

The Antioxidant and Anti-Herpes Simplex Viruses Activity of *Morus alba* Leaves Extract

Mohamed A. Dkhil,^{1,2,*} Saleh Al-Quraishy¹ and Denis Delic³

¹Department of Zoology, College of Science, King Saud University, Riyadh, Saudi Arabia

²Department of Zoology and Entomology, Faculty of Science, Helwan University, Cairo, Egypt

³Department of Biology, Heinrich-Heine-University, Duesseldorf, Germany

Abstract.- As viral resistance to available chemical drugs and virus latency duration leads to worries in herpes simplex virus type 1 and 2 infections treatment, there are growing need for new anti-herpes treatments. Therefore, the present study was aimed to evaluate the antiviral activity of *Morus alba* extract against herpes simplex virus type 1 and 2 (HSV-1 and 2) in Vero cells model and the antioxidant property of this extract using the DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free-radical assay. HSV-infected Vero cells and cell-free virus suspensions were treated with *M. alba* extract, and virus yield and infectivity were quantified by cytopathic effect (CPE) assay. The results indicated that the 50% DPPH radical scavenging property of the extract was 122.4 µg/ml. In addition, the data of the present experiment showed that *M. alba* extract at 2 µg/ml provided 66.7% inhibition of plaque of HSV-1. In addition, *M. alba* extract at 4 µg/ml provided 48.6% inhibition of plaque of HSV-2. The extracts showed 100% inhibition against HSV-1 and 2 at 5, 7.5 and 10 µg/ml. These properties suggested that *M. alba* extract may be used as a topical prophylactic and/or therapeutic agent for herpes infections. However, further studies are required to illuminate the active constituents of this extract which may be useful in developing new anti-herpes drugs.

Keyword: *Morus alba*, herpes simplex virus, antiviral, antioxidant.

INTRODUCTION

Morus alba Linn, commonly known as white mulberry and Tut in Egypt, belongs to family Moraceae. *M. alba* is a moderately sized tree grow from 3 to 6 meters tall. White mulberry is native to northern China, and is cultivated throughout the world, wherever silkworms are raised. The phytochemical analysis of this plant indicated that the plant is a good source for ascorbic acid, over 90% of which is present in a reduced form, and also is a very good source of natural antioxidants because is containing carotene, Vitamin B1, folic and folinic acids, isoquercetin, quercetin, tannins and saponins (Devi *et al.*, 2013). Moreover, the leaves of white mulberry contain triterpenes as lupeol, sterols (β -sitosterol), bioflavonoids (rutin, moracetin, quercetin-3-triglucoside and quercitrin), coumarins, apigenin, volatile oils, alkaloids and many amino and organic acids (Doi *et al.*, 2001). One of the major active constituent of *M. alba* is 1-deoxyojirimycin (Kim *et al.*, 2000). *M. alba* leaves extract has been found to generate nitric acid and

produce prostaglandin E2 and cytokines in macrophages. Andallu *et al.* (2001) and Andallu and Varadacharyulu (2003) have reported many different medicinal properties of mulberry leaves; it is used in traditional Chinese medicine as an antiphlogistic, diuretic, expectorant and antidiabetic (Mohammadi and Naik, 2008).

Herpes simplex viruses 1 and 2 (HSV-1 and HSV-2) belong to diverse family of *Herpesviridae*, causing oral herpes lesions (HSV-1) and genital lesions (HSV-2). Globally, 45% to 98% of the world population are infected or previously infected with herpes simplex virus 1 (Hong *et al.*, 2014, Alvarez *et al.*, 2015). Moreover, the most common causes of meningitis and encephalitis are HSV infection when it infects the central nervous system. Viral latency is a worry in the management of HSV treatment (Ge *et al.*, 2013). Furthermore, HSV infection may also cause lethal infections in immunocompromised patients as it reported early (Yucharoen *et al.*, 2011).

No available antiviral drug can eradicate herpes virus from the body completely, and the current antiviral drug for HSV infections management is based on nucleoside analogues that inhibit the viral DNA polymerase as acyclovir, valacyclovir, famciclovir and penciclovir and their

* Corresponding author: mohameddkhil@yahoo.com

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derivates (Donalisio *et al.*, 2013). However, treatment of herpes infection is associated with relative high side effects and emergence of drug-resistant virus strains (Alvarez *et al.*, 2015).

