Review Article

Isolation, characterization and anti-pepsin (HIV-Protease) activity of *Vernonia amygdalina* del and *Cymbopogon citratus* (de condole) stapf

Abstracts

The anti-pepsin enzyme (HIV-1) activities of crude extract of Vernonia amygdalina and Cymbopogon citratus were investigated using standard methods. UV-Visible spectrophotometer was used to detect the absorbance at 280 nm. The results of the anti-pepsin enzyme (HIV-1) activities showed that petroleum ether fraction of Cymbopogon citratus exhibited wavelength of maximum absorption (λ_{max}) ranging from 0.788 nm - 0.789 nm and its ethyl acetate fraction exhibited λ_{max} absorption ranging from 0.785 nm- 0.788 nm. Petroleum ether fraction of Vernonia amygdalina exhibited λ_{max} absorption ranging from 1.383 nm-1.399 nm while its ethyl acetate fraction exhibited λ_{max} absorption ranging from 1.384 nm-1.400 nm. The result of this work shows that both Cymbopogon citratus and Vernonia amygdalina ethyl acetate fraction have higher activity with Vernonia amygdalina being the highest. Column chromatography of the ethyl acetate fraction of Vernonia amygdalina led to the isolation of the compound which shows higher activity (1.384 nm- 1.400 nm). Available spectroscopic data using 1H NMR, 13C NMR and IR showed that the compound could be β-Sitosterol. The result of this work therefore showed that the extract from the leaves of Vernonia amygdalina could be used for the treatment of HIV-1(since it has the same sequence and family with the pepsin enzyme) and other diseases caused by targeted virus.

KEYWORDS; Bryophyllum pinnatum, Cymbopogon citratus, HAART, HIV, AIDS, Pepsin enzyme

INTRODUCTIUON

One of the most serious health challenges in the world is HIV which is a deadly disease and the major problems in the search for the cure for AIDS is the development of resistance against the provided drugs by the virus. Some laboratories in the world are actively involved in the investigation of anti-HIV agents that interfere with the stages of HIV replication cycle. There is no cure and no vaccine for AIDS .The only one hope for AIDS patients till now is highly active antiretroviral therapy (HAART).This therapy can increase the survival period of the AIDS patient by sustaining the viral load below50 copies/ml in blood serum. Since the start of the epidemic, 78 million people were estimated to have become infected with HIV and 35 million people have died of AIDS related illnesses. In 2016, an estimated 36.7 million people were living with HIV including 1.8 million children with a global HIV prevalence of 0.8% among adults. Around 30% of the same people do not know that they have the virus (UNAIDS, 2017).

The vast majority of people living with HIV are located in low and middle income countries, with an estimated 25.5 million living in sub-Saharan Africa. Among this group 19.4 million are living in East and Southern Africa which saw 44% of new HIV infections globally in 2016 (UNAIDS, 2017).

The number of HIV-related deaths has been falling since the mid-1990s, largely due to advances in HIV treatment. Although there is yet no cure for HIV, antiretroviral therapy significantly slows down the progression of the virus. The first drug approved by the U.S. Food and Drug Administration (FDA) to fight AIDS is azidothymidine (AZT) although the virus has however become adapted to the drugs since 1987. Today, HIV patients are given a combination of various drugs, of which AZT is often still the main component.

A rapid spread of the AIDS epidemic and the appearance of resistant HIV strains to the currently available drugs suggests that an effective and durable chemotherapy will require the use of innovative combination of drugs having diverse mechanism of anti-HIV activity and a continuous need for alternative inhibitors. New anti-HIV agents with such activities may be identified through a variety of approaches, one of them being screening of natural products (Singh *et al*., 2010).

Aim

The aim of the research is to determine the efficacy of *Vernonia amygdalina* and *Cymbopogon citratus* and isolation and characterization of active ingredients in inhibiting the activity of HIV1 virus using pepsin enzyme as a substitute.

