Extracts from *Dodonaea viscose* stems (Linn.) as HIV-1 inhibitors and Chemical constitutents

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ABSTRACT

This study deals with the evaluation of anti-HIV-1 effect of *Dodonea viscosa* stems extracts and also the investigation of the chemical constituents from *D. viscosa* extracts. Petroleum ether, chloroform and methanol 80% extracts of *D. viscosa* were tested for their anti-HIV-1 activity using the syncytia formation assay. Petroleum ether extract of *D. viscosa* was the most active as an anti-HIV-1 agent while the other extracts were less effective. Phytochemical analysis of the petroleum ether extract and other extracts have shown the presence of the following phytochemicals, flavonoids, tannins, triterpenes, carbohydrates and saponins while further chromatographic separation of the biocomponents from petroleum ether extract resulted in the isolation and identification of *β*-sitosterol and stigmasterol compounds. These results highlight that *Dodonea viscose* stems petroleum ether extract has potential as an anti- HIV-1 agent.

Keywords: *Dodonea viscosa*, stems, cytotoxicity, anti-HIV-1 activity, chemical constituents.

INTRODUCTION

Human immunodeficiency virus type-1 (HIV-1) is the cause of acquired immune deficiency syndrome (AIDS). Plant substances are especially explored due to their amazing structural diversity and their broad range of biological activities. Several plant extracts have been shown to possess activity against HIV by inhibiting various viral enzymes (Vermani *et al*., 2002) also medicinal plants as potential sources of new active agents not only combine the advantage of being relatively non-toxic and hence more tolerable than rationally designed drugs, but also represent an affordable and valuable source of pharmacologically active substances that can be made sufficiently available through cultivation. *Dodonaea viscosa* Linn. family: Sapindaceae is an evergreen shrub. It is a native to Australia, indigenous and wide spread throughout the tropics. *Dodonaea viscosa* has many medicinal properties and it is a traditional medicine worldwide, administered orally or as poultice to treat a great variety of ailments (Rojas *et al*., 1996). Stem or leaf infusions are used to treat sore throats; root infusions to treat colds. The stems and leaves are used to treat fever and seeds to treat malaria. The stems are used as fumigants to treat rheumatism. The leaves are used to relieve itching, fevers swellings, aches and can be used as a antispasmodic agent (Rojas et al., 1996). Leaves and roots as a painkiller to soothe toothaches and headaches (Cribb and Cribb, 1981). The flowers are used as a "home-brew "substitute to bestow a bitter flavor, and also as a tonic (Wagner et al., 1987). Previous Phytochemical studies showed that 23 flavones were isolated from seeds, bark, flowers and leaves of *D. viscosa*, (Ghisalberti, 1998), also eighteen flavonoids including glycosides of quercetin and isorhamnetin were isolated previously by (Siddiqui 1998). Large concentrations of quercetin, kaempferol and isorhamnetin in *D. viscosa* crude leaf extract were identified by (Getie *et al.*, 2000). Previous experimental studies on the plant have demonstrated antimicrobial and anti-inflammatory (Getie *et a*l., 2003), anti-ulcer (Veerapur *et al*., 2004), wound healing (Joshi *et al*., 2003), local anaesthetic and smooth muscle relaxant activities (Rajas *et al*., 1996). The present study was carried out to evaluate anti-HIV-1 effect of the extracts *Dodonaea viscose* stems and to investigate the phytoconstituents in the plant aerial parts extracts.

MATERIALS AND METHODS

Experimental:

^{1}H-NMR spectra: Varian Unity Inova 400 (400 MHz); ^{13}C-NMR spectra: Varian Unity (100 MHz) (Graz University, Austria). MS (Finnigan MAT SSQ 7000, 70 ev). (Silica gel [0.063-0.200 mm] for column chromatography), Silica gel F_{254} was used for analytical thin layer chromatography (TLC) and Sephadex LH-20 (Pharmacia Fine Chemicals). Solvents for extraction from El- Nasr Company, Egypt.

Plant Material

Stems of *Dodonea viscosa* were collected from the Orman garden, Giza, Egypt in May 2011 during flowering and the plant was identified by Dr. Mohammed El-Gebaly, Department of Botany, National Research Centre (NRC) and by Mrs. Tereez Labib Consultant of Plant Taxonomy at the Ministry of Agriculture and director of Orman botanical garden, Giza, Egypt. A voucher specimen is deposited in the herbarium of Orman garden, Giza, Egypt.