Due to the drug resistance and the high prevalence of HSV infections, there is a great demand for developing new antiviral drugs exhibiting different mechanism of action. In this context, alternative medicines have been used to treat or prevent HSV infections for a long time. Accordingly, a large number of natural products including pure compounds and standard extracts isolated from herbal medicines have been reported to exert antiviral effects on HSV-1 (Xiang *et al.*, 2008). Therefore, the present work was performed to investigate the anti-herpes viruses 1 and 2 effects of mulberry (*Morus alba*) aqueous extract.

MATERIALS AND METHODS

Viruses and cell lines

Vero cells were grown in DMEM (Dulbecco-modified Eagle's Minimum Essential Medium; Gibco, Brazil) supplemented with 10% fetal bovine serum (FBS; Gibco®, Brazil) and 80 µg/ml gentamicin. The cells were maintained at 37°C in a humidified 5% CO₂ air. Propagation of herpes simplex virus type 1 and 2 were carried out in Vero cells. Virus quantification was performed in 24-well tissue culture plates, titrated on the basis of plaque forming units (PFU) count by plaque assay (Burlison *et al.*, 1992) then stored at -80°C until use.

Plant Material

Morus alba (*M. alba*) leaves were collected from local garden in East Cairo, Egypt in the months of May-June, 2014. The samples were authenticated by a taxonomist (Dr. Jacob Thomas, College of Science, King Saud University, Saudi Arabia).

Extraction

The plant leaves were shade dried at room temperature (30±2°C) and the dried leaves were ground into fine powder using pulverizer. The powdered part was sieved and kept in deep freezer until the time of extraction. One hundred gram of

dry fine powder was extracted with 50× (weight/volume) of water at 85°C for about 3 h. The extract was filtered using Whatman No. 1 filter paper to remove any insoluble particles. The filtrate was lyophilized with a freeze-dryer-cryodo and the dried extract was at -20°C stored until used.

Determination of total polyphenols

The total polyphenolic content (TPC) of the extract was measured using Folin-Ciocalteu reagent. This step based on the oxidation and transformation of polyphenols to a blue colored complex compound with an absorbance of 750 nm (Kim *et al.*, 2003). Using gallic acid as standard for TPC, the calibration curve was prepared and measured as mg gallic acid equivalents (GAE) per mg of the sample (µg/mg).

Determination of flavonoids

For the assessment of flavonoids a colorimetric method was used (Abdel Moneim, 2013). Briefly, deionized water (1.50 ml) was added to the sample (0.25 ml) and then 5% Sodium nitrite (NaNO₂; 90 µL) was added. Six min later, after addition of 10% AlCl₃ (180 µL), the mixture was allowed to stand for another 5 min before mixing 1M NaOH (0.6 ml). The final volume was made up to 3 ml by adding deionized water. The absorbance was measured at a fixed wavelength 510 nm. Calibration curve was prepared using quercetin as standard for total flavonoids. The measurement was expressed as mg quercetin equivalents (QE) per mg of the sample (µg/mg).

Determination of DPPH radical scavenging activity

The free radical scavenging capacity of the extract was evaluated by the 2, 2-Diphenyl -1-picrylhydrazyl (DPPH) assay (Lee *et al.*, 2003). DPPH absorbed at 517 nm. Briefly, 1 ml of 0.25 mM solution of DPPH in methanol was added to 50, 100, 150 and 200 µL of sample in 950, 900, 850 and 800 µL methanol, respectively. After 20 min, the absorbance was measured at 517 nm. Ascorbic acid (Sigma®) was considered as a positive control. DPPH free radical scavenging capacity of the extract was calculated by the following equation:

$$\% \text{ DPPH scavenging} = [(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}] - 100, \text{ where } A \text{ refers to the absorbance.}$$

Cytotoxicity assay

Confluent Vero cells were subjected to different concentrations of *M. alba* (1-5000 µg/ml) for 72 h. According to Mosmann (1983), the cell viability was assessed by a MTT [3-(4,5-dimethylthiazol-2,5-diphenyl tetrazolium bromide)] assay. The 50% cytotoxic concentration (CC₅₀) was defined as the concentration of the *M. alba* extract reducing the cell viability by 50% when compared to untreated controls (Mosmann, 1983).