Objective

Extraction of crude methanol, ethyl acetate and petroleum ether fractions of *Vernonia amygdalina* and *Cymbopogon citratus.*

i. Antiviral screening of ethyl acetate fraction of the two plants.
ii. Separation of extract into different chemical components using column chromatography.
iii. Analysis of fractions collected using thin layer chromatography.
iv. Screening of isolated compounds for antiviral activity.
v. Characterization of the isolated active principle responsible for the antiviral activities using spectroscopic methods.

Materials and Methods

Fresh leaves of *Vernonia amygdalina* and *Cymbopogon citratus* were separately collected from a garden around Panteka area in Igabi Local Government, Kaduna state. They were then taken to the herbarium department of the Biological Science Department, Faculty of Science, Ahmadu Bello University Zaria for identification. The identification number of *Vernonia amygdalina* is V/N 1166 and *Cymbopogon citratus* is V/N 01882. The leaves collected were separately washed, air dried at ambient temperature and pulverized by the use of a wooden mortar and pestle. The powdered samples were then stored in a polythene bag at ambient temsperature.s

Materials

Reagents (Analytical): The reagents used include petroleum ether, methanol, ethyl acetate, ethanol, sodium chloride, acetic acid, hydrochloric acid, hemoglobin, tricloroacetic acid, n-hexane, diethyl ether, chloroform, iodine crystals and pepsin enzyme. The equipment used include percolator, rotary evaporator, steam bath, Bunsen burner,The apparatus used include round bottom flask, beakers, heating mantles, test tubes, wash bottles, wash brush, glass rod, Gallenkamp spreader, chromatography column, chromatography tank, ultraviolet lamp, capillary tubes and TLC plates.

Method of Analysis

Extraction A portion (100 g) each of the powdered plants (*Vernonia amygdalina* and *Cymbopogon citratus*) were weighed and transferred into 500 cm^3 volumetric flask and 400 cm^3 of methanol was added and allowed to stand for two weeks and then filtered. The filtrates were evaporated at 40 ^oC using rotary evaporator and the extract allowed to dry and weighed.

A portion (5.0 g) of the crude extract of *Vernonia amygdalina* was then dissolved separately in 200 cm^3 of distilled water and transferred into a separating funnel. Equal volume (200 cm^3) of petroleum ether and ethyl acetate were added and the resulting mixture shaken thoroughly and allowed to stand overnight for the two layers to separate. The two layers were separately drained off and the procedure was repeated until the petroleum ether soluble fraction was completely removed. The two layers were evaporated separately using rotary evaporator. The ethyl acetate and methanol fractions were partitioned using the same procedure. Three fractions were obtained at the end of the extraction processes (petroleum ether, ethyl acetate and methanol crude extracts respectively). This procedure was repeated on *Cymbopogon citratus* plant sample (Garba and Salihu, 2009).

Procedure for Preparation of Pepsin Enzyme Solution

Pepsin enzyme (0.5 mg) was dissolved in 1cm^{3}of 0.01M HCl to give a concentration of 0.5 mg/cm^3. The resulting solution was kept chilled and prior to assay, it was further diluted to 5 cm^3 using 0.01 M HCl to obtain a working concentration of 100 µg/cm^3.

Enzyme Pepsin Inhibition Assay

The method of Aoyagi (1978) and Singh (2010) were followed for this assay with little modifications. A portion (0.5cm^3) of pepsin enzyme solution was measured and transferred into viels, 2 cm^3 of hemoglobin and 1 cm^3 of the extracts were mixed together. The procedure was repeated for all the different extract. A standard control was also prepared using hemoglobin, enzyme and the various concentrations of the anti-retroviral drugs. A negative control was ran using only hemoglobin and pepsin enzyme whereas positive control was ran along with the standard using hemoglobin, enzyme and various concentrations of antiretroviral drugs. The mixture was allowed to incubate at 37 ºC, after 20 mins, 700 µl of 5 % TCA was added to stop the reaction. It was then centrifuged at 14000 rev/min for 5 mins and the supernatant was collected. Absorbance was recorded spectrophotometrically at 280 nm. Each sample was taken in triplicate.

