Effect of neem (Azadirachta indica A. Juss.) leaf extracts in human T lymphocytes

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Nowadays some countries provide universal access for the anti-retro-viral therapy for people living with HIV; however, this population for several reasons, only takes traditional and complementary medicine or use it to complement their conventional treatment in order to keep and improve their health, and among the popular herbal remedies used by people living with HIV are neem leaf extracts. So that, the objective of this study was to evaluate the effect of aqueous and ethanolic neem leaf extracts in human T lymphocytes. For the concentration of 1 µL of aqueous extract a cellular viability of 100% was observed, for the concentration of 10 µL of the aqueous extract a massive proliferative effect was observed of 417.89%, and for the concentration of 100 µL of aqueous extract was observed a cellular viability of 26.39%. In the case of the ethanolic extracts, they showed to be cytotoxic since the concentrations of 1 µL, 10 µL and 100 µL reduced the cellular viability to 67.17%, 51.65% and 10.22%, respectively. All the experiments were compared with the control. Due to the fact that some vegetable polymeric proteins, known as lectins such as concanavalin A and phytohaemagglutinin are polyclonal activators, also considered as mitogens due to the fact that they encourage T lymphocytes to commence cell division; we think the mitogenic effect-like that we observed in our aqueous extract may be as a consequence of the presence of lectins in it.

Keywords: Mitogenic effect, Azadirachta indica, Leaf, Lymphocytes

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The World Health Organization (WHO) defines traditional and complementary medicine (T&CM) as “The sum total of the knowledge, skills, and practices based on the theories, beliefs, and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness”, likewise the herbal medicine is stated as “Herbs, herbal materials, herbal preparations and finished herbal products, that contain as active ingredients parts of plants, or other plant materials, or combinations thereof”; in this regard, the WHO reported in the Traditional Medicine Strategy 2014–2023 that T&CM is found in almost every country in the world where the request for its services is increasing, it estimates percentages around 80% of the population living in developing countries recognize employment of T&CM since it is culturally acceptable and trusted by large numbers of people. In this way, the WHO encourages the integration of T&CM into national health systems through strategies in order to strengthen its quality assurance, safety, proper use and effectiveness.

Neem (Azadirachta indica), a popular herbal remedy native from the Indian subcontinent, that has been reported as a medicinal plant since around 10 000 - 4 000 B.C. in the annals of the ancient Siddha medicine. It is a fast growing evergreen tree used from antiquity in countries of Asia and Africa as one of the most versatile medicinal plants having a wide spectrum of biological activities; however, since the neem tree has been highly appreciated by people, the emigrants of these continents, carried it up to the places where they settled in around 30 countries in America, Australia, Europe, South Pacific Islands and The Caribbean Islands which led to an increase of its traditional use. Every part of the tree, principally roots, leaf, flowers, barks and fruits, has been used as traditional medicine for household

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remedy against various human ailments such as allergies, asthma, colic, conjunctivitis, dysmenorrhea, fever, headache, digestive disorders, renal lithiasis, psoriasis, muscular pain, parasitic diseases. Besides it has activities such as anti-ulcer, anti-bacterial, anti-viral, anti-glycaemic, anti-inflammatory, anti-oxidant, anti-carcinogenic among others  

More than 135 compounds have been isolated from different parts of neem being its major compounds known as triterpenoids; some of them are Azadirachtin A, Azadiradione, Azadirone, Epoxazadiradione, Nimbidin, Nimbin, Nimbidiol, Deacetylnimbin, Salannin, Gedunin and its derivatives. Several reviews published on the chemistry and structural diversity of these compounds.

Summarizing in spite of the effort that Governments make fundamentally to offer attention in health subjects for people exist a great attachment to its history, customs and to its systems of health. On the other hand distrust and lack of attachment to the conventional systems of health. This has generated a politics impulse and some intercultural actions in the medical land, for example by means of the WHO strategies to support and develop T&CM such as Acupuncture, Herbolar, Homeopathy, among others. In some countries these T&CM are recognized by people since the work altogether between the popular system and the conventional one generates contributions and benefits in different problems and situations furthermore they are highly accepted and efficient.

Nowadays some countries provide universal access for the anti-retro viral therapy (ART) for people living with HIV. However, this population for several reasons, beyond the aim of this study, only takes T&CM or use it to complement their conventional treatment in order to keep and improve their health. For people living with HIV, However, this population for several reasons, beyond the aim of this study, only takes T&CM or use it to complement their conventional treatment in order to keep and improve their health. Furthermore, they are highly accepted and efficient.

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 h. 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.

Evaluation of neem leaf extracts in human T lymphocytes

A saturated solution of 1000 µL was prepared with 10 mg of both aqueous and ethanolic extract in sterile water and sterile dimethyl sulfoxide (DMSO), respectively. The saturated 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).

Petri dishes (100 mm) were prepared with 8 mL of RPMI-1640 (GIBCO ® Catalog number 11875-085) at concentration of 0.125, 1.25 and 12.5 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 human T lymphocytes cell line [ATCC Catalog number CCL-119]); thus each petri dish had 2 000 000 of T cells and a final amount of 1 µL, 10 µL and 100 µL of extracts (i.e., dilution of 1:10 000, 10:10 000 and 100:10 000 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.

