

HIV-1 infection inhibition by neem (*Azadirachta indica* A. Juss.) leaf extracts and Azadirachtin

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Mexico is a country with a strong attachment to the using of traditional medicine, which is heritage of pre hispanic ethnic groups of Mexican territory that are still present all across the country. The Mexican markets, dedicated exclusively to trade medicinal plants, have facilitated the using, getting and importation of plants from other regions of the world such as neem, which has been used for many health conditions, both non- communicable and infectious diseases, such as cancer, diabetes, bacterial, parasitic and viral infections, even the infection with human immunodeficiency virus. So that, the objective of this study was to evaluate HIV-1 infection inhibition by neem leaf extracts and neem compounds. Cytotoxicity of aqueous and ethanolic neem leaf extracts and Azadirachtin and Limonene compounds was evaluated in CEM T-cells at concentrations of 1 ppm, 10 ppm (extracts); 1 µM, 10 µM (compounds). Only the aqueous extract concentration of 1 ppm and Azadirachtin at 1 µM and 10 µM concentrations allowed a cell viability of 100 % compared with controls $p > 0.05$. Based on the results of cytotoxicity we proceeded to evaluate the HIV infection inhibition with the aqueous extract at concentration of 1 ppm and Azadirachtin at both concentrations. Inhibition percentages greater than 50 % were obtained and showed to be significant compared to controls $p < 0.001$ This work evidenced the HIV infection inhibition by aqueous neem leaf extracts and Azadirachtin; even though, the antiretroviral mechanism is not completely understood. Furthermore, more studies need to be conducted in order to identify the active compound responsible for the anti HIV activity described in the aqueous extract.

Keywords: HIV-1, *Azadirachta indica*, Leaf, Azadirachtin, Aqueous extract

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Mexico is a country with a strong attachment to the using of traditional medicine, which is heritage of pre hispanic ethnic groups of Mexican territory that are still present all across the country. *Maya* people in southern¹, *Nahua* people in central territory², and *Kikapúes* people in the North are a few examples³. In addition to traditional medicine is the great diversity of plants found in Mexico, some authors estimate the number of vascular plants in 22,000 different species⁴, so it is easy to understand the popularity of herbal medicine as one of the main traditional medicines of the Mexican people⁵. The plants availability in markets stalls depends on the demand for the product⁶; thus, the Mexican markets, dedicated exclusively to trade medicinal plants, have

facilitated the using, getting and importation of plants, from other regions of the world, such as neem (*Azadirachta indica* A. Juss.) from India in Asia. Neem has been used for many health conditions, both non-communicable and infectious diseases, such as cancer, diabetes, bacterial, parasitic and viral infections, even the infection with human immunodeficiency virus (HIV)⁷⁻⁹. HIV is a virus belonging to the family *Retroviridae*¹⁰, characterized by an enzyme called reverse transcriptase capable of transcribing the genetic material of HIV from RNA to DNA¹¹, this virus is recognized as the causative agent of acquired immunodeficiency syndrome (AIDS)¹², a condition which leads to a weakening of the immune system of people living with HIV (PLHIV) and the consequent susceptibility to some opportunistic infections such as

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tuberculosis (by *Mycobacterium tuberculosis*), pneumonia (by *Pneumocystis jirovecii*), meningitis (by *Toxoplasma*), multiple infections (by *CMV*), candidiasis (by *Candida albicans*), which are the main causes of mortality specified in PLHIV in Mexico¹³.

In our working experience with PLHIV in Mexico city, we have frequently found the report of using plant extracts as traditional complementary medicine to conventional treatment of HIV¹⁴, which is consistent with other reports published^{15,16}. Among the most popular plants used by PLHIV is the neem¹⁴. Unfortunately, despite the popularity of herbal medicine among Mexican people; the clinical using of medicinal plants has not led to the development of scientific methodologies and consequently has not been incorporated into official medicine¹⁷. However, plants are a source of secondary metabolites that have been tested with activities such as antioxidant, antibacterial, antiviral, etc.; for instance, in the particular case of neem, more than 135 chemical constituents can be extracted and contain many biological activities⁸. Among these constituents are: alkaloids, flavonoids, triterpenoids, phenolic compounds, carotenoids, steroids and ketones, from them the most biologically active compound is azadirachtin¹⁸. So that, the objective of this study was to evaluate HIV infection inhibition by neem leaf extracts and neem compounds. The importance of this study for the traditional knowledge is to support the anti HIV using of the neem's compounds and extracts.