Procedure for the Preparation of Extract Solution

0.025 g of the ethyl acetate fraction and petroleum ether fraction of *Vernonia amygdalina* was measured into each of six sample bottles. 2 cm^3 of ethanol was then pipette into each of the sample bottles followed by pipetting 3 cm^3 of sterilized water to make it up to 5 cm^3.This procedure was also repeated for the ethyl acetate fraction and petroleum ether fraction of *Cymbopogon citratus.*

Procedure for Preparation of 2.5 % Hemoglobin

A portion (2.5 g) Worthington Bovine erythrocyte hemoglobin powder was weighed and dissolved into 40 cm^3 distilled water. The resulting solution was transferred into 100 cm^3 volumetric flask and diluted to the mark to obtain 2.5 % hemoglobin solution. It was blended in Waring blender at maximum speed for 3-5 mins and filtered. About 80 cm^3 of the filtrate was diluted with 20 cm^3 of 0.3 M HCl. (Anson, 1938 and Greenwell *et al.,* 1969).

Preparation of 0.1 M HCl

The stock solution of 1 M HCl was prepared by measuring 900 cm^3 deionized water in 1000 cm^3 graduated cylinder which was carefully poured into 1 L bottle. 100 cm^3 of 37 % HCl solution was carefully measured into 250 cm^3 graduated cylinder. The 37 % HCl was carefully poured into the 1 L bottle with deionized water.

Preparation of 0.3 M HCl

A volume of 30 cm^3 of 1 M hydrochloric acid solution was taken from the stock solution and added to distilled water to get the total volume of the solution to 100 cm^3.

Preparation of 0.01 M HCl

A volume of 1 cm^3 of 1 M HCl was taken and diluted up to 100 cm^3 with distilled water 1.00 L to give 0.01 M HCl.

Preparation of 10% Diethyl acetate

A volume 100 cm^3 of the diethyl acetate solution was taken and diluted up to 1000 cm^3 with distilled water to make 10 % diethyl acetate using volumetric flask.

Instrumentation

Procedure for Column Chromatography

Column chromatography was carried out using silica gel (60-200 mesh) as stationary phase and a glass column of 4.8 cm^2 internal diameter and a height of 270 cm^2 was used. Pre-adsorption of ethyl acetate fraction was done by dissolving 13 g of the fraction in 40 cm^3 ethyl acetate mixed with 180 g of silica gel and stirred using glass rod and allowed to dry. The column is plugged with glass wool to prevent loss of the stationary phase out of the bottom. The

glass wool is large enough but not so large to restrict solvent flow. It is positioned in the narrowest part of the column using a long glass rod.

The column was clamped securely and the tap closed. A layer of sand was added until it reaches the main body of the column. This gives the stationary phase an even base and prevents concentration and streaking of the bands as they come off the column and are collected. The column was packed using the slurry method by dissolving 400 g of the silica gel in the mixture of 95 % ethyl acetate and 5 % n-hexane. The fractions were eluted using 90 % n-hexane, 10 % diethyl ether, 85 % n-hexane, 15 % diethyl ether, 80 % n-hexane, 20 % diethyl ether, 75 % n-hexane, 25 % diethyl ether and 70 % n-hexane, 30 % diethyl ether as the ratio. The eluates were collected in fractions of 10 for each ratio and then analyzed using TLC. Fractions with similar TLC values were pulled together.

Thin layer Chromatography (TLC)

The TLC was carried out using commercially prepared TLC plates. The solution of the fraction (0.05 µml) were spotted on TLC plates and 25 % Petroleum ether, 50 % ethyl acetate and 25 % n-hexane were used to run the TLC and the spotted plate were carefully placed inside TLC tank. The resulting chromatogram was air dried and visualized using iodine vapor. Fractions with the same R_{fs} values were pooled together and labeled F_1-F_{10} (A1), F_{11}-F_{20} (A2), F_{21}-F_{30} (A3), F_{31}-F_{40} (A4) and F_{41}-F_{50} (A5).