Reagents

AZT (3'-azido-3'-deoxythymidine) was purchased from Sigma. All extracts were dissolved in DMSO. AZT was dissolved in RPMI-1640 and stored at -20^{o}C. HEPES (N-2(2-Hydroxyothyl) piperazine-N'-(2-ethanesufonic acid), MTT (3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), DMF (N, N'-Dimethyl formamine), Penicillin, Streptomycin sulfate, Glutamine were purchased from Sigma; 2-ME (2-Mercaptoethanol) was purchased from Bio-Rad. RPMI-1640 and fetal bovine serum (FBS) were purchased from Gibco.

Cells and virus

C8166 cells and HIV-1IIIB were kindly donated by Medical Research Council, AIDS Regent Project. The cells were maintained at 37^{o}C in 5% CO2 in RPMI-1640 medium supplemented with 10% heat-inactivating FBS (Gibco). HIV-1IIIB was prepared from the supernatants of H9/HIV-1IIIB cells. The 50% HIV-1 tissue culture infectious dose (TCID50) in C8166 cells was determined and calculated by (Reed and Muench, 1938). Virus stocks were stored in small aliquots at -70^{o}C.

Cytotoxicity assay

The cellular toxicity of the extracts on C8166 cells was assessed by MTT colorimetric assay. Briefly, 100µl of 4×10^{5} cells were plated into 96-well plates, 100 µl of various concentrations of compounds was added and incubated at 37^{o}C in a humidifed atmosphere of 5% CO_2 for 72 h. 100 µl of supernatant was discarded, MTT reagent was added and incubated for 4 h and 100µl 50% DMF-20% SDS was added. After the formazan was dissolved completely, the plates were read on a Bio-Tek ELx 800 ELISA reader at 570 nm/630 nm. 50% cytotoxicity concentration (CC_{50}) was calculated (Wang et al., 2008).

Inhibition of syncytia formation

The effect of extracts on acute HIV-1 infectivity was measured by the syncytia formation assay (Huang *et al*., 2012). In the presence or absence of various concentrations of samples, 4×10^{4} C8166 cells were infected with HIV-1 at a multiplicity of infection (MOI) of 0.015, and cultured in 96-well plates at 37 ^{o}C in 5% CO_2 for 3 days. AZT was used as a positive control. At 3 days post-infection, the cytopathic effect (CPE) was measured by counting the number of syncytia (multinucleated giant cell) in each well of 96-well plates under an inverted microscope (100×). The inhibitory percentage of syncytia formation was calculated by the percentage of syncytia number in sample-treated culture compared to that in infected control culture 50% effective concentration (EC_{50}) was calculated according to the method described by (Reed and Muench, 1938), 50% cytotoxic concentration (CC_{50}) and 50% effective concentration (EC_{50}) was determined from dose–response curve. Therapeutic index (TI of anti-HIV activity is CC_{50}/EC_{50}

1. Cell viability (% of control) = $(OD_{test}-OD_{blk})/(OD_{ctrl} - OD_{blk})\times100$
2. CPE inhibition(%) = $(1-CPE_{test}/CPE_{ctrl})\times100$

Preparation of the extracts

Finely ground stems from *Dodonea viscosa* 540 g were extracted with petroleum ether 40-60^{o}C, chloroform and methanol 80% by maceration. Each extract was concentrated to dryness to yield 9 g of petroleum ether extract, 7.5 g of chloroform extract and methanol 80% extract was 32.5 g. Each extract was tested for the presence of the phytoconstituents according to the following standard tests, Molisch 's test for carbohydrates, Shinoda test for flavonoids, forth test for saponins, Salkowski 's for terpenes and sterols, $FeCl_3$ and Mayer's reagents for detecting of tannins and alkaloids, respectively (Sofowra 1993, Trease and Evans 1989, Harborne 1973).

Phytochemical characterization

Petroleum ether (7.5 g) of *D. viscose* stems extract was subjected to silica gel column chromatography eluting with n-hexane and ethyl acetate gradually to yield compound 1 and compound 2. The chemical structures of the compounds were identified by ^{1}H-NMR and ^{13}C-NMR spectroscopic analyses.

RESULTS AND DISCUSSION

The results showed that *D. viscosa* stems extracts were minimal toxic where petroleum ether extract of *D. viscosa* was the least toxic one and the other extracts were more toxic, as well the extracts have drug ability as anti-HIV-1 agents where petroleum ether extract was the most active one as an anti-HIV-1 agent, and the other extracts were less active as anti-HIV-1 agents (Table 1, Table 2 and Table 3). Phytochemical analysis of *D. viscosa* stems extracts is shown in table 4. Chromatography separation of the active petroleum ether extract of *D. viscose* aerial parts yielded *β*-sitosterol and stigmasterol compounds.