Materials and methods

Plant material

A. indica leaves were collected in Matamoros, Coahuila, México, in November 2009, and they were identified by Dr B Serrano-Gallardo, Universidad Autónoma de Coahuila. A voucher specimen (A.i 27112009) is deposited at the Centro De Investigación Biomédica, Facultad De Medicina, Universidad Autónoma De Coahuila, Unidad Torreón, Mexico.

Preparation of neem leaf extracts

A sample of 800 gm air-dried neem leaf was blended and a sample of 256 gm of fine dust was collected. An aliquot (100 gm) was taken to do the extraction with 1 L of ethanol for 7 days at room temperature, light protected. Another aliquot (100 gm) was taken to do another extraction with hot water (60 °C) at room temperature for 1 hr. Both extracts

were filtered with Whatman® filter paper N. 40 and the filtered solutions were dried at 40 °C in a hot air oven for 7 days. Finally a sample of 6.15 gm of the ethanolic extract was stored at -20 °C and a sample of 26.09 gm of the aqueous extract was stored at -20 °C. A solution of 1000 µL at a concentration of 10 000 parts-per million (ppm) was prepared with 10 mg of both aqueous and ethanolic extract in sterile water and sterile dimethyl sulfoxide (DMSO), respectively. The solutions were vortexed for 30 s and centrifuged for 2 min the supernate was filtered in a 0.2 µM syringe filter (Acrodisc® 25 mm syringe filter w/0.2 µm supor® membrane).

Preparation of neem compounds

A stock solution of 100 µL at a concentration of 100 mM was prepared with 7.2071 mg of Azadirachtin (FW 720.71 SIGMA-ALDRICH catalog number N11107) and DMSO up to 100 µL, then a dilution 1:10 was prepared with 10 µL of the Azadirachtin stock solution and 90 µL of DMSO. The Azadirachtin dilution was vortexed for 30 s and centrifuged for 2 min and the supernate was filtered in a 0.2 µM syringe filter (Acrodisc® 25 mm syringe filter w/0.2 µm supor® membrane). The final concentration of the dilution was 10 mM.

A stock solution of 100 µL at concentration of 100 mM was prepared with 1.6 µL of Limonene (FW 136.23 and density 0.8411 gm/cm³ SIGMA-ALDRICH catalog number 334111) and DMSO up to 100 µL, then a dilution 1:10 was prepared with 10 µL of the Limonene stock solution and 90 µL of DMSO. The Limonene dilution was vortexed for 30 s and centrifuged for 2 min and the supernate was filtered in a 0.2 µM syringe filter (Acrodisc® 25 mm syringe filter w/0.2 µm supor® membrane). The final concentration of the dilution was 10 mM.

Citotoxicity of neem leaf extracts and neem compounds in CEM T cells

Petri dishes (100 mm) were prepared with 8 mL of RPMI-1640 (GIBCO® Catalog number 11875-085) at a concentration of 0.125 µL and 1.25 µL of extract either aqueous or ethanolic per mL of RPMI-1640 and 2 ml of cellular culture were added to each petri dish (concentration of 1 000 000 cells per mL of CEM T cells); thus each petri dish had 2 000 000 of CEM T cells and a final amount of 1 µL and 10 µL of extracts (i.e., a final concentration of 1 and 10 ppm respectively). Finally the petri dishes were incubated at 37 °C and an atmosphere of 5 % CO₂ for 72 hrs. The experiment was developed 3 times per triplicate each one.

In order to prepare the petri dishes (100 mm) with Azadirachtin and Limonene at concentrations of 1 and 10 μ M, 1 and 10 μ L of the dilution solution [10mM] were mixed up to 8 mL of RPMI-1640 and 2 ml of cellular culture were added to each petri dish (concentration of 1 000 000 cells per mL of CEM T cells); thus each petri dish had 2 000 000 of CEM T cells and a final concentration of 1 and 10 μ M of neem compounds: Azadirachtin and Limonene. A sample of 100 μ L of cellular culture was collected in an eppendorf tube that was mixed in a proportion 3:1 with HyClone® (Trypan blue solution. Fisher Scientific Catalog number SV3008401), i.e., 10 μ L of cellular culture with 30 μ L of HyClone, and 10 μ L were deposited into the hemocytometer to be observed at microscope. Cell titer assays were performed and viability was determined as the percentage of viable compared to the DMSO control.

