



Phytochemical screening and antiviral activity of *Marrubium vulgare*

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Aims: The present study is to investigate the phytochemical content, cytotoxicity and antiviral activity of the crude and fractions from *Marrubium vulgare*.

Methodology and results: The crude methanol extract and fractions using hexane, chloroform, ethyl acetate and *n*-butanol of *M. vulgare* whole plant were produced in this study. Qualitative phytochemical analysis revealed the presence of flavones aglycones and steroid in all preparations except for the hexane fraction. Carbohydrates, saponins were found present in the crude and ethyl acetate extracts while tannin was detected in the crude, ethyl acetate and butanol extracts. Anthraquinones and terpenes were detected in the hexane fraction. The cytotoxicity of the crude and fractions were examined using a modified MTT assay and the cell concentration that causes 50% of cell death (CC₅₀) was determined. The crude extract and fractions showed no toxicity towards Vero cells with the CC₅₀ value ranges between 140 to 400 µg/mL. Antiviral activity against herpes simplex virus type-1 (HSV-1), attachment and virus yield reduction assays were analyzed by plaque reduction assay. Only the crude extract, hexane and chloroform fractions showed antiviral activity with the selectivity indices of 3.11, 2.8 and 1.28 respectively. In this study it is revealed that the hexane fraction disrupts the early steps of cyclic replication, including HSV-1 attachment in a dose-dependent manner.

Conclusion, significance and impact of study: The crude, hexane and chloroform fractions of *M. vulgare* were not cytotoxic and have antiviral properties. Treatment with the hexane fraction inhibits viral attachment and reduces virus yield. Further study on the mechanism of *M. vulgare* must be elucidated for the potential as antiviral agent.

Keywords: *Marrubium vulgare*, phytochemical constituent, cytotoxicity, antiviral activity

INTRODUCTION

Acute and recurrent herpes simplex virus type 1 (HSV-1) infections remain an important problem due to the emergence of acyclovir (ACV) resistant virus that makes the search for novel antiviral substances imperative. Potential anti-herpes activity of bioactive compounds from numerous plant species have been investigated (Allahverdiyev *et al.*, 2004; Verma *et al.*, 2008). HSV-1 is a DNA virus that causes a variety of diseases in humans. In some instances, HSV-1 is a major threat to immune-compromised patients (Serkedjieva *et al.*, 1999; Chuanasa *et al.*, 2008). The present study thus sought to screen a medicinal plant, *Marrubium vulgare* for the safety and potential in anti-virus therapy. *M. vulgare* grows in the wild and commonly known as "Horehound" belongs to the Lamiaceae family (Masoodi *et al.*, 2008). It has been used in folk medicine and reported to possess a wide range of biological activities and pharmacological properties. This plant has been previously reported to have antihypertensive activity (El-Baradai *et al.*, 2004), exhibiting vasorelaxant (El-Baradai *et al.*, 2003a; 2003b),

anti-diabetic (Elberry *et al.*, 2011; Boudjelal *et al.*, 2012), anti-oxidant (Kadri *et al.*, 2011), anti-inflammatory, analgesic, anti-bacterial (Masoodi *et al.*, 2008; Kanyonga *et al.*, 2011) and antioedematogenic (Stulzer *et al.*, 2006) activities. However, bibliographic searches reveal that there is a dearth of information on its antiviral activity against HSV-1. In this study, various fractions of the *M. vulgare* were determined for their phytochemical constituents. The cytotoxicity and antiviral activity against HSV-1 evaluated for the crude and fractions to determine the potential as antiviral agents. The viral attachment and virus yield assays carried out for the hexane fraction to determine the preliminary mode of action.

MATERIALS AND METHODS

Plant extraction

The powder of the whole plant (including root, stem, leaves, flowers and seeds) of *M. vulgare* was purchased from Alibaba Company (Hong Kong, China). The crude extract was prepared following the method of Masoodi *et*

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al. (2008) with minor modification. The powder of the whole plant (50 g) was filled in the thimble of Soxhlet apparatus and extracted with methanol for up to 9 h. Thereafter, it was filtered and the filtrate was centrifuged at 4000 rpm for 15 min. The supernatant was collected and the solvent was concentrated and dried by rotary evaporator and kept at 4 °C until used.