Table 1: Cytotoxicity of the extracts of *D. viscose* stems in C8166 cell

The extracts	Concentration (µg/ml)	Cell viability ±SD	CC_{50} (µg/ml)
Petroleum ether	1000	16.62 ± 1.15	81.359
	200	27.43 ± 2.54	
	40	67.82 ± 2.24	
	8	82.96 ± 3.91	
	1.6	88.80 ± 3.66	
	0.32	89.16 ± 1.80	
Chloroform	1000	10.46 ± 0.32	86.407
	200	28.37 ± 2.57	
	40	69.85 ± 4.10	
	8	74.87 ± 0.16	
	1.6	93.46 ± 1.81	
	0.32	106.72 ± 1.20	
Methanol 80%	200	66.55 ± 3.36	>200
	40	92.74 ± 3.22	
	8	97.96 ± 4.38	
	1.6	98.85 ± 1.64	
	0.32	100.50 ± 8.61	
	0.064	95.72 ± 8.15	
AZT	4000	38.28 ± 0.86	1354.782
	800	86.71 ± 11.06	
	160	87.39 ± 1.77	
	32	88.60 ± 3.24	
	6.4	78.81 ± 2.57	
	1.28	80.42 ± 13.95	

Structure Elucidation of the isolated compounds

***β*-sitosterol (1):** 22 mg, white needles, ^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.37 (IH, m, H-6), 3.52 (IH, m, H-3) , 1.09 (3H, s, CH_3-19), 0.98 (3H, d, J= 6.5, CH_3-21), 0.92 (3H, t, J= 7.4, CH_3-29), 0.85 (3H, d, J= 6.7Hz, CH_3-26), 0.81 (3H, d, J= 6.7Hz, CH_3-27), 0.75 (3H, s, CH_3-18). ^{13}C-NMR(100 MHz, CDCl3): δ 140.4 (C-5), 121.5 (C-6), 71.6 (C-3), 57.2 (C-17), 56.4 (C-14), 50.3 (C-9), 46.3 (C-24), 42,8 (C-13, 4), 39.8 (C-12), 37.6 (C-1), 36.7 (C-10), 35.9 (C-20), 34.2 (C-22), 31.7 (C-8, 7), 31.4 (C-2), 29.2 (C-25), 28.4 (C-16), 26.2 (C-23), 24.5 (C-15), 23.4 (C-28), 21.1 (C-11), 19.8 (C-26), 19.5 (C-19), 19.2 (C-27), 18.6 (C-21).

Stigmasterol (2): 19 mg, white needle crystals, ^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.32 (IH, m, H-6), 5.11 (1H,dd, J= 14.2, 8.2 Hz, H-22), 5.04 (1H,dd, J= 14.2, 8.2 Hz, H-23), 3.54 (IH, m, H-3), 1.04 (3H, s, CH_3-10), 0.9 (3H, d, J= 6.5, CH_3-20), 0.84 (3H, d, J= 7.4, CH_3-27), 0.82 (3H, d, J= 7.4, CH_3-26), 0.68 (3H, s, CH_3-13). ^{13}C-NMR(100 MHz, CDCl3): δ 140.6 (C-5), 138.4 (C-22), 129.1 (C-23), 121.8 (C-6), 71.9 (C-3), 56.7 (C-17), 56.9 (C-14), 50.9 (C-9), 50.7 (C-24), 42,6 (C-13, 4), 39.6 (C-12), 37.4 (C-1), 40.2 (C-20), 36.7 (C-10), 31.4 (C-8, 7), 31.7 (C-2), 30.9 (C-25), 28.8 (C-16), 24.8 (C-15), 24.7 (C-28), 21.5 (C-11), 20.8 (C-26), 20.4 (C-19), 19.7 (C-27), 19.1 (C-21).

Table 2: Anti-HIV activity of the extracts of *D. viscose* stems in C8166 cell (Anti-HIV activity of the extracts)

The extracts	Concentration (µg/ml)	Inhibition ± SD	EC_{50} (µg/ml)
Petroleum ether	1000	100.00 ± 0.00	15.413
	200	100.00 ± 0.00	
	40	84.10 ± 1.81	
	8	26.55 ± 3.60	
Chloroform	1000	100.00 ± 0.00	68.250
	200	100.00 ± 0.00	
	40	25.15 ± 3.41	
Methanol 80%	200	100.00 ± 0.00	74.950
	40	18.01 ± 5.37	
AZT	4000	98.13 ± 0.87	5.439
	800	93.58 ± 2.13	
	160	56.74 ± 3.56	
	32	28.62 ± 4.34	

