Journal of Complementary and Alternative Medical Research

4(2): 1-9, 2017; Article no.JOCAMR.36908
ISSN: 2456-6276

Screening of *Anthocleista djalonesis* Fractions and Compounds against HIV-1 Integrase and HIV-1 Protease

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Authors' contributions

This work was carried out in collaboration between all authors. Author ISO designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors ISO and MUE managed the analyses of the study. Author TATA managed the literature searches. Authors JOI and MEK identified the compounds. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JOCAMR/2017/36908
Editor(s):
(1) Arun Singh, Professor, Community Medicine, Institute: Rohilkhand Medical College & Hospital, Bareilly International University, India.
Reviewers:
(1) Lívia Garcia Bertolacci-Rocha, Universidade Federal de Goiás, Brasil.
(2) N. J. Kadima, University of Rwanda, Rwanda.
Complete Peer review History: http://www.sciencedomain.org/review-history/22147

Original Research Article

Received 22nd September 2017
Accepted 13th October 2017
Published 4th December 2017

ABSTRACT

Aim: HIV, the virus that causes AIDS, is one of the world's deadliest diseases today. Drug resistance and narrow spectrum of available therapeutics is a main problem during HIV treatment. Therefore, new drugs effective against drug-resistant HIV strains are needed. The aim of this study was to screen *Anthocleista djalonensis* extracts, fractions and isolated compounds for *in vitro* anti-HIV-1 Integrase (HIV-1 IN) and HIV-1 Protease (HIV -1 PR) activities.
Place and Duration of Study: The study was carried out in the Department of Organic Chemistry, Rhodes University, Grahamstown, South Africa. The period was between March and July, 2016.

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Methodology: The ethyl acetate and acetone extracts of the roots of *Anthocleista djalonensis* and fractions and compounds obtained from column chromatography of acetone extract were screened for their inhibitory activity against HIV -1 Integrase using a non-radioactive ELISA-based HIV-1 integrase assay. The screening was carried out at concentration range of 10^{-5} -10^{2} µg/ml. The screening for anti-protease activity was performed using a fluorogenic octapeptide substrate, HIV-FRET (1) and a recombinant HIV-1 Protease solution.

Results: The ethyl acetate and acetone extracts showed inhibitory effects on HIV -1 Integrase with IC_{50} of 1.3001 ± 0.217 µg/mL and 0.7216 ± 0.0028 µg/mL respectively. IC_{50} values of 0.0077 ± 0.009 µg/mL, 5.0001± 0.1719 µg/mL, 3.5113 ± 0.3613 µg/mL and 0.0736 ± 0.0005 µg/ml were obtained for chromatographic fractions F-1, F-2, F-3 and F-4 respectively. The compounds Bauerenone, Bauerenol and a mixture of Stigmasterol and β-Sitosterol isolated from *A. djalonensis* had IC_{50} values of 5.6112 ± 0.8767 µg/mL, 4.8075 ± 0.0732 µg/mL and 0.8916 ± 0.0327 respectively.

Conclusion: Bauerenone, Bauerenol and a mixture of Stigmasterol and β-Sitosterol isolated from *A. djalonensis* showed significant ($P< 0.05$) inhibitory activities against HIV-1 Integrase. However, there was no activity against HIV-1 protease at 50 µg/mL by the extracts, fractions and isolated compounds.

Keywords: Anthocleista djalonensis; Bauerenol; Bauerenone; HIV-1 integrase; HIV-1 protease; stigmasterol; β-sitosterol.

1. INTRODUCTION

Human immunodeficiency Virus (HIV) /Acquired Immunodefiency Syndrome (AIDS) has caused death to more than 25 million people worldwide since its discovery in 1981 [1]. Several efforts have been made to develop inhibitors against HIV/AIDS. Despite, the tremendous progress in human medicine, adverse effects, the emergence of drug resistance and the narrow spectrum of activity have limited the therapeutic usefulness of the various inhibitors that are currently available on the market [2]. There are so many challenges associated with the treatment of HIV by patients using currently approved anti-HIV inhibitors [3-9]. This has led to continuous search for new anti-retroviral drugs with better efficacy, safety and affordability. For this reason, there is a continuing need for alternative inhibitors. One potential source of these inhibitors is the area of natural products. In 1987, an extensive evaluation of natural product extracts derived from microorganisms, plants, marine invertebrates and algae for HIV-inhibitory activity was undertaken [10]. Several natural products, mostly of plant origin have been shown to possess promising activities that could assist in the prevention and/or amelioration of the disease. Many of these anti-HIV agents have other medicinal values as well, which makes them potential novel leads for the development of new drugs that can deal with both the virus and the various disorders that characterize HIV/AIDS [2,11]. Many plants throughout the world, including some with documented medicinal properties, contain compounds which may be useful as antiviral agents. In our search for new classes of antiviral agents, extracts of *Anthocleista djalonensis* root was studied. *A. djalonensis* is used traditionally in the treatment of several diseases. There is no scientific information about the anti-HIV potential of *A. djalonensis*. In this paper we present the first anti-HIV screening of *A. djalonensis* against this virus.