Antiviral activity assay

Preincubation of the 96-well plates (for 1 h) containing confluent cell monolayers were done with increasing non-cytotoxic concentration of *M. alba* leaves extract. For each concentration, 6 wells were used. Then, cells were infected with HSV-1 or 2 (10 TCID₅₀) and incubated at 37 °C in 5% CO₂ air and observed every day up to three days for cell cytopathic effect (CPE) using a light microscope. When CPE was observed in all virus control wells, the percentage of wells with CPE was determined for each treatment concentration, as described previously. Acyclovir (Sigma®) was served as the positive control at concentration of 0.05 to 2 µg/ml.

Viral plaque assay

This assay was proceeded according to the procedures previously described by (Silva *et al.*, 2010), with minor modifications. Approximately one hundred plaque forming units (PFU) of HSV types were adsorbed for 1 h at 37 °C on confluent Vero cells (MEM; Gibco, Carlsbad, USA) and 1.5% carboxymethylcellulose (CMC, Sigma®, USA). This was either in presence or absence of different concentrations of *M. alba* leaves extract. Cell fixation and staining with naphthol blue-black (Sigma) was carried out after 72 h, then, the plaques were counted. The 50% inhibitory concentration (IC₅₀) was determined where Acyclovir (Sigma®) was used as a positive control. According to (Cos *et al.*, 2006), the obtained results were expressed as CC₅₀ and IC₅₀ values in order to calculate the selectivity index (SI = CC₅₀/IC₅₀) of each sample (Cos *et al.*, 2006).

Statistical analysis

The data were expressed as means ± SEM

from at least three separate different experiments.

RESULTS AND DISCUSSION

Table I shows the flavonoids and total polyphenolic contents of *M. alba*. The total polyphenolic content in aqueous *M. alba* extract was 52.7 µg/mg gallic acid equivalent of polyphenols/mg extract. Whereas, the flavonoids content were 22.4 µg/mg quercetin equivalents of flavonoids/mg extract. Arabshahi-Delouee and Urooj (2007) and Arfan *et al.* (2012) proved that, *M. alba* leaves obtained by methanolic, acetonic and aqueous extraction, exhibited 93, 85, and 71 mg total phenolics/g, respectively, is similar to this work.

Table I.- Total phenolics and flavonoids contents of *M. alba*.

Conditions	Total phenolics [#]	Total flavonoid [*]
Aqueous extract	52.7±1.3	22.4±2.5

(a) Flavonoids are expressed as µg/mg quercetin equivalents of flavonoids/ml juice. (b) Total phenolics are expressed as µg/mg gallic acid equivalent of polyphenols/ml juice. Data are represented as means ± SEM of three independent experiments each performed in duplicate.

The antioxidant property of *M. alba* leaves extract was determined by measuring the DPPH• radical scavenging capacity. Figure 1 shows the dose-response curves for the DPPH radical-scavenging capacity of the *M. alba* leaves extract. The obtained results proved that, the DPPH radical scavenging capacity was 122.4 µg/ml. The investigated feature reveals good antioxidant attributes significantly (Iqbal *et al.*, 2012). The relation between total polyphenols and antioxidant property has been widely examined in many herbs. As previously reported by (Li *et al.*, 2009, Kim *et al.*, 2014), our results showed that the antioxidant property of *M. alba* significantly increased with the presence of a high total polyphenolic content. Also, the antiradical capacity against the DPPH radical was determined by Zhang *et al.* (2011) for the presence of anthocyanins compounds (cyanidin-3-glucoside and cyanidin-3-rutinoside) isolated from *M. alba*.

