International Journal of Molecular and Clinical Microbiology 1 (2014) 398-405

International Journal of
Molecular and Clinical Microbiology

Study of Anti-Retroviral Effects of Salix Aegyptiaca L Herbal Extract on HIV-1 in-vitro

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ARTICLE INFO

Article history:
Received 12 January 2014
Accepted 3 May 2014
Available online 1 June 2014

Keywords:
Human immunodeficiency virus1; Salix aegyptiaca L; single cycle replicable virion (SCR); Gaussian, HF, Hyper Chem

ABSTRACT

Human immunodeficiency virus (HIV) infection is the major problem in the world. Nowadays, new anti-retroviral drugs are extracted from medicinal plants, make an important role to supersede synthetic drugs with different side effects. In this study, we studied the Salix aegyptiaca L extract, Iranian herb, as an anti-HIV drug with anti-retroviral effects. We used a single cycle replicable (SCR) HIV-1 system by co-transfection of HEK 293T with pmzNL4-3, psPAX2 and pM2G.2. Cytotoxicity and cytopathic protection assay were performed by XTT method. Inhibition of p24 Ag production level assay was done using quantitative enzyme linked immunosorbent assay (ELISA). Salix aegyptiaca L inhibited HIV-1 induced CPE in HELA cells with SI value of 22.2. Finally, we use informatics analysis such as Gaussian, Hyper Chem , HF and B3LYP methods and 6-31G, 6-31G** and 6-311G** basis sets. These results suggest that Salix aegyptiaca L has potent anti-HIV activity and may be a promising candidate for AIDS.

1. Introduction

HIV-1 that infects the human T lymphocytes is the etiologic agent of acquired immune deficiency syndrome (AIDS) (Barre-Sinoussi et al., 1983). Thirty three million people are infected with this virus in the world and 5,700 people die each day (Anglaret, 2008). However, no promising vaccine or effective therapy against HIV-1 is available. HIV-1 infection is becoming a serious health problem in the developing as well as Middle East countries (Cheemeh et al., 2006)

HIV-1-based self-inactivating (SIN) lentiviral vectors have been already introduced as a model of such replication-deficient virions that contain a large deletion within the U3 region of their 3'LTR (Miyoshi et al., 1998). This mutation is transferred to the 5'LTR during the reverse transcription, which leads to the transcriptional inactivation of LTR in the provirus (Miyoshi et al., 1998). This mutation reduces the chance of producing the replication-competent virions and prevents the occurrence of recombination between vector and wild type HIV-1 within the infected cells. These SIN virions capable of efficient transduction of target cells with desirable genes are generally accepted as the safe lentiviral vectors for gene therapy experiments (Miyoshi et al., 1998; Zufferey et al., 1998; Mukherjee et al., 2007). However, a screening of drugs against HIV-1 requires the

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examination of a number of novel compounds with the potential replication inhibitory effect. Obviously, the replication-competent but safe HIV-1 virions are needed for this assessment.