Table 3: The summary of cytotoxicity and anti-HIV-1 activities of the extracts of *D. viscose* stems

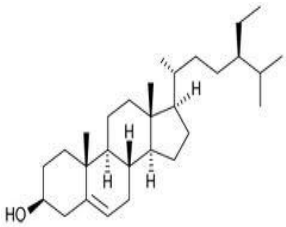
The extracts	Method	CC_{50} (µg/ml)	EC_{50} (µg/ml)	Therapeutic index (TI)
Petroleum ether	MTT	81.359	—	5.28
	CPE	—	15.413	
Chloroform	MTT	86.407	—	1.27
	CPE	—	68.25	
Methanol 80%	MTT	>200	—	>2.67
	CPE	—	74.95	
	CPE	—	11.577	
AZT	MTT	1354.782	—	249086.60
	CPE	—	5.439 ng/ml	

Cytotoxicity of the *D. viscosa* stems extracts was carried out by using MTT colormetric methods. The results showed that *D. viscosa* stems extracts were minimal toxic and showed anti-HIV-1 activity. Petroleum ether extract of *D. viscosa* had the least cytotoxic effect, it was significantly different from that of the other extracts (Table 1). The anti-HIV-1 activity assay was performed by synctia formation. Petroleum ether extract showed anti-HIV-1 activity and its therapeutic index (TI) value was the highest (table 2, table 3) with comparison with AZT. These results may be explained by the presence of phytochemicals in the petroleum ether extract, triterpenes and/or sterols (Table 4) and also the bioactive compounds isolated from the extract, *β*-sitosterol and stigmasterol. triterpenes as oleanolic acid was identified as an anti-HIV principle which was isolated from several plants, including *Rosa woodsii* (leaves), *Prosopis glandulosa* (leaves and twigs), *Phoradendron juniperinum* (whole plant), *Syzygium claviflorum* (leaves), *Hyptis capitata* (whole plant), and *Ternstromia gymnanthera* (aerial part). It inhibited HIV-1 replication in acutely infected H9 cells with an EC50 value of 1.7 microg/mL, and inhibited H9 cell growth with an IC50 value of 21.8 microg/mL with therapeutic

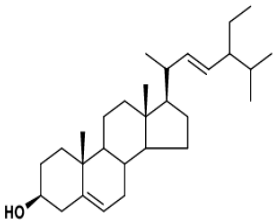
index (T.I) = 12.8, also ursolic acid showed anti-HIV activity (EC50 2.0 microg/mL), but it was slightly toxic (IC50 6.5 microg/mL, (TI) =3.3 (Kashiwada *et al*., 1998). The significant of Antibacterial, anti-HIV-1 protease and cytotoxic activities of aqueous ethanolic extracts from *Combretum adenogonium* is due to the presence of the active principles, β-sitosterol and stigmasterol in this plant extract (Novatus *et al*., 2012).

Table 4: Phytochemical Analysis from the *D. viscose* stems extracts

Constituents	Petroleum ether	Chloroform	Methanol 80%
Triterpenes and /or Sterols	++	+	+
Carbohydrates and/or glycosides	-	-	+
Flavonoids	-	+	+
Coumarins	-	-	-
Alkaloids and/or nitrogenous compounds	-	-	-
Tannins	-	-	+
Saponins	-	-	+
(+) presence of constituents, (-) absence of constituents			



β-Sitosterol



Stigmasterol

Fig.1: Chemical compounds isolated from petroleum ether extract of *D. viscose* stems

CONCLUSION

In this paper, *D. viscose* stems were extracted with petroleum ether, chloroform and methanol 80% solvents by maceration method and each extract was tested for its ability to act as anti-HIV-1 agent. Petroleum ether was the most active as anti-HIV-1 agent and the isolation and identification of the active principles from this active extract yielded two compounds, β-sitosterol and stigmasterol and so anti-HIV-1 activity from petroleum ether extract is may be due to the presence of these bioactive components and thus *D. viscose* stems petroleum ether extract could provide a chemical reservoir of anti-HIV agent.

Acknowledgements

This work was supported in part by grants from 973 Program of China (2009CB522306), the Key Scientific and Technological Program of China (2012ZX10001-006) and NSFC (81001462, 81102483).

Conflict of interest

There is no conflict of interest associated with the authors of this paper.

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How to Cite this Article:

Khaled Rashed, Meng-Ting Luo, Lin-Tao Zhang and Yong-Tang Zheng, 2013. Extracts from *Dodonaea viscose* stems (Linn.) as HIV-1 inhibitors and Chemical constitutents. *Sci. Res. Rept.,* **3**(2):91-96.