

The antiviral activity of Egyptian *Magnolia grandiflora* on Human Immunodeficiency Virus (HIV)

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Introduction

The human immunodeficiency virus type-1 (HIV-1), belonging to the retrovirus family, is the infectious agent that causes acquired immunodeficiency syndrome (AIDS) [1]. The current chemotherapeutic strategies focus on inhibition of one of the three critically essential enzymes (protease, reverse transcriptase, or integrase) in the HIV life cycle [2]. A recent strategy has been shown to inhibit the HIV-1 LTR promoter by G-quadruplex ligands [3]. Also, a range of protease inhibitors such as Ritonavir, Saquinavir, and Indinavir have been developed and applied in clinical trials against AIDS. However, the problem is the rapid development of HIV resistance to the drugs [4, 5]. In addition, the high cost of these drugs make them inaccessible to HIV infected people in developing countries. So, cheap, effective, and safe antiviral agents to suppress HIV are strongly desired. The natural sources are the best candidate for this purpose. Several medicinal plants have been reported to inhibit HIV infection [6, 7].

Magnolia grandiflora L. (magnoliaceae), grows in the south-eastern states of the USA and Mexico. It is used in traditional medicine for treatment of blood pressure, heart disturbances, abdominal discomfort, muscle spasm, infertility, epilepsy and the aqueous extracts of the flowers and leaves exhibited cardiovascular effects [8, 9].

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ABSTRACT

Objective: This study was aimed to evaluate the *in vitro* antiviral activity of two extracts, methanol and aqueous, from the leaves of *Magnolia grandiflora* against the human immunodeficiency virus (HIV).

Methods: The cytotoxic and antiviral assays were performed by using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) reduction colorimetric assay and TZM-bl reporter cell line, respectively.

Results: Our results suggested that both the extracts showed antiviral activity against HIV infection by interfering with the HIV life cycle in infected cells. The methanolic extract showed higher antiviral activity than the aqueous extract with SI= 2.74 and 1.84, respectively.

Conclusions: Our results demonstrate that *in vitro* infection with HIV can be effectively treated by methanol and aqueous extracts of *Magnolia grandiflora* leaves.

KEY WORDS: *Magnolia grandiflora*
HIV
Antiviral
in vitro
MTT

Neolignan, magnolol, honokiol, and 3,5'-diallyl-2'-hydroxy-4-methoxybiphenyl exhibited antibacterial activities [10]. Neolignan, magnolol and honokiol [11] exhibited several medicinal functions such as the inhibitory effects on skin tumour promotion [12]. It is listed in United State Pharmacopoeia and Pharmacognosy texts as bitter tonics, antimalarials and diaphoretics [13] for a cold, headache, and stomachache [14]. It is used as an anticonvulsant for treatment of fever, diarrhea, rheuma, arthritis and antitumor [15-17]. It is interesting to note that *M. grandiflora* contains a number of sesquiterpene lactones and neolignans to possess anti-inflammatory properties and treatment of pain [18, 19]. Dichloromethane extracts and sesquiterpene lactones from leaves and the stem bark exhibited antifungal activity, a nematicidal activity and herbicide activity [20, 22]. On the other hand, the methanol extract *Magnolia grandiflora* L. leaves showed antiviral activities against the

herpes, simplex-1 and poliovirus. Four aporphine alkaloidal compounds exhibited cytotoxicity against tumor cell lines Hela, HEPG2, and U251 [23]. The essential oils from leaves displayed antifungal, antimicrobial and antioxidant activities [24]. Chemical studies on *Magnolia grandiflora* have reported the presence of alkaloids [11, 23, 25], glycosides [26-28], sesquiterpenes and sesquiterpene lactones [29-37], neolignan, magnolol and honokiol [10, 13, 38], volatile constituents [39, 40] and other compounds [41]. In this study we evaluated the antiviral activity of methanolic and aqueous extracts of *M. grandiflora* against HIV infection *in vitro*.

Materials and Methods

Plant material

Magnolia grandiflora leaves were obtained from Zoo, Giza, Egypt and were kindly identified with the help of Mrs Tersea Labib, taxonomist at Orman Botanical Garden, Giza. A voucher specimen from the plant was deposited at the Herbarium of the National Research Centre, Cairo, Egypt (Registration No. 13512).

