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Evaluation of Activity of Cassia Occidentalis *Leaf Extracts*

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ABSTRACT

Air dried leaves of *Cassia occidentalis* were ground and percolated with 95 % ethanol. The ethanol extract (CO1) was partitioned sequentially with n – hexane, chloroform, ethyl acetate and methanol (60 %). These were labeled CO1-1, CO1-2, CO1-3 and CO1-4 respectively. Each of these fractions was screened for the presence of secondary metabolites. The fractions were subjected to antiretroviral bioassay. Fraction CO1 was found to be the most active against the most active against the virus. CO1 (10 g) was further subjected to column chromatography which yielded two pure white powdery solid compounds named CO1-01A (62.15 mg) and CO1-01B (6.18 mg). Secondary metabolites such as alkaloids, flavonoids, reducing sugars, steroids, tannins and saponins were found to be present. CO1 fraction at a low concentration of 1.25 mg/cm^3 showed an antiretroviral activity of 94.13 % compared to Nevilast-30 (an active drug) with antiretroviral activity of 96.19 %, used as positive control. ^{1}HNMR, ^{13}CNMR, FTIR and mass spectra of the compound CO1-01A suggest its structure to be 2, 3-dichloro-1, 4-dioxane. This study supports the traditional use of *Cassia occidentalis* leaves for the treatment of HIV/AIDS in different regions of the world.

Keywords: Antiretroviral, *Cassia occidentalis*, HIV/AIDS, Phytochemical, Secondary metabolites

Introduction

Human Immunodeficiency Virus (HIV) is an RNA retrovirus infection agent that causes Acquired Immunodeficiency Syndrome (AIDS), a disease that leaves a person vulnerable to life threatening infections. Two types of these virus were identified by scientists HIV- 1 which is the cause of AIDS worldwide and HIV- 2, found mostly in West Africa (UNAIDS, 2000). Acquired Immunodeficiency Syndrome was first reported in 1981 in the U. S. A, and by 1982 clusters of AIDS patients appeared among homosexuals also in the U. S (Arora and Arora 2008, Trust 2010). In a more recent perspective, doctors make diagnosis of AIDS in patients with CD_4 count below 200 cells per micro litres of blood regardless of the associated illness they may have (Aledort *et al.* , 1993).

Progress has been made in research on natural products which effectively inhibit HIV-1 replication. Many active compounds were isolated from medicinal plants which include; aerial parts of *Coleus parvifolius* which showed 4 out 11 ethanol extracted isolates to exhibit inhibitory activities against HIV-1 (Tewtrakul *et al., 2003*). Minerals such as selenium and zinc, beta-carotene, vitamin E and mushroom (Suzuki *et al.*, 1989) also inhibit the replication of HIV.

Cassia occidentalis is a small tree growing 5-8 meters in height. The leaves are compound, composite and peripinnate with 5-8 pairs of leaflets usually oval. Inflorescence occurs at auxiliary or terminal, yellow, short cluster of flower. Fruit is a pod, narrow, flat slightly curved about 15 cm long with 10-12 seeds brownish at maturity (Mann, 2003).

Traditionally the root, leaves, flowers and seeds of *Cassia occidentalis* are used as laxative and purgative (Todd, 1967). It is vermifuge, anticonvulsant and used against chicken pox (Mann, 2003). Other uses include febrifuge and extrusion of guinea worm (Iwu, 1993). Previous studies have shown that its leaves exhibit invitro antibacterial, antimalarial and antihepatotoxic properties (Gasquet, 1993; Percez, 1994; Saraf, 1994). Phytochemically the aqueous extract of *Cassia occidentalis* contained tannins, anthraquinones, sterols, cardiac glycosides, saponins and alkaloids (Muyibi *et al*., 2000).

The orthodox medications were expensive and not readily affordable by the common man. Furthermore, the drugs have devastating side effects, hence rejected by most of the users. Consequently, search for agents from natural origin such as plants or animals with immune modulation and antiretroviral potentials may assist in the fight against the major calamity since 20th century.