Spectroscopic Analysis

The isolated compound was subjected to spectroscopic analysis using IR, 1 H NMR, 13 C and GC-MS.

RESULT AND DISCUSSION

Table 1 showed the various yields of the crude extracts and the quantities obtained from the two plants (*Vernonia amygdalina* and *Cymbopogon citratus*). Petroleum ether and ethyl acetate extracts of *Vernonia amygdalina* gave 7.00 g and 13.50 g respectively while the petroleum ether and ethyl acetate extracts of *Cymbopogon citratus* gave 5.60 g and 7.72 g respectively.

Table 1 Yields of the extract from 500 g of each sample.

Plants	Solvents of extraction	Weight of extracts
Vernonia amygdalina	Petroleum ether	7.00 g
	Ethyl acetate	13.50 g
Cymbopogon citratus	Petroleum ether	5.60 g
	Ethyl acetate	7.72 g

Table 2 activity of *Vernonia amygdalina* and *Cymbopogon citratus* on Pepsin Enzyme (HIV Protease) Inhibition

Plant extract	Solvent	Conc (mg/cm^3)	Absorbance			
			1st	2nd	3rd	Ave
Cymbopogon citratus	Pet Ether	0.5 mg	0.789	0.790	0.785	0.789
		0.25 mg	0.791	0.792	0.786	0.789
		0.0125 mg	0.790	0.791	0.787	0.788
	Ethyl acetate	0.5 mg	0.787	0.788	0.790	0.788
		0.25 mg	0.786	0.787	0.789	0.787
		0.0125 mg	0.786	0.784	0.786	0.785
Vernonia amygdalina	Pet Ether	0.5 mg	1.650	1.387	1.111	1.399
		0.25 mg	1.654	1.400	1.112	1.389
		0.0125 mg	1.662	1.411	1.124	1.383
	Ethyl acetate	0.5 mg	1.652	1.389	1.111	1.400
		0.25 mg	1.655	1.402	1.113	1.390
		0.0125 mg	1.663	1.412	1.124	1.384
Hemoglobin and extract (Control C1)			0.778	0.777	0.778	0.778
Lamivudine (Anti-retroviral Drug C2)		0.5 mg	0.780	0.781	0.782	0.784
		0.25 mg	0.783	0.782	0.782	0.782
		0.0125 mg	0.783	0.783	0.785	0.781

In Table 2, The Absorbance increases with increase in concentration. The absorbance of the plant extract is higher than the absorbance of the control (C1) and the anti-retroviral drug (C2). The wavelength of maximum absorption of hemoglobin is 2.114.

Table 2 shows the effect of the activity of *Vernonia amygdalina* and *Cymbopogon citratus* on Pepsin enzyme (HIV Protease) inhibition. The results show that Petroleum ether fraction of *Cymbopogon citratus* gave the wavelength of maximum absorption ranging from 0.788-0.789 and its ethyl acetate fraction gave wavelength of absorption ranging from 0.785-0.788. The results of Petroleum ether fraction of *Vernonia amygdalina* shows wavelength of maximum absorption ranging from 1.399-1.383 and its ethyl acetate fraction ranging from 1.4 - 1.384.The result of the positive control where no extract was added gave wavelength of maximum absorption at 0.778 and the result of anti-retroviral drug gave wavelength of maximum absorption ranging from 0.784-0.781. The results therefore show that both petroleum ether and ethyl acetate fractions of *Vernonia amygdalina* had higher absorbance indicating that it was more active in suppressing the activity of the enzyme on hemoglobin. The activity of both petroleum ether and ethyl acetate fraction of *Cymbopogon citratus* were similar with the activity of anti-retroviral drug and positive control indicating that *Cymbopogon citratus* has little or no effect on the activity of pepsin enzyme. This has been shown from studies that the different categories of antiretroviral can only suppress HIV replication in about 80% of cases. They inhibited the different steps in the replication of the virus but did not eliminate it from the body (Thompson *et al*., 2010). Similar report was given on the potency of plant extract on HIV Protease by Govindappa *et al.* (2011).From the results above, the petroleum ether and ethyl acetate extracts of *Vernonia amygdalina* have proven potential inhibition of the pepsin enzyme activity due to the different phytochemical constituents present in the plant extract in line with observation made by Ojiako and Nwajo (2006).