Plant fractions were prepared following the method of Ilango *et al.* (2009) and Ramkumar *et al.* (2007) with slight modifications. Dried powder (200 g) was dispensed in the thimble of a Soxhlet apparatus and extracted with distilled water and methanol (1:2) for up to 15 h. The methanol from the resulting extract was removed by rotary evaporation and the liquid part successively partitioned (3×50 mL) with hexane, chloroform, ethyl acetate and *n*-butanol yielding four fractions. These fractions were filtered and concentrated under reduced pressure. Subsequently, the obtained aqueous fraction was lyophilized.

Phytochemical analysis

Phytochemical analysis of the crude extract and four fractions was established according to standard procedures used to test for the presence of alkaloids, carbohydrates, saponins, tannins, flavones glycones, steroids, terpenes and anthraquinone as described by Kasolo *et al.* (2010) and Edeoga *et al.* (2005).

Cells and virus

Vero cell lines from the stock collection of the Virology Laboratory, School of Biosciences and Biotechnology were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% fetal bovine serum, penicillin/streptomycin (100 U/L) and non-essential amino acids. Cell culture was maintained at 37 °C under a humidified 5% CO₂ atmosphere. A clinical isolate of HSV-1 was propagated in Vero cells and incubated at 37 °C until the cytopathic effect develops. The titer of virus was estimated and stored at -80 °C until used.

Cytotoxicity assay

Evaluation on the crude and fraction cytotoxicity towards Vero cells was performed using MTT [3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide] assay. Vero cells (2×10⁵ cells/mL) were prepared in 96 well tissue culture plates, after a 24 h period of incubation at 37 °C under a humidified 5% CO₂ atmosphere. The medium was removed from the wells, and 100 µL of dilutions of the crude extract and fractions (70 µg/mL to 600 µg/mL concentration prepared in cell culture medium) were added to each well. Control cells were added with only 100 µL of the medium. The plates were incubated for 3 days under the same conditions mentioned above. Subsequently, the medium was removed and the cells washed by phosphate buffer saline (PBS) followed by the addition of 100 µL of DMEM and 10 µL of MTT to each well. The plates were then incubated for 3 h. The MTT

solution was then removed and 130 µL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals, followed by gentle shaking for 15 min. Absorbance was determined spectrophotometrically on a multi-well plate reader at 540 nm. The CC₅₀ was defined as the cytotoxic concentration of each extract that reduced the absorbance of treated cells to 50% when compared with that of the control cells (Akanitapichat *et al.*, 2005; Capua *et al.*, 2010). CC₅₀ values were calculated using regression analysis.

Antiviral activity

Vero cell line (2×10⁵ cells/ mL) was prepared in 24 wells (500 µL in each well). When the cells is 80-90% confluent, the medium was removed from the wells. HSV-1 at 50 pfu and DMEM were added subsequently to the cells and incubated for 1 h. The medium was removed and different concentrations of crude extract and fractions (concentration is less than the CC₅₀ values) were added to the methyl cellulose (MC) which was applied to the cells. This was then incubated for two days. After the plaques appeared, the plates were stained with crystal violet and shaken for about 30 min, followed by gentle washing. The plates were left to dry and the number of plaques was counted using an inverted microscope. The IC₅₀ was defined as the concentration of the extracts that inhibit 50% of the plaques in virus infected cells compared to control (Yew *et al.*, 2002). The mean number of plaques was used to calculate the percentage of plaques inhibited. A curve relating percent plaque inhibition against extract concentrations was used to calculate the inhibitory concentration that elucidated a 50% reduction of cell viability (IC₅₀). The experiments were performed in triplicates with the mean values represent three experiments

Attachment assay

This assay was carried out by mixing virus and the hexane fraction with different concentrations and incubated at room temperature one minute. Vero cell monolayer was grown in 12 well culture plate and then pre-chilled at 4 °C for 1 h. The medium was aspirated and the cell monolayer was infected with 100 a plaque-forming unit (PFU)/well of HSV-1 in the absence or presence of different concentrations of hexane fraction, then incubated at 4 °C for another 3 h. The medium was aspirated to remove virus inoculum and cell monolayer was washed with PBS and overlaid with medium containing 1% methylcellulose. The cell monolayer was incubated for a further 48 h before it was fixed and stained. The percentage inhibition was calculated by the following formula:

Percentage inhibition =

$$1 - \frac{(\text{Average number of plaques})_{\text{test}}}{(\text{Average number of plaques})_{\text{control}}} \times 100\%$$

Virus yield reduction assay

Monolayers of Vero cells grown in 12 well plates were infected with 100 pfu/well of HSV-1 for 2 h at 37 °C in 5% CO₂. The inoculum was removed and hexane fraction at concentrations 20-100 µg/mL in DMEM with 5% FBS was added. At 30 h after viral infection, the infected cells were lysed by freezing and thawing three times. The supernatant was obtained by centrifugation at 3000 rpm for 10 min at 4 °C. A serial dilution of each supernatant was prepared in DMEM and the surviving virus titer was determined by the plaque assay (Saddi *et al.*, 2007)

RESULTS AND DISCUSSIONS

Phytochemical content

The presence of active compounds from different fractions of *M. vulgare* is shown Table 1. Carbohydrates, saponins, tannins, flavones aglycones and steroids were present in crude and ethyl acetate fractions. Alkaloids were absent in all extract and fractions. Herbs in the Family *Lamiaceae* have a long history of treating diverse diseases. Natural compounds purified from these herbs have demonstrated inhibitory effects on HSV-1 replication, targeting nearly every stage of the HSV-1 life cycle (Pushpa *et al.*, 2013). These compounds vary in chemical composition and include alkaloids, polysaccharides, polyphenolics,

flavonoids, coumarines, phenolics, tannins, carvacrol, triterpenes and anthraquinones. Abadi *et al.* (2013) and Verma *et al.* (2012) reported that *M. vulgare* essential oils contains major components including tetramethyl heptadecan-4-ol, germacrene d-4-ol, α-pinene, phytol, dehydro-sabina ketone, piperitone, δ-cadinene, 1-octen-3-ol and benzaldehyde. Terpene compounds isolated from the oils includes linalool, 1-8-cineole, thymol, marrubiin (Zawislak, 2012).

In this study, the methanol crude extract of *M. vulgare* showed the highest level of inhibitory effect on HSV-1 followed by the hexane fraction. Phytochemical analysis showed that the crude and the hexane fraction contain compounds of terpenes and anthraquinones. According to Astani *et al.* (2011), sesquiterpenes were able to suppress viral infection by 40-98% and directly inactivate herpes virus by interfering with virion envelope structures or mask viral structures that are necessary for adsorption or entry into host cells. Soares *et al.* (2012) also identified the meroditerpenoid atomaric acid from the *Styopodium zonale* extracts as anti-HSV-1. According to Aref *et al.* (2011) triterpenes showed activity against HSV-1 and their mode of action was found at all stages of multiplication. Sydskis *et al.* (1991) demonstrated that the anthraquinones acted directly on the envelope of HSV-1, resulting in the prevention of virus adsorption and subsequent replication.

Table 1: Phytochemical screening of the crude and fractions of *Marrubium vulgare*.

Test Sample	Carbohydrates	Alkaloids	Saponins	Tannins	Flavones aglycones	Steroids	Anthraquinone	Terpenes
Crude	+	–	+	+	+	+	+	+
Hexane fraction	–	–	–	–	–	–	+	+
Chloroform fraction	–	–	–	–	+	+	–	–
Ethyle acetate fraction	+	–	+	+	+	+	–	–
<i>n</i> -butanol fraction	–	–	–	+	+	+	–	–

+, present; –, absent

Cytotoxicity

Table 2 shows the CC₅₀ values of the crude extract and the fractions towards Vero with the value of more than 20 µg/mL indicating that the samples were non cytotoxic (Moo-Puc *et al.*, 2009; Chandrashekar *et al.*, 2011). Thus, the antiviral activity is now worth to be demonstrated for the potential as antiviral agents.

