Anti-HIV, pro-inflammatory and cytotoxicity properties of selected Venda plants

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The use of medicinal plant species among rural communities has played a role in the development of various traditional medical systems. Such development has also accelerated the exploration of different plant species in order to correlate traditional practices to scientific findings. In the Vhembe district (Limpopo, South Africa), a vast majority of traditional medicine is indigenous based and knowledge of the pharmacological activities of plants is solely grounded on the understanding by traditional healers. The anecdotal claims by traditional healers to treat HIV using these plants and their safety are being investigated in this study. RAW 264.7, U937, MeWo and Vero cells were treated to various concentrations (50, 100, 125, 250 μg/mL) of Elaeodendron transvaalense, Cassia abbreviata, Ornithogalum ornithogaloides, Ochna holstii, Lannea edulis, Elephantorrhiza elephantina, Coccinia rehmanni and Jatropha zeyheri for anti-inflammation and cytotoxicity testing. Reverse Transcriptase (RT) assay was used for the anti-HIV activity of the plants. There was no anti-inflammatory activity observed for the plants tested. However, in the absence of LPS stimulation there was an increase of NO production indicating that the extracts might have pro-inflammatory properties. The cytotoxicity observed in human tumor cancer cell lines U937 (p < 0.0001) and MeWo (p < 0.0001) was more pronounced with O. ornithogaloides and E. transvaalense. However, cytotoxicity of the same extracts was not observed in both resting and activated macrophages (RAW cells). With the exception of J. zeyheri, all the extracts tested, induced reverse transcriptase inhibition with some of the extracts showing significant inhibitory activity (p < 0.0001). There was no cytotoxicity on mouse macrophage cell line. The study demonstrated the inhibitory potential of selected Venda plants and their pro-inflammatory properties however; further studies are needed in order to rule out other effects.

Keywords: Plant extract, Anti-inflammatory, Anti-HIV, Pro-inflammatory, Traditional medicine, Venda, South Africa

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Traditional medicine is regarded as a very efficient alternative health care system especially in areas characterized by high rate of unemployment and lack of efficient services in government hospitals. The use of medicinal plants can also be attributed to their accessibility, affordability and cultural beliefs. Medicinal plants are widely used in many third world countries for their benefits in treating a wide variety of ailments. A number of natural products and other chemical substances constitute over 50 % of drugs with antioxidant, antiviral, antibacterial and antifungal activities. The selection of plants based on ethnomedicinal usage increases the probability of finding therapeutic agents as opposed to random plant selection. Traditional medicine is mainly used in making infusions and decoctions that can either be taken orally or applied topically. Some of the herbs are used to cleanse or drive away evil spirits. The traditional knowledge is thought to be basic and can be practiced without any form of training although there is urgent need to document the indigenous knowledge in order to save them from over-harvestation, habitat degradation and also illegal collection.

The traditional knowledge of plants in relation to their use in the Vhembe district has not been well

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explored scientifically or documented for the benefit of future generations. Several ethnobotanical surveys have brought a highlight on the fact that rural communities heavily depend on traditional medicine for treatment of many ailments10-13.

A number of studies have shown the diverse range of biological and pharmacological properties of *Elaeodendron transvaalense* R.H. Archer, *Cassia abbreviata* Oliv., *Ornithogalum ornithogaloides* (Kunth.) Obem, *Ochna holstii* Engl., *Lannea edulis* (Sond.) Engl., *Elephantorrhiza elephantina* (Burch.) Skeels, *Coccinia rehmannii* Cogn. and *Jatropha zeyheri* Sond14-19. However, the anti-inflammatory and anti-HIV properties of these plants are not known nor reported.

Chronic inflammation is a series of complex, non-ending processes that result in the development of many serious diseases. Several mediators of inflammation include chemokines and cytokines20. Preliminary results regarding the anti-inflammatory potential of plant extracts and plant-derived compounds are usually acquired from *in vitro* studies utilizing cultures of cells involved in inflammatory processes such as cells of monocyte origin, models of endothelial cells and hepatocytes. This kind of research comprises experiments that determine the abilities of the compounds to limit the synthesis of common mediators of inflammation exemplified by pro-inflammatory cytokines, chemokines, cell adhesion molecules or nitric oxide21.