HIV-1 infection inhibition in CEM T cells

CEM T cells were infected with a clinical isolate from a donor (PLHIV), estimating a multiplicity of infection (MOI) of 0.5. Post-infection, the cells were incubated for up to 7 days with 1 μ L of aqueous extracts [10 000 ppm] (i.e., a final concentration of 1 ppm), 1 μ L and 10 μ L of Azadirachtin [10 mM] (i.e., a final concentration of 1 μ M and 10 μ M) whereas solvents used to prepare extracts were used as negative controls. The virus load in the supernatant of CEM T cells treated with neem leaf extracts and Azadirachtin was estimated by measuring p24 protein using an ELISA kit (Perkin Elmer), following the instructions of the manufacturer. Per cent inhibition in HIV infection was calculated by dividing the p24 concentration in the presence either neem leaf extracts or Azadirachtin by p24 value observed in control (infected cells without any treatment), multiplied by hundred and the obtained value was subtracted from hundred.

Statistical analysis

Statistical calculations and graphs were carried out with the SPSS 21 for windows software package and Graph Pad Prism 6.01 for windows. Results are expressed as the mean $\pm$ standard error of the mean (SEM) of triplicate experiments. Z-test for proportion differences was employed being significant P values < 0.05.

Results and discussion

Citotoxicity of neem compounds (Azadirachtin and Limonene [1 and 10 μ M]) and neem leaf extracts (aqueous and ethanolic [1 and 10 ppm]) were

evaluated in CEM T cell as shown in Fig. 1. Neither Limonene nor ethanolic extract showed a cellular title over 80 %; in the case of the aqueous extract (10 ppm) a massive proliferative effect was observed that was consistent with previous reports ($p < 0.001$)¹⁹. Only Azadirachtin at both concentrations 1 and 10 μ M, and aqueous extract at a concentration of 1 ppm showed a cellular title of 100 % when compared with the control $p > 0.05$. Based on the results of cytotoxicity, Azadirachtin and aqueous neem leaf extract were evaluated against HIV and percentages of HIV infection inhibition over 50 % were found to be significant compared to their control $p < 0.001$ as shown in Fig. 2.

Neem is a traditional and popular remedy among various ethnic groups and different parts of the plant as seed, bark, fruits and leaf have been reported with biological activities against human ailments; even