Widespread awareness of HIV disease began with a brief report in 1981, published in the Morbidity and Mortality Weekly Report of a rare pneumonia *Pnuemocystis carinii* (now known as *Pnuemocystis jiroveci*) followed by other unusual infections in five young homosexual men in Los Angeles (MMWR, 1981).

CD_4 responses to HIV are important in viral control and are associated with lower HIV viral loads. The fact that HIV infects CD_4 cells themselves is an evolutionary strategy with a number of consequences. Depletion of CD_4 lymphocytes is the hallmark of HIV infection, and predicts individuals risk for infection with opportunistic pathogens as other complications of HIV diseases (Haynes *et al.,* 1996). Progress has been made in research on natural products which effectively inhibit HIV-1 replication. Many active compounds were isolated from traditionally used medicinal plants which include the methanol *Acacia nilotica* bark and pods, the leaves of *Euphorbia granulate* the stem bark of *Maytenus senegalensis* and aqueous extracts of *Acacia nilotica* pods and stem bark of *Maytenus senegalensis* showed considerable inhibitory effects against HIV-1 protease. Chinese medicinal herbs with antiviral activity *Prunella vulgaris* and *Rhizoma cibotta* extracts of these two plants significantly decreased after they were passed through polyamide resin mini column (Liu *et al.,* 2002), which are able to bind polyphenols including tannins, an HIV-1 inhibitor with multiple mechanism of action.

This research aimed at evaluating the phytochemical constituents and antiretroviral activity of *Cassia occidentalis* leaves extract.

Materials and Methods.

Collection and identification of plant material.

Fresh leaves of *Cassia occidentalis* were collected at Thomas dam in Dambatta Local Government Area of Kano State, Nigeria on 31st October, 2009. The plant was authenticated at Department of Biological Sciences, Faculty of Science, Bayero University Kano, Nigeria.

Extraction and Partitioning

Two hundred grams (200 g) of the air dried and ground samples of *Cassia occidentalis* (leaves) was percolated with 800 cm^3 of 95 % absolute ethanol according to Fatope *et al.,* (1993). After regular shaking, the sample was filtered and the residue obtained was repercolated for seven days with about 400 cm^3 of 95 % absolute ethanol and combined. It was concentrated at 40 ^oC under reduced pressure using Gallenkamp rotavapor and weighed. The crude ethanol extract (8 g) was dissolved in 60 % aqueous methanol (200 cm^3) in a separating funnel and sequentially partitioned with n-hexane, chloroform and ethyl acetate (100 cm^3x 3) each. The extract were concentrated using rotavapor, weighed and labeled as CO1, CO1-1, CO1-2, CO1-3 and CO1-4 representing crude ethanol extract, n-hexane, chloroform, ethyl acetate and aqueous methanol fractions respectively. The soluble fractions were stored in a freezer before phytochemical screening and antiretroviral bioassay.

Phytochemical screening of the extract

The fractions obtained were characterized and screened for the presence of alkaloids, flavonoids, reducing sugars, steroids, tannins and saponins. The screening was performed according to the standard methods of El-olemy *et al.,*(1994) , Sofowora (1984) , Brain and Turner (1975) and Harbone (1975).

Antiretroviral Bioassay

HIV positive blood samples were collected from Hasiya Bayero Hospital and used immediately for the antiretroviral assay.

Serial dilution was used in preparing concentration from different fractions; the final concentrations prepared by dilution were 1.50, 2.50, 5.00 and 10.00 mg/cm^3 using dimethylsulphoxide (DMSO) as the solvent. 25 µl of the various concentration of the extract mixed with RPMI culture media was added to 25 µl of the HIV positive lymphocyte (blood) sample and introduced into a vial. The initial CD_4 cells count of the HIV positive blood sample was first determined by the CD_4 machine. The mixture was allowed to incubate for 30 minutes at 37 ^0C. Finally the CD_4 cells count for the various mixtures of the soluble fractions were determined using the CD_4 machine (Bashir M 2010).