In many countries, the use of anti-HIV medicinal remedies and other natural products as part of HIV/AIDS regimen is an extremely delicate and controversial issue22. Many of the HIV-infected people in South Africa have access to antiretroviral drugs, but those who believe in natural products take the concoctions simultaneously. Recently, medicinal plants have become a paramount source of discovering pharmacologically active compounds. The discovery of higher plants as source of newer and efficient drugs has resulted in production of about 25 % drugs currently in use23. The possibility of anti-HIV drugs from plant origin could somehow overstate to reduce side effects associated with the use of antiretroviral drugs associated with the use of ARV. The traditional remedies have been reported effective in the treatment of different ailments including cancer, diabetes, HIV/AIDS and other chronic as well as minor infections (Interviews). The increase has been seen among individuals with moderate and advanced HIV disease24. There are several medicinal plants used worldwide to improve the immunological disorders. The modulation of immune in curing diseases has grown to be a very interesting field25.

Infusions, decoctions, tincture of herbal plants are used traditionally for treatment of various ailments though their efficacy and mechanism of action has not been scientifically tested. It is of great importance to understand and establish such substantial information to avoid cascading toxic effects from the phytochemicals or to uncover newer drug candidates that can be employed as future drugs. Thus, the aim of the present study was to assess the anti-inflammatory properties, cytotoxicity and anti-HIV activities of 8 indigenous medicinal plants used in Venda to validate various claims by traditional healers on their ability to heal certain diseases.

**Material and methods**

**Plant materials and preparation of extracts**

Different plant parts were collected for each of the selected medicinal plants within the Thulamela (22,89 22°S; 30,62 00°E) and Mutale local municipality (22,51 08°S; 30,80 39°E) in Vhembe district of the Limpopo province between August 2014 and December 2014 (Fig. 1). The collected plants were identified using their vernacular names and later confirmed by the taxonomic rank at the Department of Botany (University of Venda) using their respective IPNI (Table 1). The samples were deposited in the departmental herbarium and assigned voucher numbers. Identification of the plant species was also confirmed with the database from the plant list (www.theplantlist.org Accessed on 18/10/2016) and the International Plant Names Index (www.ipni.org Accessed 18/10/2016).

![Map showing Vhembe district municipality comprising of 4 local municipalities](image)
The collected plant parts were naturally dried in the laboratory for 5 days at room temperature and powdered (Buchi, Switzerland). The powdered samples were kept in air tight containers until required. The powders were extracted by maceration with hot water filtered using a Buchner funnel, through Whatman number 1 filter paper (Sigma Aldrich, St Louis, MI; USA). The filtrate was concentrated through freeze drying (FTS systems, Stone Ridge, NY; USA). Unless indicated, plant extracts were solubilized in DMSO (Sigma Aldrich, St Louis, MI; USA) to a stock concentration of 50 mg/mL and stored at 4 °C until required for further use.

**Cell culture**

The Murine macrophage RAW 264.7 cells (ATCC® TIB 71), human tumor cell line U937 (ATCC® CRL1593.2), Mewo (ATCC® HTB 65), Vero (ATCC® CCL 81) were maintained in Dulbecco’s modified Eagle Media (DMEM; Sigma Aldrich®; St Louis, MI; USA). The media were supplemented with 10 % foetal bovine serum (Sigma Aldrich®, St Louis, MI; USA) and 0.01 % Gentamycin Sulfate (Sigma Aldrich, St Louis, MI, USA).