2. MATERIALS AND METHODS

2.1 Collection of Plant Material

The root of *A. djalonensis* was obtained from Zaki Biam in Benue State. The plant was identified by Mr Ibe Ndukwe of the Forestry Department, Michael Okpara University of Agriculture Umudike. A voucher specimen was deposited in the Herbarium of Michael Okpara University of Agriculture Umudike, Nigeria. The roots were dried under a shade for three weeks and were milled at the Chemistry Department, University of Agriculture Makurdi using Thomas model 4 Willey Mill.

2.2 Extraction of Plant Material

The pulverized plant material (1.2 Kg) of *A. djalonensis* roots was macerated in methanol for one week and concentrated on a rotary evaporator at 35℃ to give a thick residue of 93.6 g of a light brown extract.

2.3 Fractionation of Crude Extract

The 93.6 g was extracted successively with hexane (4 x 100 mL), ethyl acetate (4 x 100 mL), acetone (4 x 100 mL) by maceration. The extracts were concentrated individually with rota vapor to give AF01, AF02, AF03, respectively with AF00 as the crude extract.

2.4 *In vitro* HIV-1 Integrase Assay

The effect of the ethyl acetate and acetone extracts were tested using a non-radioactive HIV-1 Integrase Assay Kit (Express Biotech International, USA) was used to measure the inhibitory effects of *A. djalonensis* on HIV-1 integrase activity. The protocol by Kapewangolo [12] was followed. Streptavidin coated 96-well plates were coated with a double-stranded HIV-1 LTR U5 donor substrate (DS) oligonucleotide containing an end-labelled biotin. Full-length recombinant HIV-1 integrase protein was then loaded onto the oligo substrate. Ethyl acetate/acetone extracts, and Chicoric acid (positive control) were added to the enzyme reaction and then a different double-stranded target substrate (TS) oligonucleotide containing 3′-endmodification was added to the reaction mixture. The use of Chicoric acid as a positive control was a modification of the protocol. The horseradish peroxidase (HRP) labelled antibody was directed against the TS 3′-end modification and the absorbance due to the HRP antibody–tetra-methylbenzidine peroxidase substrate reaction was measured at 450 nm using a plate reader (SpectraMax M2).

2.5 *In vitro* HIV-1 Protease Assay

The HIV-1 PR assay as adopted by Klos [13] was performed by cleavage of a fluorogenic substrate, Arg-Glu (EDA-NS) –Ser-Gln-Asn-Tyr-Pro_Ile-Val-Gln-Lys (DAB-CYL)-Arg in a microtiter plate format for rapid Screening of activity. A small quantity (45 µl) HIV-1 protease was added to a total of 2 ul reactions buffer (0.1M sodium acetate, 1 M Nacl, 1 mM EDTA, 1 mM dithiothreitol, 10% dimethyl sulfide, 1 mg/mL bovine serum albumin,pH4.7) containing 50 µl substrate in the presence or absence of plant extract. After incubation at 37℃ for 2 h, The fluorescence intensity was measured kinetically every 30s over a period of 10 min at an excitation wavelength of 355 nm and an emission wavelength of 460 nm, at a temperature of 37℃, using a Fluoroskan Ascent FL microplate reader (Thermolabsystems). The reaction rates were determined by the gradient of the initial linear portions (usually the first 5–10 min) of the plot of RFI (relative fluorescence intensity) as a function of time. Negative controls included were HIV-1 PR with only assay buffer, HIV-1 PR enzyme with DMSO (2%) in assay buffer and substrate alone. Positive controls included HIV-1 PR with a general acid-protease inhibitor, a potent HIV PR specific inhibitor ritonavir (Bachem Feinchemikalien AG, Bubendorf, Switzerland). The percentage inhibition of HIV-1 PR was calculated as a percentage of a control with only the solvent (2% DMSO).

$$\% \text{ Inhibition} = [(A0-A1)/A0] \times 100$$

Where A0 is absorbance of negative control

Sample A1 is absorbance of test sample.

