, lower mortality and sero-reversion [28-32]. The observed anti-retroviral activity of the formulation in chronic infection of OM-10.1 cell therefore may not be unconnected with the significant reduction of the viral load in HIV patients in human studies by Onifade *et al*. [21-23]. Also, the documented capacity of the various formulations of NS to stimulate the immune system [33] as well as the anti-HIV-1 activity in OM-10.1 cell suggests its therapeutic potential in the management of immunosuppressive condition like HIV/AIDS. Further studies will be carried out to determine the active principle in a-zam.

Conclusion

The dose dependent reduction of p24 antigen in OM-10.1 cell is an indication that a-zam has the capacity to block virus replication and therefore has a potential for anti-retroviral activity in chronic infection state. Therefore, a-zam should be exploited as a source of novel anti-HIV-1 agent for treating chronic infection in the nearest future.

Limitation of the study

The present study was conducted using the crude drug after dissolution in warm water and filtration with 0.22µm Millipore filter. Further experiments will be done with the bioactive component of a-zam, to reduce the formulation's cytotoxicity as well as aid the identification of the active principle.

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