**Anti-inflammatory activity**

RAW 264.7 cells were seeded at a density of 25 000 cells/wells in 96-well microtiter plates (NUNC, Rochester, NY, USA) and allowed to attach overnight in a Heracell VIOS CO2 Incubator (Thermo Fisher, Waltham, MA USA). Plant extracts (50 and 100 µg/mL) in complete DMEM medium were added to replenish the spent culture medium in each well. In order to stimulate macrophages, 50 µL of LPS (10 µg/mL) containing medium was added to specific wells whereas for unstimulated macrophages, 50 µL of complete medium without LPS (Sigma Aldrich, St Louis, MI; USA) was added to the other wells. A well known inhibitor of nitric oxide aminoguanidine (Sigma Aldrich, St Louis, MI, USA) served as a positive control. The plates were further incubated for an hour. To quantify the production of Nitric oxide (NO) in both stimulated and non-stimulated cells, 50 µL of the spent culture medium was transferred to new 96 well plates and an equal amount of Griess reagent (Roche Diagnostics, Risch-Rotkreuz, Switzerland) was added. Absorbance was measured (VersaMax ELISA Microplate Reader, Sunnyvale, CA, USA) at 510 nm and results were compared to the respective controls. Cell viability was also assessed using the MTT assay (Sigma Aldrich, St Louis, MI, USA) to rule out toxicity as a contributory factor to NO production.

**Cytotoxicity assay**

The assay is based on the reducing power of cells on MTT\textsuperscript{26}. The assay was carried out in 96-well plates (NUNC, Rochester, NY, USA). Three cell lines were seeded at a density of 10 000 cells/well (U937) or 6 000 cells/well (MeWo/Vero) in 96-well plates and incubated overnight at 37 °C in a humidified 5 % CO2 incubator (Thermofisher, Waltham, MA, USA). Samples were tested in 2 concentrations, 125 µg/mL and 250 µg/mL for U937cells and 50 µg/mL and 100 µg/mL for the MeWo/Vero cells for optimal results as obtained from Prof Van Der Venter’s Lab (Bioassaix, Nelson Mandela Metropolitan University, PE, South Africa). After addition of the plant extracts, the plates were further incubated for 48 h at 37 °C. Melphalan (Glaxo-Smithkline, Brentford, UK) was used a positive known toxic chemical.
At the end of the incubation period, the medium was removed from the adherent plate (MeWo/Vero) and replaced with fresh DMEM containing MTT at a final concentration of 0.5 mg/mL and for the suspension plate (U937 cells), 20 μL of MTT was added per well to also get a final concentration of 0.5 mg/mL. The plates were again incubated for a further 4 h to allow reduction of MTT and then the formed crystals were solubilized by adding DMSO with gentle shaking for 15 min. Absorbance was read at 560 nm using a multiwall scanning spectrophotometer (Multiscan MS, Thermo Labsystems; Waltham, WA, USA). Data were obtained for triplicate wells. The percentage of inhibition was calculated as previously reported as per the equation:

\[
\% \text{ Cell viability} = \frac{\text{Absorbance value of treated cells} \times 100}{\text{Absorbance value of control cells}}
\]

Where, absorbance\text{control} is the absorbance of cells treated with DMSO and Absorbance\text{treated} represents the absorbance of cells with extract.

**Reverse transcriptase inhibition**

Reverse transcriptase enzyme is a potential therapeutic target against retrovirus infection since it plays a vital role in the conversion of viral RNA to cDNA. The assay is based on the incorporation of dioxigenin and biotin labelled nucleotides into the new DNA synthesis. A commercially available kit was used and the assay was conducted as per the manufacturer’s instructions (Roche Diagnostics; Risch-Rotkreuz, Switzerland). The detection and measurement of the synthesized DNA as a variable for RT activity follows an ELISA protocol: labeled DNA strands binds to the surface of streptavidin-coated microplate modules. Then, an antibody to digoxigenin, conjugated to peroxidase is added and binds to the digoxigenin-labeled nucleotides. The peroxidase substrate ABTS is added to catalyze the cleavage of the substrate and produce a colored reaction product. The absorbance of the samples is determined using a microplate reader (Multiscan MS, Thermo Labsystems; Waltham, WA, USA), and is directly comparable to the level of RT activity in the sample.