Both Control A0 and test sample were subtracted by blank prior to calculation

2.6 Bioassay–guided Isolation of *Anthocleista djalonensis*

A preliminary bioassay-guided fractionation of most active extract (the acetone extract) was performed. An acetone solution of the extract was very viscous, presumably due to the presence of mucilages and polysaccharides. About 30 g of concentrated acetone extract was fractionated using chromatography column. Packaging was carried out using silica gel (Merck 70-30 mesh, bed surface area 500 m^2/g pore volume 0.75 cm^2) column (12 cm diameter and 62 cm lenght). Best eluent (hexane, dichloromethane and methanol) were used as mobile phase with a gradient system. Eluate was collected in several bottles of 50 mL, each of which was given a number then analyzed by TLC. This was done by spotting extract on TLC plates pre-coated with silica gel (Merck, TLC grade, with gypsum binder) using hexane, dichloromethane and ethyl acetate as solvent system. TLC bands were visualized under ultraviolent light (at 254 nm and 365 nm), by exposure to iodine and by spraying with 5% phosphomolybdic acid in ethanol, concentrated H_2SO_4, and anisaldehyde in H_2SO_4 using a gun spray. Four fractions labeled F-1, F-2, F-3, and F-4 were obtained. Further purification afforded three compounds. The antiviral assays were used to monitor the purification.

2.7 Statistical Analysis

Data represent the mean±standard deviation (SD) of the indicated number of experiments.

Graphs were prepared by Prism software. Statistical analysis of the data was carried out by one way ANOVA (Graph Pad Prism 5.02 Software). A value of $p< .05$, $p<0.01$, $p<0.0001$ were considered to be significant, very significant and highly significant, respectively. Linear regression analysis was used to calculate IC_{50}.

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 *In vitro* HIV integrase assay

The acetone extract demonstrated the highest activity against HIV integrase with IC_{50} value of 0.726 ± 0.0028 µg/mL, While IC_{50} value of 1.3001± 0.2171 µg/mL was recorded by ethyl acetate extract (Fig. 1). The results presented here showed that these extract possess anti-HIV properties of therapeutic interest. The fractionation of pharmacologically active compounds from the acetone extract of *A.djalonensis* (F-1, F-2, F-3 and F-4) revealed significant ($P < 0.05$) activity on HIV Integrase. Inhibition of HIV-1integrase was observed for fractions F-1, F-2, F-3, and F-4 with IC_{50} of 0.0077 ± 0.0009 µg/mL, 5.0001 ± 0.1719 µg/mL, 3.5113 ± 0.3613 µg/mL and 0.0736 ± 0.0005 respectively (Fig. 1). The chemical structures of the isolated compounds (Fig. 2) were determined by spectroscopic and chemical methods and their structure elucidation will be published elsewhere. All compounds exhibited significant ($P<0.05$) anti HIV-1 integrase activity (Table 2). IC_{50} value of 5.6112 ± 0.8767 µg/mL, 4.8075 ± 0.0732 µg/mL were observed for compound 1, 2 and a mixture of compound 3 & 4 with IC_{50} of 0.8916 ± 0.0327 µg/mL exhibiting the highest inhibitory activity on HIV-1 integrase.

3.1.2 *In vitro* HIV protease assay

There was no anti HIV-1 protease (PR) inhibitory activity at 50 µg/mL of the ethyl acetate, acetone extracts and fractions (F-1, F-2, F-3, and F-4) of root from *A. djalonensis*. Likewise no compound showed inhibitory activity on HIV-1 protease at 50 µg/mL (Table 1).

3.2 Discussion

In the search for anti-HIV active agents from natural products, many attempts at screening traditional medicines have been made [14-17]. However, *A. djalonensis* plant has not been investigated for their antiviral activity. The phytochemical screening of the root extract revealed the presence of saponins, flavonoids, tannins, reducing sugar, steroids, phlobatanins, volatile oils, alkaloids and terpenoids [17,18]. There are a few reports on some of their biological activities such as antibacterial and anti-malarial activities [19-21].

In this report, we investigated plant extracts/fraction and compounds from *A. djalonensis* used traditionally for treatment of several diseases targeting their inhibitory effects on HIV-1 IN and HIV-1 PR enzymes. The results showed that the ethyl acetate and acetone extracts /fractions of *A. djalonensis* had inhibitory effects on HIV-1 Integrase. This may be attributed to the chemical constituents as reported in the plant as above. However, they did not cause disfunction to the PR enzyme in an *in vitro* assay at the doses that potently blocked viral infectivity. This may reflect specific activity against PR, suggesting a protease-independent mechanism of action.