Column Chromatography

Silica gel (50-200 mesh) about 300 g was mixed with about 500 cm^3 of petroleum ether (60-80) to form the slurry. The column (125-2.5 cm) outlet was blocked using a clean cotton wool. The slurry was poured into the column through a funnel from the top, with addition of some petroleum ether till all the silica gel was poured into the column. Conical flask was placed at the bottom of the column for collecting the drained solvent. When the silica gel settled, the column was washed with 500 cm^3 of Chloroform and fresh petroleum ether each. The column was loaded by mixing about 20 g of silica gel with 10 g of CO1 until the mixture became non-sticky powder; this was then loaded into the column through a funnel.

The column was run by eluting solvents in order of increasing polarities as follows; Petroleum ether (60-80) (1000 cm^3), a mixture of petroleum ether-chloroform in a ratio (1:1, 1000 cm^3) (1:3, 1000 cm^3) each, chloroform (1000 cm^3), mixture of chloroform-ethyl acetate in a ratio (4:1, 1000 cm^3), (3:2, 1000 cm^3), (1:1, 1000 cm^3), (2:3, 1000 cm^3), (1:4, 1000 cm^3) each, ethyl acetate (1000 cm^3), mixture of ethyl acetate-methanol in a ratio (4:1, 1000 cm^3), (3:2, 1000 cm^3), (1:1, 1000 cm^3) each and methanol (1000 cm^3). The eluents collected in fractions of 100 cm^3 receiving bottles were evaporated to dryness and analyzed on TLC plates to determine purity and identical ones with equal R_f values were pooled together (Sharma and Achaya, 1988).

Results and Discussion

Air dried leaves of *Cassia occidentalis* percolated with 95 % absolute ethanol yielded a crude ethanol extract CO1 (11.85 %), which after partitioning into n-hexane, chloroform, ethyl acetate and methanol yielded CO1-1, CO1-2, CO1-3 and CO1-4 respectively. The weight, colour, texture, and percentage recovery of the crude ethanol and partitioned fractions are as in table 1 as follows.

Table 1: Weight of crude ethanol and partitioned fractions obtained.

Fraction	Weight (g)	Colour	Texture	Percentage recovery (%)
CO 1	23.70	Dark Green	Sticky	11.85
CO1-1	2.10	Orange	Solid	26.25
CO1-2	2.00	Yellow	Solid	25.00
CO1-3	2.50	Dark Green	Sticky	31.25
CO1-4	1.95	Brownish	Gummy	24.38

Key: CO1; crude ethanol fraction, CO1-1; n-hexane soluble fraction, CO1-2; chloroform soluble fraction, CO1-3; ethyl acetate soluble fraction and CO1-4; aqueous methanol soluble fraction.

Phytochemical screening revealed the presence of alkaloids, tannins, glycosides, flavonoids and saponins in *Cassia occidentalis* methanol, n-hexane, chloroform and aqueous leaf extract while no steroids was detected in all the four extract as shown in table 2. (Saganuwan and Gulumbe, 2006).

Table 2: Results of phytochemical analysis.

Fractions	Alkaloids	Flavonoids	Reducing Sugars	Steroids	Tannins	Saponins
CO1	+	-	+	-	+	+
CO1-1	-	-	-	-	-	-
CO1-2	+	+	-	-	-	+
CO1-3	+	-	+	-	+	+
CO1-4	+	-	-	-	-	+

Key: + = present, - = absent.

Generally, at a lower concentration of the various fractions the replication of the virus in the human lymphocytes was inhibited, this clearly shows that the human immune system can be boosted or the immunity of the CD_4 cells boosted. Higher concentration of 10.00 mg/cm^{3}of n-hexane, ethanol and chloroform did not show any response Ethanol extract at a 1.25 mg/cm^3 inhibited the replication of the virus and increased the CD_4 cells count from 305 cells/µl to 325 cells/µl. (It showed an activity of 94.13 %). On comparison Nevilast-30 (an active drug) at a lower concentration of 0.193 mg/cm^3 increases the CD_4 cells count from 582 cells/µl to 605 cells/µl (It showed an activity of 96.19 %) used in the positive control. Table 3 shows the CD_4 cells count of fractions at various concentrations.