Medicinal plants make an important part of traditional medicine in most of the countries, and in the establishment of the therapeutic value of new approaches has a special place. In this study, we showed that herbal extract as an anti-HIV drug has better efficacy and fewer side effects than a synthetic drug. This plant is known with the scientific name of *Salix aegyptiaca L* and its extract made by petals of the plant, which contains 200 types varies compositions. *Salix aegyptiaca* L. (musk willow) is a dioecious plant belongs to the family of Salicaceae traditionally has been cultivated in Iran, Turkey, Turkmenistan and Afghanistan. In some areas of Iran such as Kashan, Shiraz and Azerbaijan, it grows well, the extract of that has dark brown color with bitter taste and penetrating smell. The major components of this plant are carovone, phenolic flavonoids (16%), carvacrol (9%), cedreneoxide (16%), Geranol (10%), citronellol (4%), p-metoxybenzene (2.3%) (Karimi et al., 2011). There are many industrial and traditional systems that are involved in cultivating musk willow (Bidmeshk in Persian) in different regions of Iran. The aqueous extract and essential oil (EO) of *Salix aegyptiaca* L. (SA) are being used in confectionary and flavorful syrups. In Iranian traditional medicine, SA has been employed as laxative, cardioprotective, nervonic, sedative, hypnotic, somnolent, aphrodisiac, orexiogenic, carminative, gastroprotectant, anthelmintic and vermifuge. The decoction of leaves or barks of SA have been used as an anthelmintic and vermifuge remedy. The decoction of SA's leaves in honey still is used as a nervonic functional food. The decoction of leaves of SA plus sugar has been used among Iranian and Turkish people for maladies like depression, neuropathic pain and rheumatoid arthritis. The Salix family is famous due to its endogenous salicylate compounds e.g., salicylic acid and acetyl salicylic acid (ASA, Aspirin®). This class of compounds exert anti-inflammatory effects throughout the inhibition of cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) leading to the inhibition of prostaglandin synthesis (Yu et al., 2002). The anti-inflammatory and anti-nociceptive properties of extracts of willow family may be related to its phytochemicals such as salicin, myricetin, kaempferol, quercetin, rutin and luteolin (Qin and Sun, 2005). These compounds have immunomodulatory and anti-inflammatory activities by inhibiting pro-inflammatory cytokine production and their receptors (Qin and Sun, 2005). The considerable myricetin, rutin and catechin content of musk willow extracts could potentially contribute to the anti-inflammatory functions of willow extracts (Enayat and Banerjee, 2009). According to Grecian medicine, SA has warm humor nature and ethnic herbalists prescribed it for cholelithiasis, cholecystitis, arthritis and rheumatism. The EO of SA is febrifuge and is dubbed among Iranian people for its calming effect on heart and possibly its antihypertensive effect. Gaussian is one of the most widely used quantum chemical program packages for molecular applications, and is used both in industry and in many scientific areas of research. We have calculated the geometric parameters of the compounds in the ground state the using the Hartree-Fock (HF), Becke's three-parameter hybrid method with the Lee, Yang, and Parr correlation functional methods (B3LYP), Becke's exchange functional in combination with the Lee, Yang and Parr correlation functional methods (BLYP), Becke's three parameter exchange functional combined with gradient corrected correlation functional of Perdew and Wang's 1991 (B3PW91) , and 6-31G, 6-31G* and 6-31+G basis set (Adamo and Barone, 1998; Burke et al., 1998). In the present study we investigated the possible inhibitory activity of Salix aegyptiaca L against HIV-1 replication using the recombinant HIV-1 system. Salix aegyptiaca L has a significant anti-retroviral activity at some concentrations.

2. Materials and Methods

2.1. Extraction

The petals of *Salix aegyptiaca L* were collected from Kashan and crushed to powder using grinding machine. The powdered plant

material (0.5 kg of Salix aegyptiaca L seeds) was moisten with 1.5 liters mixture of analytical grade methanol, ethyl acetate and hexan (Merck 1.1.1) for 72 hours in a round bottom flask, on water bath attached to the reflux water condenser. After filtering and concentrating under vacuum, the crude extract (yellow reddish) was obtained. Organic extract were evaporated to concentrate by friz-dried method and semi solid extract was stored in refrigerator. one gram of the dried components were dissolved in 10 ml of the DMSO and used as a anti-HIV drug.

2.2. Inhibition of HIV p24 core antigen production (HIV replication) assay

HeLa cell line which was used as the target was infected by HIV in this experiment, were seeded at 6×10^4 cell per well in 24-well plates, 600ng p24 SCR HIV virion was used to infect each well. After 2 hours of virus adsorption, cells were washed three times with pre-warmed DMEM (used in a wide range of mammalian cell culture application) to remove free virus particles. A total volume of 500 µl/well fresh medium with various concentrations of *Salix aegyptiaca L* distilled with DMSO and incubated for 48 hours. Nevirapine (HIV1/2 RT inhibitor) was used as positive control. After 48h p24 Ag assay was performed on the supernatants by quantitative p24 ELISA method (HIV P24 ELISA, CELL BIOLABS) according to manufacturer's protocol.