**Statistical analysis**

All experiments were done in triplicate and data were expressed as mean ± SEM. Student t-test was used for comparison of results obtained for the 8 plant extracts. Differences in the data were considered statistically significant when \( p < 0.05 \).

**Results**

**Anti-inflammatory**

The anti-inflammatory potential of the extracts was evaluated on RAW 264.7 cells, Fig. 2a illustrates that none of the samples could reduce the nitrate levels below the untreated LPS-stimulated cells. In Fig. 2b, even in the absence of LPS stimulation there appeared to be an increase in nitrate levels as compared to the controls. In Figs. 3a&b, the cytotoxicity towards both resting and stimulated macrophages was not significantly reduced.

**Cytotoxicity**

The cytotoxic effects of aqueous extracts of 8 medicinal plants were evaluated on U937, MeWo and Vero cell lines using MTT assay (Fig. 4). In all the 3 cell lines tested, a similar cytotoxic effect was obtained. Cytotoxicity exhibited by O. ornithogaloides and E. transvaalense in Vero cell line was highly significant \( (p < 0.005) \). L. edulis at the highest tested concentration was seen to be significantly toxic \( (p = 0.007) \). In U937 cells, C. abbreviata and Lannea edulis exhibited a similar toxic effect. O. holstii and L. edulis \( (p < 0.007) \) also showed a similar toxic effects in the MeWo and Vero cell lines.

**Fig. 2 — Effect of plant extracts on the production of nitrate and cell viability in LPS-stimulated (a) stimulated macrophages (b) RAW macrophages. Amino guanidine (AG), an inhibitor of iNOS expression serves as positive control to confirm the functionality of the assay.**
Reverse transcriptase inhibition

With the exception of J. zeyheri, all extracts induced inhibition of RT activity (Fig. 5). *C. rehmannii* significantly inhibited RT activity ($p < 0.0001$) slightly above 40% followed by *E. elephantina* ($p < 0.0002$) and *E. transvaalense* ($p < 0.0008$). Overall, 3 of the plant extracts showed inhibition < 20% at the low concentration level, 4 of the extracts had inhibition between 20 – 40% and *J. zeyheri* had no observable activity.

Discussion

Medicinal plants are known to produce many types of phytochemicals which possess a variety of biological activities. The pharmacological and immunologic activities of the selected plant extracts have not been rigorously investigated. The screening of the different plant extracts was carried out in order to investigate the anti-inflammatory potential of the...
extracts as well as determining the cytotoxicity against the macrophage cell line. None of the 8 plant extracts could reduce nitrate levels below the untreated LPS stimulated levels (Fig. 2), a symbol that they are lacking any anti-inflammatory activity. For most of the samples, there was a measurable increase in nitrate levels relative to LPS stimulated controls. The observed slight viability decrease in O. ornithogalooides revealed a correlation because the NO synthesis level was also attenuated. The decrease in cell viability was also observed in the un-stimulated macrophages with moderate toxic effects from the same plant extract as seen in activated macrophages (Fig. 3). NO is an intracellular mediator/free radical that can be produced in various mammalian cells. It is a crucial and flexible molecule involved in neurotransmission, acute and chronic inflammation and host defense mechanisms.30-32

For in vitro or in vivo experiments, a variety of stimuli could lead to massive production of NO once the macrophages are activated; this may benefit in pathological processes. Some studies have reported that low NO concentrations can protect the immune cells from death whereas unrestricted production can cause death not only to targeted cells. It is now acknowledged that cell death can play an important role in host defense by preventing replication of intracellular organisms and, at simultaneously releasing signaling elements (e.g. microparticles) which can activate other cell types to increase antiviral or anti-bacterial activity. This type of death is regulated and programmed, with the immunological outcome determined by downstream pathways which may be induced.