Table 3: The CD_4 cells count for the various fractions of the leaf extract of *Cassia occidentalis.*

Fractions count	Concentration (mg/cm^3)	CD_4 cells (cells/µl)
CO1 (455)	1.25	324
	2.50	295
	5.00	300
	10.00	-
CO1-1 (1019)	1.25	335
	2.50	365
	5.00	-
	10.00	-
CO1-2 (296)	1.25	256
	2.50	224
	5.00	260
	10.00	-
CO1-3 (3646)	1.25	625
	2.50	575
	5.00	614
	10.00	286
CO1-4 (022)	1.25	492
	2.50	508
	5.00	515
	10.00	474

Key: - no response by the machine, Blood Sample No 022 Initial CD_4 cells Count 459 cells/µl, Blood Sample No 296 Initial CD_4 cells Count 247 cells/µl, Blood Sample No 455 Initial CD_4 cells Count 305 cells/µl, Blood Sample No 1019 Initial CD_4 cells Count 350 cells/µl, Blood Sample No 3646 Initial CD_4 cells Count 582 cells/µl, **CD_4 cell count for positive control (Nevilast-30 an active drug) measured on blood sample No 3646 was 605 cells/µl.**

The isolates CO1-01A and CO1-01B were washed thoroughly with chloroform to remove a light brown liquid which was soluble in chloroform. The residue was rinsed with more chloroform and allowed to dry in a fume cupboard; white substances CO1-01A (62.15 mg) and CO1-01B (6.18 mg) were obtained. CO1-01A and CO1-01B give a single spot with their R_f values as given on table 4 below

Table 4: Purification of CO1-01A and CO1-01B

Isolate (mg)	Solvent System	R_f value	Mass
CO1-01A	EA: ME (9:1)	0.85	62.15
CO1-01B	EA: ME (3:2)	0.76	6.18

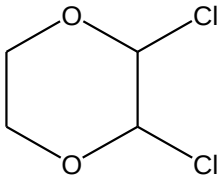
___Key:- EA: Ethyl acetate and ME: Methanol

The ^1H, ^{13}C NMR, IR and Mass spectral data were combined in proposing the structure of the compound. Two different electronic environments were observed in the ^1H NMR. The chemical shift at δ 4.00 indicates ($O-CH_2$) (4H, s) protons, and chemical shift at δ 7.40 indicates (O-CH-Cl) (2H, s) protons, ^{13}C NMR analysis indicate the presence of four (4) carbon atoms in two different chemical environments of a dioxane. Signal at δ 40.70

indicates O-CH-Cl, and signal at δ 40.49 indicates O-CH_2.

Infrared absorption spectral studies were used to identify the functional group for the compound. Prominent vibrational/ rotational frequencies were observed in the following regions. C-O stretching of a dioxane at a signal of 1024.24 cm^{-1} and a dichloro C-Cl stretching was vividly observed at 757.09 cm^{-1}.

The proposed structure of the isolated pure compound CO1-01A was given as follows with the IUPAC name "2, 3-dichloro-1, 4-dioxane".



The ethanol extract (CO1) contain tannins which is a polyphenols and it inhibits the replication of virus just like the Chinese medicinal herbs *Prunella vulgaris and Rhizoma cibotta.* The ethanol extract at a lower concentration of 1.25 mg/cm^3 showed an antiretroviral activity of 94.13 % compared to Nevilast-30 (an active) drug which had an antiretroviral activity of 96.19 % used as a positive control. The ethanol extract of *Cassia occidentalis* (leaves) contains secondary metabolites tannins which is a polyphenols and has the potential of inhibiting the viral replication for curing HIV. Therefore this research work establishes the scientific basis of *Cassia occidentalis* as an ethno medicinal plant that can be used in treatment of HIV/AIDS.

Recommendation

It is recommended that further work be carried out on all the soluble fractions of the plant in order to isolate more active antiretroviral compounds and secondary metabolites.

Conclusion

Based on the research carried out it clearly indicated that *Cassia occidentalis* leaves extract has a medicinal value in treatment of HIV/AIDS, or can be used in suppressing the replication of virus.

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