The activities demonstrated by the different types of extracts may be attributed to the diversity of structures and/or the uneven distribution of chemical constituents within these extracts, each of them with a different degree of inhibitory activity and specificity against the virus and/or its essential enzymes. The result from further purification of the acetone extract afforded a pentacyclic (compound 1: Bauerenone), a bauerane (Compound 2: Bauerenol) triterpenoids and a sterol (compound 3&4; Mixture of Stigmasterol and β-Sitosterol). This is in agreement with the presence of terpenoids and steroids in *A. djalonensis* as reported above. Plant sterols have been found to have immune modulating activity in animal models and human clinical trials [22]. Terpenoids have also been reported as one of the natural products recognized to control infections caused by HIV [23-30]. Sun et al. [31] reviewed plant-derived terpenoids and analogues with respect to their anti-HIV activity, structure-activity relationships, and mechanism of action. The active compounds included diterpenoid lactones, phenolic diterpenes, atisane and kaurane diterpenes, phorbol diterpenes, triterpene glycosides, friedelane triterpenes, taraxerane triterpenes, ursane triterpenes, lanostane triterpene, lupane triterpenes, secoring A triterpenes, degraded triterpenes, and cucurbitacin triterpenes There are some studies on the mechanism of action of triterpenoids published in the literature [11-33] and many examples where a molecular target

was discovered [34]. This target is typically a protein which the active molecule interacts with, and the disruption of this protein's normal function results in the observed biological activity. The high activity demonstrated by Bauerenone, Bauerenol and a mixture of Stigmasterol and β-Sitosterol on HIV integrase could be attributed to the impairment of the integrase normal function. The very similar IC_{50} values 5.6112 ± µg/mL and 4.8075 ± 0.0732 µg/mL showed by Bauerenone and Bauerenol respectively indicates that the basic skeleton of the compounds were responsible for the binding to the targeted enzymes. Mbouanqouere et al. [35] reported Bauerenone and Bauerenol as α-glucosidase inhibitors. Recently, Glucosidases inhibitors are of considerable interest because of anti-HIV (Human Immunodeficiency Virus) activity demonstrated by the natural competitive inhibitors – nojirimycin, and for its potential development as lead in the treatment of the AIDS (Acquired Immunodeficiency Virus) [36]. Inhibition of HIV-1 Integrase by mixture of Stigmasterol and β-Sitosterol [37] and its derivative [38] has also been reported. It is evident that β-Sitosterol facilitates the development of a potentially protective immunity against HIV [39]. The activity of the Bauerenol, Bauerenone and a mixture of Stigmasterol and β-Sitosterol may be highlighting positive new leads for drug development against HIV-integrase. However, the mechanism of action of these compounds is worthy of further investigation. Extensive study needs to progress on considering the compounds as potential therapeutic agents against HIV-1 Integrase.

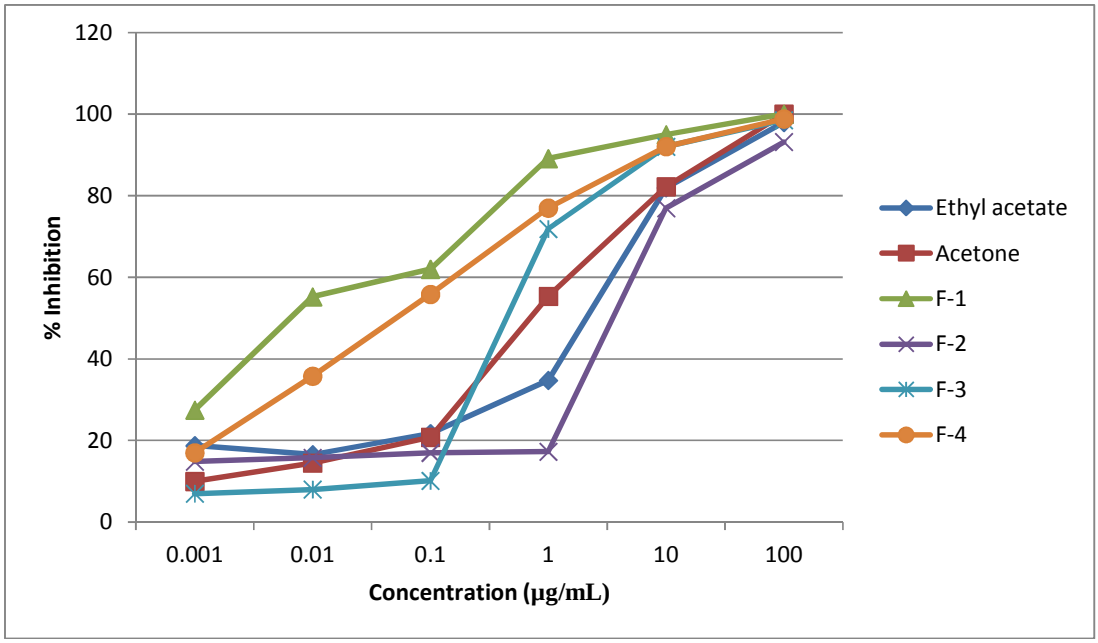


Fig. 1. The graph showing inhibitory activity of extracts and fractions on HIV -1 integrase at various concentrations