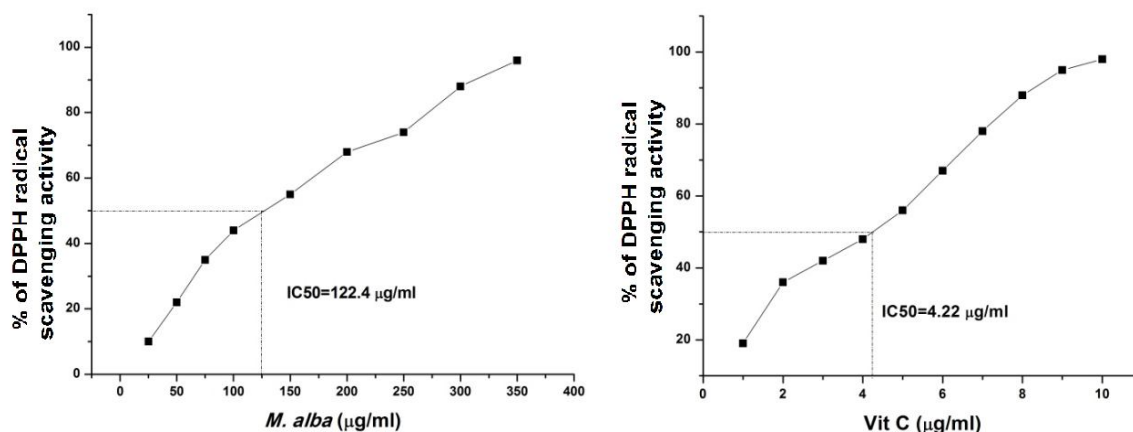


Fig.1. Antiradical activities of *Morus alba* leaves extract and ascorbic acid against DPPH radical. Examination of the cytotoxicity of *M. alba* extract was performed in the range of concentrations up to 5000 µg/ml. The maximum non-cytotoxic concentrations were read individually from the obtained survival curves.

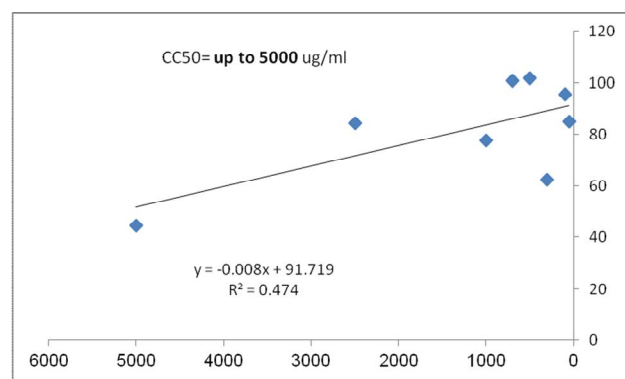


Fig. 2. The cytotoxic effect of *Morus alba* leaves extract. CC_{50} (the concentration of the 50% *M. alba* cytotoxic effect). Data are expressed as means \pm SEM of two independent different experiments. Each experiment was performed in triplicate.

Examination of the cytotoxicity of *M. alba* extract was performed in the range of concentrations up to 5000 µg/ml. The maximum non-cytotoxic concentrations were read individually from the obtained survival curves.

Cytotoxicity of *M. alba* leaves extract was determined in seeded Vero cells by the MTT assay. According to the results of this experiment (Fig. 2), *M. alba* extract has cytotoxicity up to 5000 µg/ml indicating the non-toxicity effect of *M. alba* extract.

To study the antiviral activity of *M. alba* extract against HSV types-1 and 2, cytopathic

inhibitory assay was done and the data was depicted in Table II. Based on CPE of the virus-infected seeded Vero cells, *M. alba* extract exerted strong antiviral activity against both of HSV-1 and HSV-2 at high concentration, 10 µg/ml. In this manner, the CPE was showed at 3.5 µg/ml.

Table II.- The antiviral activity of *Morus alba* leaves extract.

<i>Morus alba</i>	Cell Cytopathic Effect (CPE)	
	HSV-2	HSV-1
1 µg/ml	+	+
2 µg/ml	+	+
3 µg/ml	-	-
5 µg/ml	-	-
10 µg/ml	-	-

Plaque inhibition assay was carried out to determine the IC₅₀. As shown in Figure 3, *M. alba* extract at 2 µg/ml provided 66.7% inhibition of plaque of HSV-1 and showed 100% inhibition against HSV-1 at 3.5, 5 and 10 µg/ml.

The *in vitro* anti-HSV-2 activity of the *M. alba* extract was investigated (Fig. 4). The HSV-2 strain was more sensitive to the samples. According to the present results, *M. alba* leaves extract at 2 µg/ml provided 48.6% inhibition of plaque of HSV-2 and showed 100% inhibition against HSV-2 at 4.5, 5 and 10 µg/ml. the anti-HSV-2 and anti-HSV-2

could have been due to the binding of a phytochemical phenolic compound with the protein coat of the virus and arrestment absorption of the extracts into the Vero cells.