Table 3 Activity of pooled fraction on pepsin enzyme (HIV-Protease) inhibition

Solvent of elution	Pooled fraction	Color	RF Value	Weight
n-hexane-Diethyl ether 95%-5%	F_1,F_2,F_3,F_4,F_5,F_6 F_7,F_8,F_9F_{10}(A1)	Colorless	0.06	0.113
n-hexane-Diethyl ether 90%-10%	$F_{11},F_{12},F_{13},F_{14},$ $F_{15},F_{16},F_{17},F_{18},$ F_{19},F_{20}(A2)	Light green	0.61	0.158
n-hexane-Diethyl ether 85%-15%	$F_{21},F_{22},F_{23},F_{24}$ $F_{25},F_{26},F_{27},F_{28},$ F_{29},F_{30}(A3)	Green	0.36	0.751
n-hexane-Diethyl ether 80%-20%	$F_{31},F_{32},F_{33},F_{34},$ $F_{35},F_{36},F_{37},F_{38},$ F_{39},F_{40} (A4)	Dark green	0.12	0.167
n-hexane-Diethyl ether 75%-25%	$F_{41},F_{42},F_{43},F_{44},$ $F_{45},F_{46},F_{47},F_{48},$ F_{49},F_{50} (A5)	Dark green	0.25	0.176

The table 3 shows the result of the effect of the activity of the pooled fraction of the ethyl acetate fraction of *Vernonia amygdalina* on Pepsin enzyme inhibition. These results show that the absorption ranges from 1.443 – 1.450. At lower concentration of 0.25 mg, the absorbance of the fraction was 1.450 nm which shows higher activity compared to the wavelength of maximum absorption of ethyl acetate fraction at 0.5 mg which is 1.400. It therefore shows that the pooled fractions have higher ability in suppressing the activity of the enzyme, as the wavelength of maximum absorption was very close to the initial wavelength of maximum absorption of the hemoglobin which is 2.114.

list 1 : Column chromatography of the ethyl acetate extract of *Vernonia amygdalina* (leaves), Pooled Fractions and their Weights

Fraction	Concentration (mg/cm^3)	Wavelength of Max Absorbance (nm)
A3 ($F_{21} – F_{30}$)	0.25 mg 0.125 mg 0.0625 mg	1.450 1.448 1.443

The table showed the color, R_f values and the weights of *Vernonia amygdalina* extract obtained from the pooled fractions. The colour of the fractions range from colorless to light green and dark green. Thin layer chromatography of the fractions indicate that some are similar. Similar fractions were therefore pooled and recorded respectively. Fractions $F_1,F_2,F_3,F_4,F_5,F_6,F_7,F_8,F_9$ and F_{10} were pooled together as they had similar R_f values (0.06) and coded A_1, fractions $F_{11},F_{12},F_{13},F_{14},F_{15},F_{16},F_{17},F_{18},F_{19}$ and F_{20}were pooled together as they had similar R_f values (0.61) and coded A2, fractions $F_{21},F_{22},F_{23},F_{24},F_{25},F_{26},F_{27},F_{28},F_{29}$ and F_{30} were also pooled together as they had similar R_f values (0.36) and coded A3,fractions $F_{31},F_{32},F_{33},F_{34},F_{35},F_{36},F_{37},F_{38},F_{39}$ and F_{40} were pooled together as they had similar R_f values (0.12) and coded A4, fractions $F_{41},F_{42},F_{43},F_{44},F_{45},F_{46},F_{47},F_{48},F_{49}$ and F_{50} were also pooled together as they had similar R_f value (0.25) and coded A5.Thin layer chromatography of pool fraction $F_{21},F_{22},F_{23},F_{24},F_{25},F_{26},F_{27},F_{28},F_{29}$, and $F_{30}$was further carried out and it gave a single spot of R_f value 0.36, indicating high level of purity, thus it was subjected to enzyme pepsin inhibition assay and spectroscopy analysis.