2.3. Cytopathic inhibition assay

Effect of *Salix aegyptiaca L* for reducing the lytic activity of HIV virions replication was assayed using HeLa cells. 20×10^3 HeLa cells were infected with 1800 ng P24 VSVG-SCR virion in each well of 96 wells plate containing 60 µl fresh medium and different concentrations of Salix aegyptiaca L. The BMS- 232632 (HIV-1 protease inhibitor) was used as positive control. After 16 hour, 250 µl fresh complete medium was added to each well. 100 µl of each wells was replaced with fresh medium every 48 hours during days after infection. 3 days after infection, 50 µl of XTT (Roche) was added to each well and incubated for 4 hours at 37°C. Plate was read using Bio-Tek ELx 800 ELISA reader at 450 nm/630 nm.

2.4. Computation Methods

Stage 1: Start ChemDraw and construct molecules. Save the results as a ChemDraw file.

Stage 2: Reopen this file using Chem3D and perform an energy minimization. Then save the results as a gjc file.

Stage 3: Reopen this file using Gaussian98 and the calculations were performed using the Gaussian® 98 program suite. Calculations were performed using an all-electron linear combination of atomic orbital Hatree–Fock (HF). The optimizations of p24 and Salix aegyptiaca L are carried out including exchange and correlation contributions using Beckẻs three parameter hybrid and Lee-Yang-Parr (LYP) correlation [B3LYP]; including both local and non local terms. We have geometric optimization calculation at the HF/6-31G, HF/6-31G**, HF/6-311G**. We have also performed a geometric optimization calculation at the B3LYP/6-31G, B3LYP/6-31G** and B3LYP/6-31G** level.

3. Results

3.1. Cytotoxicity effects of Salix aegyptiaca . L on HeLa cell

Firstly the cytotoxicity of SA was evaluated on HeLa cells using the XTT assay. The data indicated that SA had no cytotoxic effect on the HeLa cells at concentrations of 100 µg/ml (Table 1). The concentration of CC50 of SA for HeLa cells were in the range of 1000 µg/ml.

3.2. Inhibition of HIV p24 production (replication assay) by Salix aegyptiaca L extract

HIV replication assay is performed by means of VSVG pseudo-typed SCR (single cycle replicable) HIV-1 virions which are able to replicate only for one cycle. This experiment showed the capability of test drug to inhibit the replication of HIV virions from entry to release of the host cells. At the concentration of 100µg/ml SA inhibited the production of HIV-1

p24 Ag by more than 80% (Table 2) while the cytotoxicity of the test drug at this concentration is less than 1% (Table 1).

3.3. Cytopathic inhibition assay

We examined the activities of SA to protect the HeLa cells from HIV-1-induced cell lytic effects. The concentrations of the test compound leading 50% inhibition of HIV-induced CPE (IC50) were determined by dose-response curves (not shown). The IC50 of SA in HeLa infected cells was 45µg/ml while its CC50 was 1000 µg/ml so this gave a selectivity index (SI) of >22.2 (Table 3).

3.4. Compiotional results

In this work, we have calculated parameters like isotropic (σiso) and anisotropic (σaniso) shielding, NMR determinant and distance matrix determinant by using HF method with 6-31G,6-31G*, 6-31+G basis set and B3LYP methods with 6-31G basis set. (Table 4)

4. Discussion

Due to the increasing emergence of drug resistant viruses in HIV-infected patients and cellular toxicity of conventional anti-HIV drugs, various studies have been carrying out to develop new anti-retroviral agents (Merluzzi et al., 1990) which has been recently attracted great attention on account of its organic and non-organic elements with possible anti-viral activities. We were motivated to study on the probable anti-retroviral activities Salix Aegyptiaca L in vitro as the interfering and suppressive effects of various elements with HIV enzymes have been previously reported. Comparing the effects of current synthetic anti-retroviral drugs for clinical use like Nevirapine (RT inhibitor), the SI value of Salix aegyptiaca L, it seems to be much weaker than that of current clinical drugs.