A sample of 100 µl 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 µL of cellular culture with 30 µL of Hyclone, and 10 µL were deposited into the hemocytometer to be observed at microscope.

Results and discussion

Evaluation of neem leaf extracts, aqueous and ethanolic, were done in human T lymphocytes (cell line CCL-119). For the concentration of 1 µL of aqueous extract a cellular viability of 100%
was observed, for the concentration of 10 µL of the aqueous extract a massive proliferative effect of 417.89% was observed, and for the concentration of 100 µL of aqueous extract was observed a cellular viability of 26.39%. In the case of the ethanolic extracts, they showed to be cytotoxic since the concentrations of 1 µL, 10 µL and 100 µL reduced the cellular viability to 67.17%, 51.65% and 10.22%, respectively (Table 1). All the experiments when compared with the control.

We believe that the proliferative effect that is observed in the aqueous extract concentration of 10 µL may be a consequence of lectins that might be present in it, since the methods employed to obtain and purify lectins are based in the solubility of these proteins in either water or saline; and the fact that lectins are polyclonal activators of T lymphocytes, where the mechanism involved in this kind of activation is through the polyclonal activator attachment with many complexes of the T lymphocyte receptor (TCR) independently of the TCR specificity; thus activating the T lymphocyte in a similar way than the complexes of the major histocompatibility complex (MHC) and a peptide presented for antigen presenting cells (APC)\textsuperscript{18}.

The polyclonal activators are used to activate in vitro T lymphocytes from human blood or lymphoid tissue of experimental animals. These activators can also be employed to activate T lymphocytes of unknown specificity, because they can lead to a detectable response from a mixed population of naïve T lymphocytes, even when the percentage of a specific T lymphocyte for a concrete antigen is too low to induce a detectable response.

Some lectins, vegetable polymeric proteins such as Concanavalin A and Phytohaemagglutinin, are polyclonal activators also considered as mitogens due to the fact that they encourage a cell to commence cell division, these mitogens are frequently employed to the study of the T lymphocytes because of its attachment to specific saccharide components in the membrane glycoproteins of T lymphocytes, including the TCR and CD3; consequently, they induce the T lymphocyte activation\textsuperscript{18}.

This mitogenic effect of aqueous A. indica leaf extracts in human T lymphocytes may provide a supportive explanation for the results found by Mbah in 2007 and Anyaehie in 2009 who reported an extract of A. indica in a clinical trial that increased T lymphocytes CD 4 in 60 persons living with HIV (p<0.001); and an increase of T lymphocytes CD 4 in people living with HIV, compared with people living with HIV in anti-retro viral therapy; respectively\textsuperscript{19,21}. Besides, neem oil extracts have also been reported with lymphocyte mitogenic activity\textsuperscript{22}.

An ethnobotanical study of Maria Francis in 2014 supports the therapeutic potential of Indian plant species such as Azadirachta indica A. Juss. This study found that many different parts of the medicinal plant species are used as medicine but the most commonly used plant part was leaf. This was in agreement with earlier findings published elsewhere. Since the therapeutic properties are easier to elicit from this soft raw material\textsuperscript{23}. Thus, we focused into an easy way of getting the neem leaf in Mexico, so given the report of The Sonora Market as one of the most important places in Mexico City for selling and buying medicinal plants (having sales of about 160 tons per month; even considered as one of the most important places in Latin America for management of traditional medicine)\textsuperscript{24}, we randomly asked in several venues of the market, dedicated to selling herbs and medicinal plants, for the neem leaf and we found easily to get it for around 20 US dollars per Kilogram.

In summary this study reports the proliferative effect of aqueous neem leaf extracts at a concentration of 10 µL which leads to a percentage of cells over 417 ± 10.19% when compared with the control, this mitogenic effect may be as a consequence of the presence of lectins in the extracts. However more investigation need to be done in order to identify the molecules involved.

**Conclusion**

As previously mentioned, people living with HIV use T&CM to complement their conventional treatment in order to keep and improve their health. Thus, the traditional significance of this study to society is to provide evidence of the effect of neem leaf extracts

<table>
<thead>
<tr>
<th>Extract amount</th>
<th>Aqueous extract Mean (SEM)</th>
<th>P value*</th>
<th>Ethanolic extract Mean (SEM)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 µL</td>
<td>100 (2.79)</td>
<td>0.317</td>
<td>67.17 (11.88)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>10 µL</td>
<td>417.89 (10.19)</td>
<td>0.001</td>
<td>51.65 (3.54)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>100 µL</td>
<td>26.39 (8.06)</td>
<td>&lt;0.001</td>
<td>10.22 (4.06)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*Z-test for proportions difference comparing each extract proportion of viable cell titer with its negative control.
in human T lymphocytes. On the other hand the significance of this study to the researchers is the mitogenic effect of the aqueous neem leaf extract that might be used to the study of human T lymphocytes since we found an increase of viable cell titer.

**Statistical analysis**

Statistical calculations were carried out with the SPSS 21 for Windows software package. Results are expressed as the mean ± standard error of the mean (SEM) of 9 independent experiments. Z-test for proportion differences was employed being significant P values < 0.05.

**Conflicts of interest**

The authors declare no conflict of interest.

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**References**