Spectroscopic Analysis

The FTIR spectrum of compound A3 (Beta-Sitosterol) shown in Plate 3 was obtained by analyzing the sample on Agilent FTIR Technologies. The IR spectrum showed a strong absorption at 3302.4 cm^{-1} which was due to polymeric hydroxyl (OH) compound. Absorption at 2847.7 cm^{-1} was assigned to C-H structure. The presence of methyl (CH_3) asymmetric stretching was confirmed by the presence of the peak at 2952 cm^{-1}. Absorption at 1654 cm^{-1} was due to alkenyl stretch and the presence of methylene (C-H) bend was further confirmed by the presence of the peak at 1481 cm^{-1}.
The 1H NMR and 13C NMR spectra of A3 shown in appendix 1 and 3 were obtained by analyzing the sample on Agilent – NMR Technologies. The 1H NMR spectrum showed that H-3 proton appeared as a single peak at 3.303 ppm which is a hydroxyl group. The signal at 4.192 ppm was due to an olefinic proton. The signal at 1.652 ppm was due to methyl protons and the peaks at 2.089 ppm and 2.033 ppm was due to methylene protons.

The 13C NMR spectrum of compound A3 (Fig 5) has shown recognizable signal at 140.307 ppm (C-5) which is quartenary carbon and 121.245 ppm (C-6) is olefinic carbon. Signals at 77.388 ppm (C-3), 56.121 ppm (C-14), 50.996 ppm (C-17), 39.929 ppm (C-9), 45.781 ppm (C-24), 33.098 ppm (C-20), 31.601 ppm (C-8) and 29.658 ppm (C-25) was due to methine carbon. The peaks at 42.247 ppm (C-4), 39.929 ppm (C-12), 31.910 ppm (C1), 31.865 ppm (C-22), 31.608 ppm (C-7), 29.681 ppm (C-2), 25.987 ppm (C-16), 26.232 ppm (C-23), 26.002 ppm C-15), 24.281 ppm (C-28) and 23.025 ppm (C-11) were methylene carbon. The signals observed at 21.050 ppm (C-26 and 27), 19.382 ppm (C-21), 18.995 ppm (C-19), 11.957 ppm (C-29) and 11.957 ppm (C-18) were assigned to methyl carbons. The values at 11.957 ppm (C-18) and 18.995 ppm (C-19) are angular carbon atoms while signals at 36.476 ppm (C-10) and 42.247 ppm (C-13) are assigned to carbons attached to the methyl groups.