The results of this study showed that the quantity of HIV-1 p24 Ag in the supernatant of the *Salix aegyptiaca L* treated culture was significantly low even if the test drug was added at 12 hours after infection, and the manner of effect was comparable to that of Nevirapine, a potent viral RT inhibitor, which suggests that HIV protease and maturation of virus is a possible target of *Salix aegyptiaca L*. Furthermore, the mechanism of action of *Salix aegyptiaca L* seems not to be inhibition of the early stages of HIV-1 life cycle.

Regarding to the whole study the exact mechanism of anti-HIV activity of *Salix aegyptiaca L* is still unclear. So, our findings just presented a hint on the possible mode of the action of *Salix aegyptiaca L* against HIV-1 replication.

Table 1. Effects of herbal drugs (SA) on the viability of Hela cell lines which treated with various concentrations (1000, 100, 10µg/ml)of SA for 72 hours. Viability of cells was determined using XTT assay.

	ELISA OD (450nm)	**Mean OD**	**Cell Viability %**
Un-treated (1 µl DMSO) cells	1.3 , 1.2 , 1.4	1.3	100%
Drug Salix A (1 mg)	0.7 , 0.65	0.67	51%
Drug Salix A (100µg)	1.4 , 1.3	1.3	100%
Drug Salix A (10 µg)	1.3	1.3	100%
Drug Salix A (1 µg)	1.3	1.3	100%

Table 2. Inhibition (%) of HIV p24 Core Antigen Production (HIV Replication) after treatment with three different concentrations of herbal drugs

	P24 OD	Mean p24 OD	HIV Inhibitory %
Uninfected cells	0.04 , 0.03	0.035	---
Virus control (without drug)	1.44, 1.46, 1.16	1.35	0
Nevirapine (Anti -retroviral drug)	0.03, 0.05	0.03	100%
Drug Salix A (1 mg)	0.02 , 0.02	0.02	100%
Drug Salix A (100µg)	0.29 , 0.25	0.27	80%
Drug Salix A (10 µg)	0.84 , 0.97	0.9	33%
Drug Salix A (1 µg)	1.26	1.26	7%

Table 3. Anti-retroviral activities of some herbal extracts on HIV-1 virion

SI [c] (CC50/IC50)	IC50 [b] (µg/ml)	CC50 [a] (µg/ml)	host cell	compound
22.2	45 (µg/ml)	1000 (µg/ml)	Hela cells	B

[a] CC50 is the drug concentration of 50% cytotoxic effect.
[b] IC50 is the concentration of the drug required to inhibit 50% of virus-induced CPE.
[c]Selectivity index (SI) = CC50/IC50