Furthermore, it is shown that even in the absence of stimulation there was significant increase in NO production (Fig. 2b), this may indicate that the extracts have pro-inflammatory properties. The nature of the extracts with regard to their polysaccharide content is known to have immunomodulatory activity. In this case we concluded that extracts prepared from succulent or bulbs have greater chances of producing pro-inflammatory effects. However, to rule out or confirm such activities further confirmatory tests are to be conducted. To the best of our knowledge, this is the first report demonstrating the pro-inflammatory properties of the chosen medicinal plants to date.

Our results demonstrated the high level of selective inhibitory effects from the 8 extracts on all 3 cell lines (Fig. 4). However, the toxicity was more pronounced in the Vero cell line with decreased cell viability by over 80% at both concentrations tested as compared to the known inhibitor (Melphalan). The varying difference in the level of toxicity might be due to the cell specificity of the extracts. The extracts of O. ornithogalooides and E. transvaalense displayed more toxicity on the Vero, U937 and MeWo cell lines (79% inhibition). These two plants have toxicity similar to a known toxic chemical and this pattern is seen in all the 3 cell lines. The similarity in toxicity levels could be due to the phytochemical constituents or compound(s) which are responsible for the activity. Over 50% of the plant extracts exhibited some extent of inhibitory effects, the values were statistically significant in both Vero and MeWo cell lines. A study done on C. abbreviata showed that methanolic extracts displayed elevated toxicity as observed in brine shrimp lethality test with LC$_{50}$ of 12.7 µg/mL. The ethanol extracts from the same plant were however less toxic with an IC$_{50}$ of 39.6. According to another study done, the methanolic extract of E. elephantina exhibited an LC$_{50}$ f 1.8 which is considered toxic. However, the individual effects of chloroform, ethyl acetate and methanol fractions exhibited lower toxicities (LC$_{50}$: 10, 7.9, and 15 respectively). To the best of our knowledge, no data could be found in the literature in support of the toxicity findings of O. ornithogalooides and E. transvaalense. However, the toxicity observed in the 2 cancer cell lines (U937 and MeWo), is important when identifying compounds for the development of anti-cancer drugs. A study done on the leaves extracts of Holarrhena antidisenterica showed significant antitumor potential from the chloroform fraction. Such findings emphasize the fact that traditional medicine can be great source of newer anti-tumor agents.

In addition, the 8 extracts tested demonstrated anti-HIV activities. A previous study has classified four levels of RT enzyme inhibition for medicinal plant extracts; with activity below 20 % being considered insignificant, 20–40 % low, 40–70 % moderate, and 70–100 % high. According to this classification the activities demonstrated by 6 extracts are considered low despite high statistical significant inhibition (p < 0.001) obtained for C. rehmannii and E. elephantina at both 50 and 100 µg/mL (Fig. 4). The overall percentage inhibition ranged from 11 – 43 % (Fig. 5). C. rehmannii had RT inhibition slightly over 40%. The ethanolic extracts of C. abbreviata displayed a
against HIV-1 RDDP than the methanolic extract. The aqueous root extract of *E. transvaalense* showed good inhibitory activity of 64% and 76% at 1 µg/mL in the NF-kβ assay. In the same study, acetone extract from the same plant displayed no activity.

A study has shown that the aqueous root extract of *E. transvaalense* displayed greater inhibitory effect against HIV-1 RDDP than the methanolic extract. It was revealed that the chloroform and ethyl acetate extracts of *E. transvaalense* showed good inhibitory activity of 64% and 76% at 1 µg/mL in the NF-kβ assay. In the same study, acetone extract from the same plant displayed no activity.

In summary, for the first time, our findings presented here provide evidence that these crude extracts selected from the traditionally used medicinal plants in Venda are precious source of new potential pro-inflammatory and anti-HIV compounds that are yet to be extensively explored. However, the two extracts (*O. ornithogaloides* & *E. transvaalense*) with excessive toxicity are to be excluded from any further testing.

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Declaration

The authors declared no conflict of interest.

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