Extraction

For preparation of methanol extract, air dried fine powdered *Magnolia grandiflora* leaves (2kg), were extracted with methanol in percolator at room temperature till exhausted and evaporated till dryness in rotary evaporator at 40 °C, the extract yield was 13.84%. For preparation of aqueous extract, air-dried, powdered of *Magnolia grandiflora* leaves (1kg) were extracted with water in percolator till exhausted at room temperature and concentrated under reduced pressure at 40 °C, then lyophilized, to give a yield of 9.35%.

Cytotoxicity Assay in TZM-bl Cells

Cytotoxicity of alcohol and water extracts of *M. grandiflora* were tested in parallel to antiviral assays by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) as described previously by Perrone et al. [42]. In brief, TZM-bl Cells were seeded in 96-well plate (1.5 x 10⁴/well). After 24 h, different dilutions of test extract were added to cell lines in triplicate wells and incubated for 48 h at 37°C. After removal of the extract solutions, MTT (Sigma-Aldrich, Milan, Italy) solution (5mg/ml in PBS) was added to wells. After 4 h at 37°C, MTT solution was

carefully discarded from wells and replaced by a solubilizing solution (SDS 10%, HCl 10mM) to solubilize the formazan crystals. After incubation period of 24 h at 37°C, the optical density was measured spectrophotometrically at 620nm. The 50% cytotoxic concentration (CC₅₀) was defined as the concentration of test compound that was able to reduce the absorbance of the mock-infected cells by 50%. The 50% inhibitory concentration (IC₅₀) was defined as the concentration of test compound that inhibits 50% of LTR-Luciferase signal. The selectivity index (SI) is the relative effectiveness of the tested compound in inhibiting viral replication compared to inducing cell death (CC₅₀ value/IC₅₀ value).

Antiviral Assay in HIV-1 Infected TZM-bl Cells

HIV-1 infectivity was measured using the TZM-bl reporter cell line (obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH, from Dr. J.C. Kappes, Dr. X. Wu, and Tranzyme Inc.). TZM-bl is a HeLa cell line stably expressing large amounts of CD4 and CCR5 and containing integrated copies of the luciferase and β -galactosidase genes under control of the HIV-1 promoter. TZM-bl were grown in DMEM supplemented with 10% FBS. Cells (1 × 10⁴ cells/well) were seeded in 96-well plates and grown overnight. Cells were next infected with HIV-1 NL4-3 strain at a MOI of 0.1, treated with 5-fold serial dilutions (concentration range 1000-7.8 µg/ml) of alcohol and water extracts of *M. grandiflora* and incubated at 37 °C. After 48 h, cells were washed with PBS (1×) and HIV-1 production was assessed following the LTR-luciferase signal using the britelite plus Reporter Gene Assay System (PerkinElmer, Waltman, MA, USA) according to the manufacturer's protocol. The 50% inhibitory concentration (IC₅₀) was defined as the concentration of test compound that inhibit 50% of LTR-Luciferase signal. The selectivity index (SI) is the relative effectiveness of the tested compound in inhibiting viral replication compared to inducing cell death (CC₅₀ value/IC₅₀ value).

Determination of total phenolic and total flavonoids Reagents:

For determination of the total phenolic and flavonoid compounds in the extracts: known weight of the extract was dissolved in 100 ml of 80% methanol or water.

Estimation of total phenolic

The total phenolic content in the extracts was determined by Folin–Ciocalteu according to the method described previously by Singleton et al [43] and Meda et al [44], with some modification. Briefly, 1 ml of the extract was mixed with 7.5 ml of 0.2 N Folin–Ciocalteu reagent (Sigma Chemical Co.; St. Louis, Mo.; USA). After 15 min at room temperature, the mixture was neutralized with 1 ml of 20% Na₂CO₃ prepared in distilled water, mixed well by a vortex. After additional 30-min reaction, the absorbance of the mixture was measured at 760 nm with a (UV–VIS spectrophotometer). Concentration of the total phenolic was estimated from a standard concentration curve where chlorogenic acid (Sigma) was used as standard to produce the calibration curve. The mean of three readings were used and the content of total phenolic was expressed as milligrams of chlorogenic acid per 1 gram of the extract (mg /1 g extract).