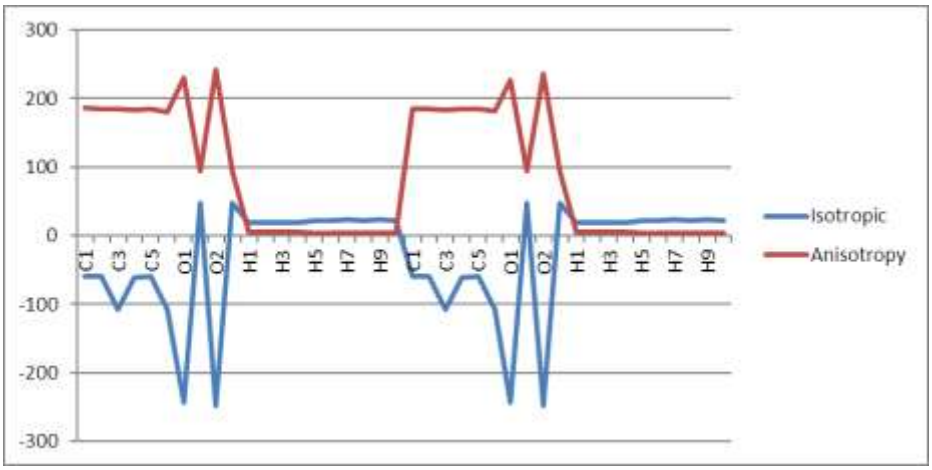

Figure 1. isotropic and anisotropy of salix in b3lyp/6-31g

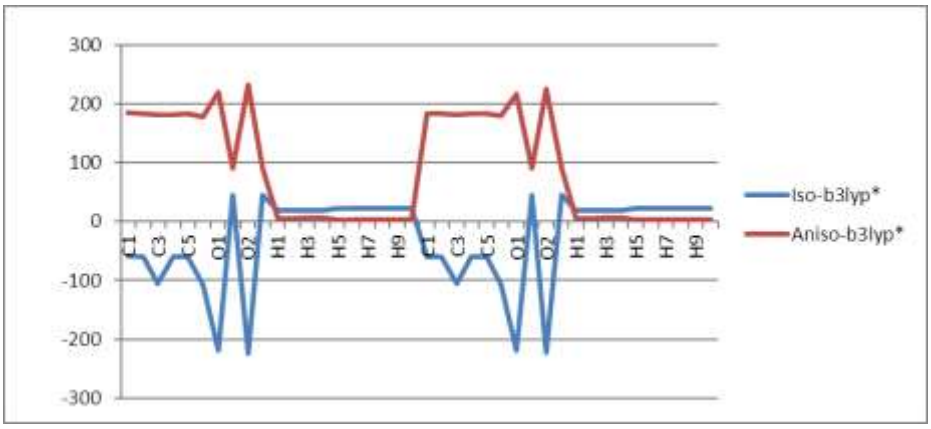

Figure 2. isotropic and anisotropy of salix in b3lyp/6-31g*

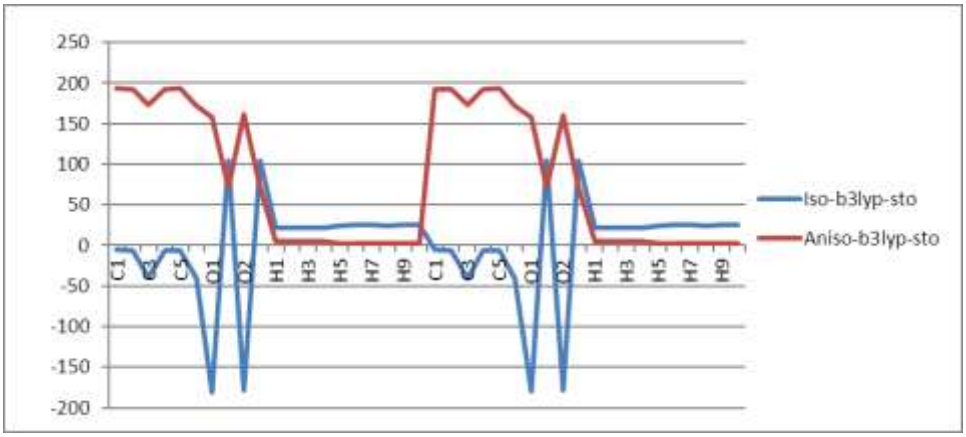

Figure 3. isotropic and anisotropy of salix in b3lyp-sto

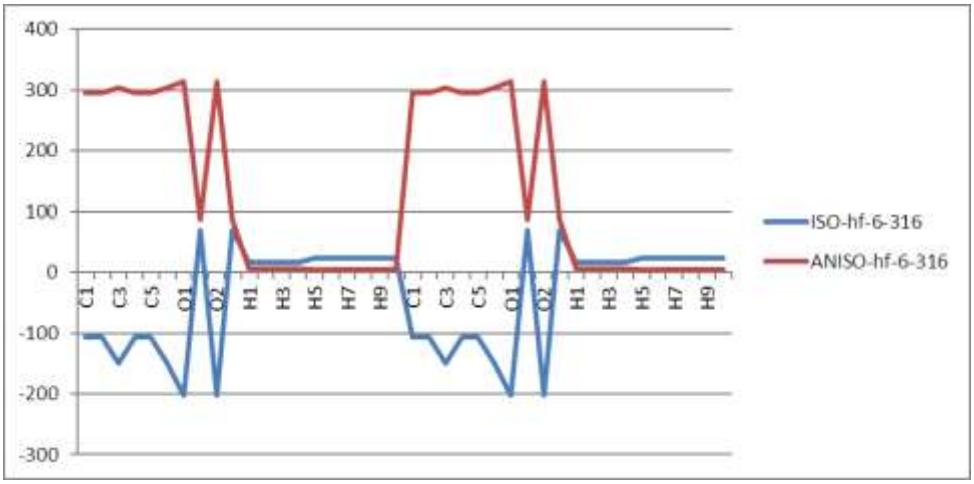

Figure 4. isotropic and anisotropy of salix in hf -6-31g

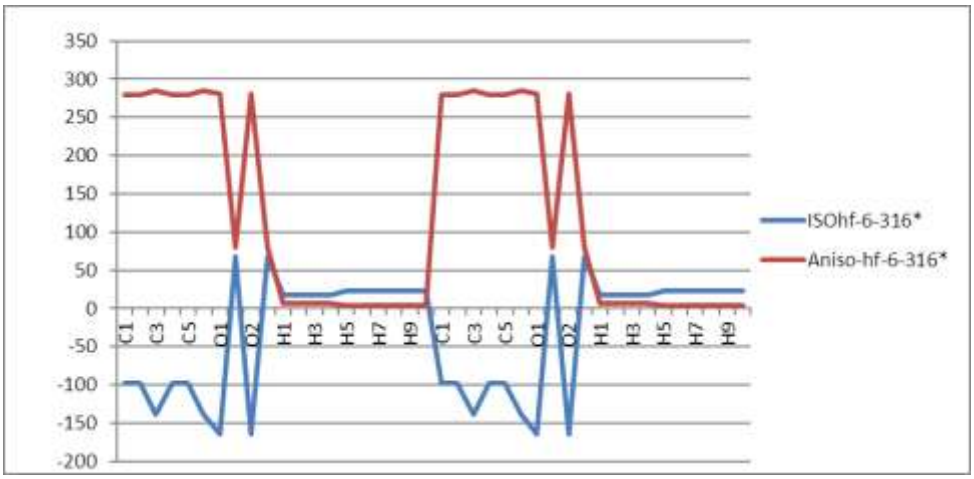