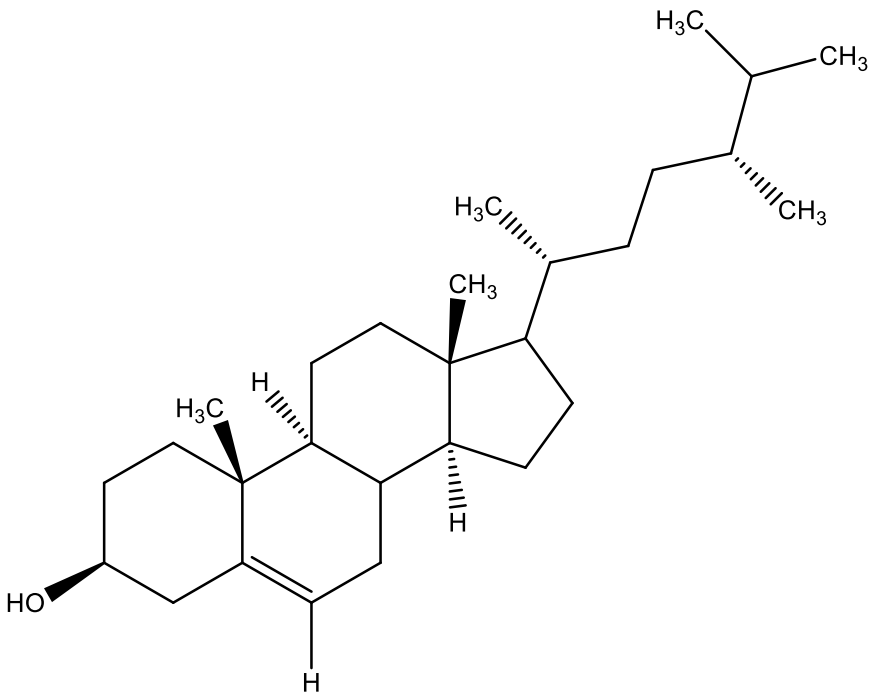

Figure 1 Beta- Sitosterol

Table 4 Chemical shift and the position of carbon atoms in compound A3

Carbon atom	13C NMR Experimental	13C NMR Literature	1H NMR Experimental	1H NMR Literature	Nature of Carbon
C-1	31.91	36.72			CH_2
C-2	29.68	29.71			CH_2
C-3	77.39	71.97			CH
C-4	42.25	42.35			CH_2
C-5	140.31	140.94			C=C
C-6	121.25	121.32	4.19	5.38	C=CH
C-7	31.61	31.71			CH_2
C-8	31.60	29.24			CH
C-9	39.93	50.02			CH
C-10	36.48	36.16			C
C-11	23.03	24.32			CH_2
C-12	39.93	39.82			CH_2
C-13	42.25	40.45			C
C-14	56.12	56.90			CH
C-15	26.00	24.32			CH_2
C-16	25.99	28.90			CH_2
C-17	51.00	56.03			CH
C-18	11.64	12.06	1.65	1.29	CH_3
C-19	19.00	19.06			CH_3
C-20	33.10	39.82			CH
C-21	21.05	23.12			CH_3
C-22	31.87	138.40			CH_2
C-23	26.23	129.34			CH_2
C-24	45.78	51.26			CH
C-25	29.658	34.01			CH
C-26	21.05	21.12			CH_3
C-27	21.050	22.82			CH_3
C-28	24.281	25.32			CH_2
C-29	11.957	12.06			CH_3

Taking the information above into consideration (1H NMR, 13C NMR and Infra-red spectroscopy), Compound A3 isolated from the leaves extract of *Vernonia amygdalina* is proposed as beta-Sitosterol. The structure of the isolated compounds were identified on the basis of spectroscopic methods and by comparing their physical properties reported in the Literature (Luhata *et al.*, 2015)

CONCLUSION

Vernonia amygdalina and *Cymbopogon citratus* show inhibition against Pepsin enzyme by inhibiting the activity of the enzyme to the hemoglobin. The extracts of the two plants showed higher activity compared to the anti-retroviral drugs since they were able to inhibit the activity of the enzyme more than the drug. The compound isolated (β- Sitosterol) shows high activity against the pepsin enzyme. (HIV-1) which belongs to the same family, hence the compound may inhibit the activity of HIV-1.

RECOMMENDATION

i. Other plants that have not being tested against Pepsin enzymes (HIV-1) could be tested to determine their efficacy against the virus.

ii. Other method of testing the activity of the enzymes on other substrate should also be explored using this extract.
iii. Effort should be geared towards administering the plant extracts to infected people especially during the early stage of infection.

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