Estimation of total phenolic

The total phenolic content (TPC) was determined by Folin–Ciocalteu according to the method described previously by Singleton et al [43] and Meda et al. [44] with some modification. Briefly, 1.0 ml. of the sample extract was added to 7.5 ml. of redistilled water and 0.5 ml. of Folin–Ciocalteu reagent (Sigma Chemical Co.; St. Louis, Mo.; U.S.A.) was added. After 15-min equilibration, the mixture was neutralized with 1.0 ml. of 20% Na₂CO₃, mixed well by a vortex. After a 30-min reaction, the absorbance of the mixture was measured at 760 nm with a UV–VIS spectrophotometer. Chlorogenic acid was used as a standard curve, the mean of three readings were used and results were expressed as milligrams of chlorogenic acid equivalent per gram of the extract (mg /1 g extract).

Estimation of total flavonoid:

The total flavonoid content (TFC) was determined according to the method as adopted by [44, 45]. Briefly, 3 ml. of the extract was mixed with 3 ml. of AlCl₃ (2 % in methanol) solution and the mixture was allowed to stand for 30 min. The absorbance was measured at 415 nm with a UV–VIS spectrophotometer. Rutin was used as a standard and the mean of three readings were recorded, the total flavonoids was expressed in mg as rutin equivalent per 1g of the extract (mg /1g extract).

Statistical analysis

Quantitative data were statistically represented as mean $\pm$ standard division (S.D.). The therapeutic index (TI) of methanol and water extracts were calculated as the ratio of CC₅₀ / IC₅₀. Comparison between control and test samples on the virus inhibition was done using One-way analysis of variance (ANOVA) test. A probability value (*p* value) $\leq$ 0.05 was considered significant. All statistical calculations were done using computer program SPSS (Statistical Package for Social Science) statistical program version (16.0). Graphs were done using SPSS statistical program version (16.0) and Microsoft Excel program version 2010.

Results

Cytotoxicity of methanol and water extracts of *M. grandiflora* leaves on TZM-bl Cells

The cytotoxic effect of the plant extracts on TZM-bl Cells was investigated by MTT assay at CC₅₀. TZM-bl Cells were incubated with DMEM media in the presence or absence of different concentrations of each extract for 2 days and by treatment of cells by MTT reagent we demonstrated that both extracts have low cytotoxic effect on TZM-bl Cells with CC₅₀ > 1000 μ h/ml (Table 1).

Table 1. *In vitro* anti-HIV activities of *Magnolia grandiflora* extracts.

Extract	CC ₅₀ (μ g/mL) ^a	IC ₅₀ (μ g/mL) ^b	SI ^c
Methanol	> 1000	364.40	> 2.74
Aqueous	> 1000	544.69	> 1.48

^a CC₅₀: 50% cytotoxic concentration required to kill half of viable cells.

^b IC₅₀: 50% effective concentration required to protect half of infected cells

^c SI: selective index (CC₅₀/ IC₅₀).

Antiviral activity of methanol and water extracts of *M. grandiflora* leaves on TZM-bl Cells

Using different non-toxic concentrations of each extract, our results suggested that the both extracts possess antiviral activity against HIV infection where the methanol extract showed higher selective index than the water extract with SI = 2.74 and 1.84, respectively. The results are summarized in Table 1.

Content of total phenolic and flavonoid compounds in methanol and water extracts of *M. grandiflora*

As shown in table 2, our results demonstrated that the methanol extract contains high percentage of total phenolics (18.28 %), and total flavonoids (1.44 %) compounds than water extract, which contains percentage of total phenolics (12.56 %) and total flavonoids (0.98 %) compounds.

Table 2. The content of total phenolics and flavonoids in the methanol and water extracts of *M. grandiflora*.