Figure 5. isotropic and anisotropy of salix in hf -6-31g*

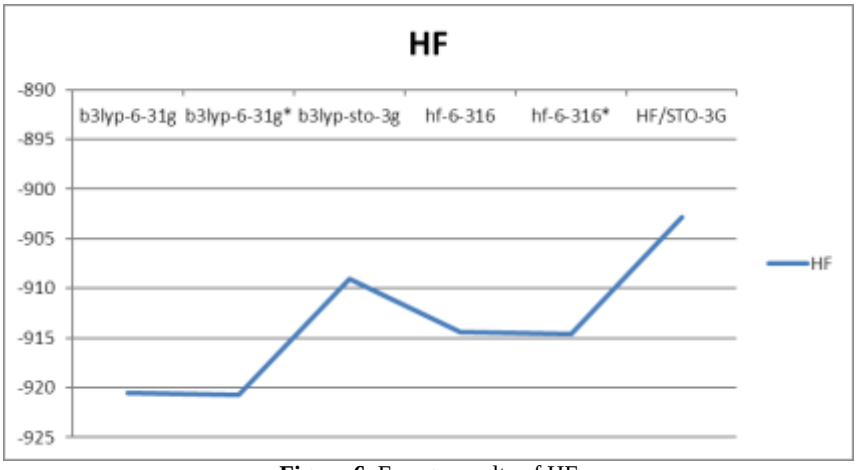

Figure 6. Energy results of HF

Table 4. Energy results of HF

Basis set	DIPOL	Quadrupole	HF
HF-STO-3G	5.0031	1.11E+07	-902.874
HF/6-31G	10.3076	2.41E+07	-914.381
HF/6-31G*	9.2128	4.19E+07	-914.629
b3lyp/6-31g	7.981	2.98E+07	-920.577
b3lyp/6-31g*	7.2967	2.58E+07	-920.734
b3lyp/sto-3g	4.3269	7.66E+06	-909.022

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