Table 1. Inhibitory activities of extracts and fractions at various concentrations on HIV-1 protease

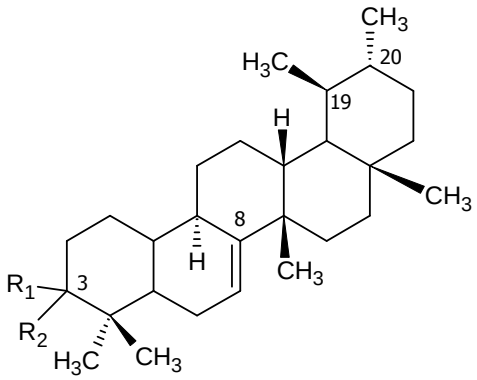
Sample name	% Inhibition at various concentration (µg/mL)						IC_{50}
	0.001	0.01	0.1	1	10	50	
Ethyl acetate	-	-	-	-	-	-	n.a
Acetone	-	-	-	-	-	-	n.a
F-1	-	-	-	-	-	-	n.a
F-2	-	-	-	-	-	-	n.a
F-3	-	-	-	-	-	-	n.a
F-4	-	-	-	-	-	-	n.a
Ritanovir*	2.98 ± 0.31	7.67±0.71	15.67±0.28	46.09±0.32	94.21±0.31	97.01±0.63	0.131±0.001

*Key: The results are Mean ± SD (n = 2), * positive control, - no inhibition, n.a = no activity*

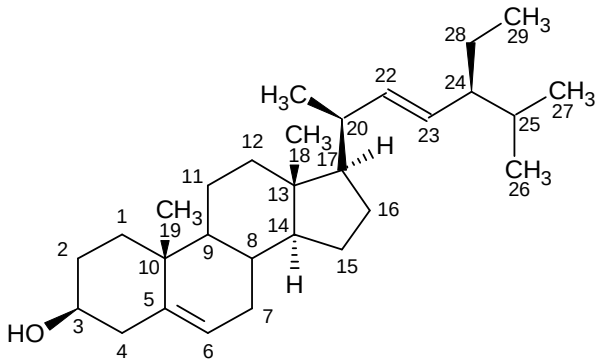
Table 2. IC_{50} values of Bauerenone, Bauerenol, Mxture of Stigmasterol and β-Sitosterol and Chicoric acid (positive control) on HIV-1-Integrase

Compounds	IC_{50}
Bauerenone	5.6112 ± 0.8767
Bauerenol	4.8075 ± 0.6732
Mxture of Stigmasterol and β-Sitosterol	0.8916 ± 0.0327
Chicoric acid*	0.0007 ± 0.0001

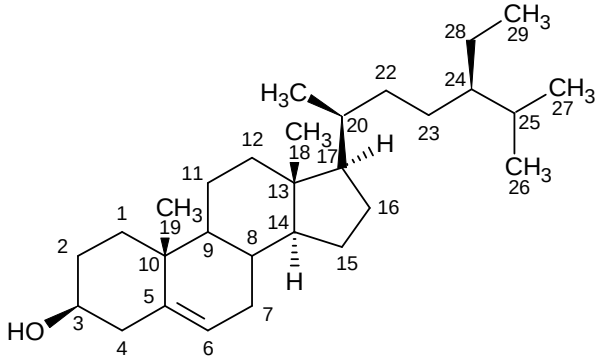
** Positive control*



1. **Compound 1: R1, R2 = O (Bauerenone)**
2. **Compound 2: R1=H, R2 = OH (Bauerenol)**



Compound 3: Stigmasterol



Compound 4: β-Sitosterol

Fig. 2. Structures of compounds isolated from acetone extract of *A. djalonensis*

4. CONCLUSION

The isolated compounds from the roots of *A. djalonensis* have been shown to be active against HIV -1 Integrase.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

ACKNOWLEDGEMENTS

The authors thank Professor Rui Krause and his Research group at Department of Chemistry, Rhodes University for allowing us the use of his laboratory.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/22147