Extract	Total phenolics	Total flavonoids
Methanol extract	18.28%	1.44%
Water extract	12.56%	0.98%

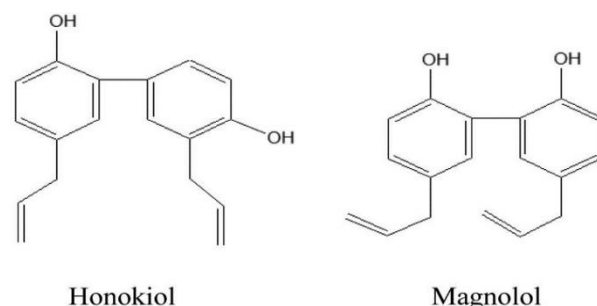
Discussion

HIV infection and AIDS remain a unique case in the infectious diseases history and the development of an effective drug against HIV become one of the priorities of global interest in public health. In the present study, we have investigated the antiviral activity of methanol and water extracts against HIV *in vitro*. Our results demonstrated that the both extracts showed antiviral activity against HIV infection, where the methanol extract showed the higher antiviral effect than water extract. This finding may be attributed to presence of several compounds such as flavonoids, alkaloids, tyramine, phenolic alcohols, terpenes, sesquiterpene lactones, and glycosides [18, 23, 46] in the methanol extract. The reaction of these constituents with each other in methanol extract can lead to synergistic effect and thereby higher antiviral activity than the purified individual compounds [47]. Mechanistically, these extracts may be inhibited the HIV infectivity by affecting on the viral life cycle inside the infected cell.

Our current study is the first report to demonstrate that *M. grandiflora* possess antiviral activity against HIV infection. Mohamed et al [23] reported that the methanolic leaf extract of *M. grandiflora* have high to moderate inhibitory effects against herpes simplex Virus type 1 and moderate and poliovirus type 1, respectively. Other species within the Magnolia genus, such as *M. fargesii* exhibited inhibitory effects against HIV-1 protease whereas other two species, *M. officinalis* and *M. obovate*, were not [48]. Also, Magnolia Kobus showed inhibitory effects on reverse transcriptase and protease of HIV-1 [49]. Kawahara et al [50] stated

that the aqueous extract of *Magnolia obovate* showed potent antiviral activity against rotavirus induced diarrhea due to presence of quercitrin and rutin compounds in this plant. Several species of Mangolia genus, including *M. grandiflora*, have reported to contain Honokiol and Magnolol compounds [51- 53]. Honokiol exhibited antiviral activity against hepatitis C virus infection by targeting two steps of HCV life cycle, cell entry and virus replication [52]. Also, it showed antiviral activity against HIV-1[54, 55]. Recently, the honokiol derived from Magnolia tree was shown to inhibit the viral replication, protein expression, and endocytotic of serotype 2 dengue virus [56]. Whereas, Magnolol exhibited antiviral activities against several viruses such as HBV, influenza virus A [57,58]. The chemical structure Honokiol and Magnolol compounds are presented in figure 3. So, we are strongly suggest that the antiviral activity of the *M. grandiflora* against HIV may be attributed to presence of such these compounds.

Figure 3. Structure of honokiol and magnolol.



On the other hand, the phenolic and flavonoid compounds have been reported to possess antiviral activity against wide range of RNA viruses such as SARS-coronavirus, influenza A (H3N2) and B viruses, coxsackie virus B3, rotavirus [62], rabies virus, dengue virus, influenza A (H1N1) and herpes simplex virus type 1 (HSV-1), Parainfluenza-3 virus, rhinoviruses, human immunodeficiency virus (HIV) [59-69]. These findings promoted us to determine the presence of these compounds in our tested extract. These findings promoted us to detect the presence of these compounds in our tested extracts. We demonstrated that the methanol extract contains high mounts of total phenolics and flavonoids compounds than water extract. So, the higher antiviral activity of the methanol extract may be due to the presence of these compounds in high contents than water extract.

In conclusion, the current study has shown that the methanol and aqueous extracts from *M. grandiflora* leaves suppress the replication of HIV in infected cells, may be by interfering with viral life cycle. Further studies about their specific mechanism of action in HIV infection and their anti-HIV *in vivo* should be explored.

Conflict of Interest

We declare that we have no conflict of interest.

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