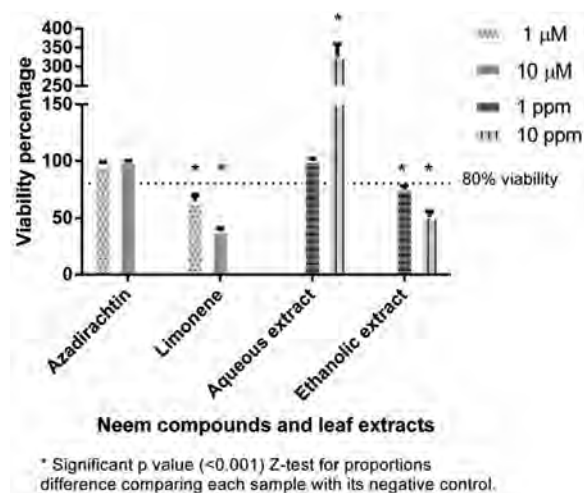


Fig. 1 — Cytotoxicity in CEM T-cells

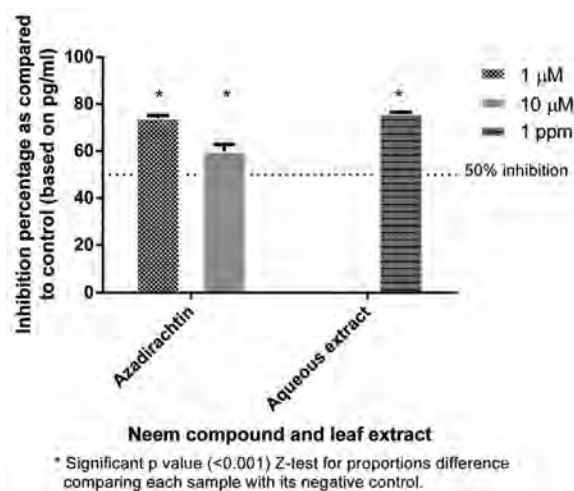


Fig. 2 — HIV infection inhibition

though, the responsible compounds have not been identified^{20,21}. The highly active anti retroviral therapy (HAART) available since 1996, is a cocktail of anti retrovirals currently used as the standard treatment to combat HIV infection; the mechanism of action of HAART is a combination of three drugs that inhibit virus replication in the host in multiple steps of the viral cycle. It is important to mention that the HIV reverse transcriptase enzyme has no proofreading activity, leading to high rates of mutation in the genetic material of the virus consequently causing drug resistance²² when infection is only treated by only one drug as observed at the beginning of the epidemic. In this context, the combination of three drugs in the anti retroviral cocktail reduces the odds of generating drug resistance; thus, prolonging anti retroviral treatment activity which results in a reduction in the viral load of the host and facilitates the restoration of the immune response of people; so, our findings should be interpreted with caution. Awah *et al.*, reported an hydroacetone extract that inhibited the HIV infection and cell-to-cell transmission, as shown in a syncytia formation assay. A dose-dependent inhibition of syncytia formation was observed. Also the neem hydroacetone extract exhibited retrotranscriptase inhibitory activity⁹. According to these reports of the involvement of neem extracts in the inhibition of syncytium and considering that syncytia formation is mediated by interaction of HIV glycoprotein gp120 with the host cell CD4 receptor; seems plausible that the mechanism of action in the inhibition of HIV infection may involve the participation of lectins, this mechanism of action would also be consistent with the induction of the mitogenic-like effect observed in the increased concentrations of T cells observed at 10 ppm of the aqueous extract¹⁹. However we believe that in the extracts exist a variety of compounds that could be acting at different steps of HIV replication cycle, since other anti HIV activity reports have been published although the mechanism of action has not been fully described²³.

Conclusion

Plant products have attracted attention as possible anti HIV drugs targeting on different steps of the viral cycle, such as viral attachment and entry and on enzymes that play a crucial role during the viral replication. It is noteworthy that from neem have been isolated over 135 compounds that may have antiviral potential as reported in various types of virus. So that,

this work evidenced the HIV infection inhibition by aqueous neem leaf extract and Azadirachtin; even though, the antiretroviral mechanism of this extract and compound is not completely understood. Furthermore, more studies need to be conducted in order to identify the active compound responsible for the anti HIV activity described; and the mechanism involved.

Conflicts of interest

The authors declare no conflict of interest.

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References

- 1 Hopkins A & Stepp JR, Distribution of Herbal Remedy Knowledge in Tabí, Yucatan, Mexico, *Econ Bot*, 66 (3) (2012) 249-254.
- 2 Iorio S, An ethnographic study of medical practices and knowledge in the Nahuatl contest (Naupan, Puebla, Mexico), *Med Secoli*, 26 (3) (2014) 793-820.
- 3 Robles-Zepeda RE, Coronado-Aceves EW, Velázquez-Contreras CA, Ruiz-Bustos E, Navarro-Navarro M & Garibay-Escobar A, *In vitro* anti-mycobacterial activity of nine medicinal plants used by ethnic groups in Sonora, Mexico, *BMC Comple Altern Med*, 25 (13) (2013) 329-334.
- 4 Villaseñor JL, Los géneros de las plantas vasculares de la flora de México, *Boletín de la sociedad botánica de México*, 75 (2014) 105-135.
- 5 Taddei-Bringas GA, Santillana-Macedo MA, Romero-Cancio JA & Romero-Téllez MB, Acceptance and use of medicinal plants in family medicine, *Salud Publica Mex*, 41 (3) (1999) 216-20.
- 6 Aarland, Rayn Clarenc; Peralta-Gómez, Susana; Sánchez, Cesar Morales; Parra-Bustamante, Francisco; Villa-Hernández, Juan Manuel; León-Sánchez, Fernando Díaz de; Pérez-Flores, Laura J; Rivera-Cabrera, Fernando; Mendoza-Espinoza & José Alberto, A pharmacological and phytochemical study of medicinal plants used in Mexican folk medicine, *Indian J Tradit Knowle*, 14 (4) (2015) 550-557.
- 7 Kumar VS & Navaratnam V, Neem (*Azadirachta indica*): prehistory to contemporary medicinal uses to humankind, *Asian Pac J Trop Biomed*, 3 (7) (2013) 505-514.
- 8 Biswas K, Chattopadhyay I, Banerje RK & Bandyopadhyay U, Biological activities and medicinal properties of neem (*Azadirachta indica*), *Curr Sci*, 82 (11) (2002) 1336-1345.
- 9 Awah FM, Uzoegwu PN & Ifeonu P, *In vitro* anti-HIV and immunomodulatory potentials of *Azadirachta indica* (Meliaceae) leaf extract, *Afr J Pharm Pharmacol*, 5 (11) (2011) 1353-1359.
- 10 Ondarza RN, Retrovirus, In: *Virus y Enfermedad. Spanish version*, edited by Trillas, (Mexico city, Mexico), 2011, 140-170.
- 11 Pepin J, Appendix: clasification of retroviruses, In: *The origins of AIDS*, edited by Cambridge university press, (United Kingdom), 2011, 282.