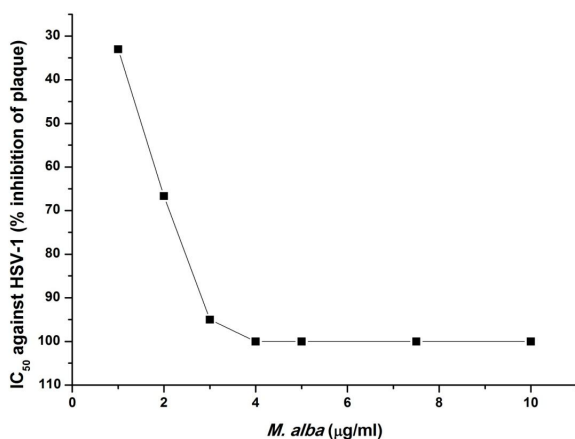


Fig. 3. The anti-herpes virus 1 activity of *Morus alba* leaves extract. IC₅₀ (concentration of the sample needed to inhibit 50% virus-induced CPE). Data are means±SEM of two different independent experiments; each one was performed in triplicate.

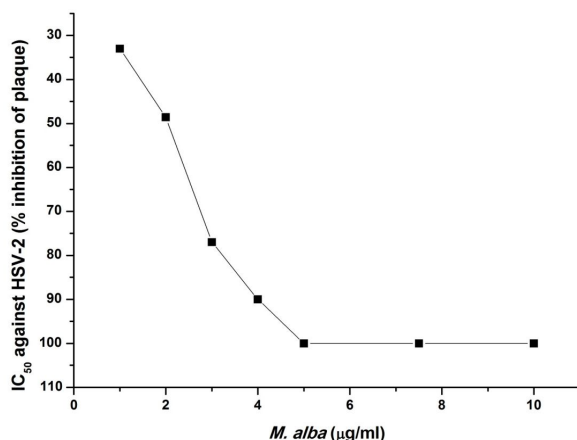


Fig. 4. The anti-herpes virus 2 activity of *Morus alba* leaves extract. IC₅₀ (concentration of the sample needed to inhibit 50% virus-induced CPE). Data are means±SEM of two different independent experiments, each one was performed in triplicate.

Several medicinal plants products are potential sources of functional foods and have various bioactivities like immunomodulatory and

anti-cancer activities. Also, a number of medicinal plants had been reported to contain compounds possessing strong antiviral activity (Patharakorn *et al.*, 2010). Several studies showed that polyphenols (Kuo *et al.*, 2002; Chattopadhyay and Naik, 2007), triterpenes and saponins (Simoes *et al.*, 1999), polysaccharides (Marchetti *et al.*, 1996) and anthraquinones (Sydiskis *et al.*, 1991) isolated from several plants inhibit the herpes viruses replication. Furthermore, a large number of plant-derived and synthetic anti-herpes virus agents have also been described (Jassim and Naji, 2003; Ikeda *et al.*, 2000) and several works is in progress to identify plants and their active components having anti-herpes virus activity.

Park *et al.* (2003) demonstrated that mulberry extracts are rich in phytochemicals and have antimicrobial potential against harmful pathogens. In this context, 14 compounds were isolated from *M. alba* and these compounds were tested against HIV. The Result showed that the ethanolic extract of *M. alba* contains flavonoids like morusin, morusin 4'-glucoside and kuwanon H showed activity against HIV (Shi-De *et al.*, 1995). Furthermore, Du *et al.* (2003) demonstrated that two prenylated flavonoids, namely leachinone G (IC₅₀ = 1.6 µg/mL) and mulberroside C (IC₅₀ = 75.4 µg/mL), from seven known compounds isolated from the rootbark of *M. alba* L., were active principles for anti-HSV-1 activity. This evidenced that *M. alba* leaves extract has strong antiviral activity that could interfere with virus adsorption into host cells and also with some intracellular activity. The minimum inhibitory concentrations of the extract were investigated. However, it remains to be shown whether this inhibitory effect is due to impairment of viral proteins concerned with host cell receptor binding, adsorption, and/or virions penetration.

CONCLUSION

In conclusion, *Morus alba* leaves extract could be used as a good candidate in the development of new effective antiviral drugs against HSV-1 and HSV-2. Further studies are necessary to identify the active principle responsible for this activity.

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