- 12 Flint SJ, Enquist LW, Krug RM, Racaniello VR & Skalka AM, Multiple facets of Human Immunodeficiency Virus Pathogenicity, In: *Principles of virology, molecular biology, pathogenesis*, edited by ASM Press, (Washington DC, USA), 2000, 631.
- 13 Estadísticas: Salud en números: DGIS, Dirección General de Información en Salud, 2016. (<http://www.dgis.salud.gob.mx/contenidos/sinais/estadisticas.html>) (Accesed April 2016).
- 14 Pedroza-Escobar D, Sevilla-González MDLL, Escobar-Ávila EAD & Serrano-Gallardo LB, Using of medicinal plants among people living with HIV, *J Plant Dev Sci*, 8 (7) (2016) 311-314.
- 15 Rivera JO, González-Stuart A, Ortiz M, Rodríguez JC, Anaya JP & Meza A, Herbal product use in non-HIV and HIV-positive Hispanic patients, *J Nat Med Assoc*, 97 (12) (2005) 1686-1691.
- 16 Herrera-Arellano A, Jaime-Delgado M, Herrera-Alvarez S, Oaxaca-Navarro J & Salazar-Martínez E, The alternative medicine used as complementary in patients positive for HIV, *Rev Med Inst Mex Seguro Soc*. 47 (6) (2009) 651-8.
- 17 Schlaepfer L & Mendoza-Espinoza JA, Las plantas medicinales en la lucha contra el cáncer, relevancia para México, *Revista Mexicana de la Asociacion Farmaceutica*, 41 (4) (2010) 18-27.
- 18 Verkerk RHJ & Wright DJ, Biological activity of neem seed kernel extract and synthetic azadirachtin against larvae of *Plutella xylostella*, *Pesticide Sci*, 37 (1) (1993) 83-91.
- 19 David PE, Serrano-Gallardo LB, Sevilla-González MDLL, López-García S, Escobar-Ávila EAD, Luna-Herrera J & Vega-Menchaca MDC, Effect of neem (*Azadirachta indica* A. Juss.) leaf extracts in human T lymphocytes, *Indian J Tradit Knowle*, 15 (2) (2016) 219-222.
- 20 Sharma P, Tomar L, Bachwani M & Bansal V, Review on Neem (*Azadirachta indica*): Thousand problems one solution, *Int Res J Pharm*, 2 (12) (2011) 97-102.
- 21 Pathak GP, Vineet S, Gurdeep S & Amitoz K, Neem (*Azadirachta indica*): traditional medicine for house-hold remedy against various human and animal ailments: Review, *World J Pharm Pharmaceut Sci*, 2(5) (2013) 3854-3860.
- 22 Mansky LM & Temin HM, Lower *in vivo* mutation rate of human immunodeficiency virus type 1 than that predicted from the fidelity of purified reverse transcriptase, *J Virol*, 69 (8) (1995) 5087-5094.
- 23 Talwar GP, Raghuvanshi P, Misra R, Mukherjee S & Shah S, Plant immunomodulators for termination of unwanted pregnancy and for contraception and reproductive health, *Immunol Cell Biol*, 75 (2) (1997) 190-192.