Antiviral activity

Hexane fraction displayed antiviral activity compared to other fractions as 80% of inhibition was achieved at 100 µg/mL and an associated IC₅₀ value of 50 µg/mL (Table 2). Both crude extract and chloroform fraction indicated a reduction in the number of plaques by 72% and 55% at 150 µg/mL and IC₅₀ values respectively being 90 µg/mL

and 140 µg/mL. On the other hand, no activity was detected in the ethyl acetate or *n*-butanol fractions. The selective index (SI) was determined to associate the cytotoxicity and the antiviral potential of the tested crude and fractions as shown in Table 2. In this study, only the crude extract, hexane fraction and chloroform fraction displayed safe antiviral activity. The effective concentrations of crude, hexane, chloroform fractions were 150 µg/mL, 100 µg/mL and 150 µg/mL respectively. We isolated the pure compound and tested for antiviral activity (data not shown). However, when compared with the methanolic crude of the whole plant, chloroform and hexane fractions in the cytotoxicity assay, it was found to be less active in antiviral activity with the selective indices was less than 2 (1.7). Thus in this study, we continue to evaluate the fraction with selective index more than 2 and higher (Aschenbrenner and Venable, 2009).

Table 2: Cytotoxicity (CC₅₀), inhibition concentration (IC₅₀) and selective index (SI) of crude and fractions of *Marrubium vulgare*.

Test sample	CC ₅₀	IC ₅₀	SI
Crude extract	280 µg/mL	90 µg/mL	3.11
Hexane	140 µg/mL	50 µg/mL	2.8
Chloroform	180 µg/mL	140 µg/mL	1.28
Ethyl acetate	400 µg/mL	NI	ND
<i>n</i> -butanol	350 µg/mL	NI	ND

NI, No inhibition in plaque reduction assay
 ND, Not determined

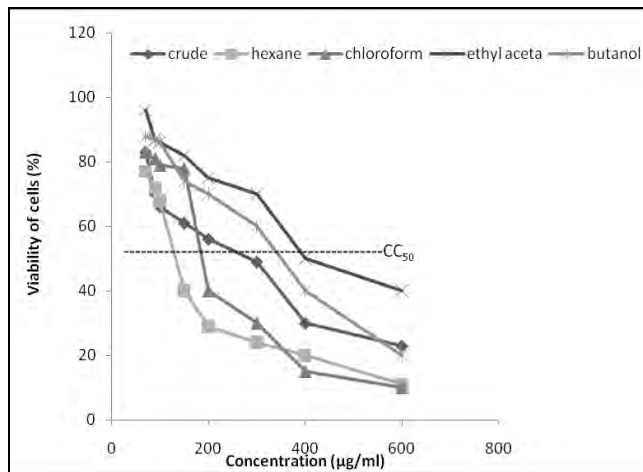


Figure 1: Viability of cells of crude and fractions of *Marrubium vulgare*.

In this study, the selective index or SI of crude, hexane and chloroform fractions were more than 1 thus can be accepted and at least not dangerous. For use as topical agents, drugs should not have SI less than 1. Most drugs have a selective index of 2 or higher. Drugs with SI of 5 or 10 are safer drugs (Aschenbrenner and Venable 2009; Omonike *et al.*, 2013).

The hexane fraction was effective when it was added in parallel with the virus to cells. This observation suggests that the hexane fraction might disrupt the early steps of cyclic replication, including obstruction of viral attachment. HSV-1 attachment was inhibited by hexane fraction in a dose-dependent manner at 100 µg/mL, 90 µg/mL and 80 µg/mL with the percentage of inhibition was 44%, 33% and 18% respectively.

The complete HSV-1 replication cycle takes approximately 18 to 20 h (Bacon *et al.*, 1996) but in this study the replication cycle takes place in 30 h. Figure 2 shows the virus yields of HSV-1 in Vero cells as control or treated with varying concentrations of hexane fraction. Hexane fraction reduced the virus yields in a dose dependent manner. The highest concentration of 100 µg/mL reduced the yields to less than 1×10^2 pfu/mL. A 90 µg/mL, the fraction reduced the virus yields to 2.4×10^2 pfu/mL and at 80 µg/mL the virus yield was reduced to the half amount estimated in control cells.

Antiviral activities of some *Lamiaceae* species have been reported in previous studies. The extract of *Thymus vulgaris* has been reported to show high level of antiviral activity against HSV-1 and HSV-2 (Behravan *et al.*, 2011). Several compounds such as carvacrol, thymol, anthraquinones, especially the polyphenolics, polysulphonates and monoterpenes derived from *Lamiaceae* have been shown to possess antimicrobial or antiviral activity. Most extracts exhibit their effects on viral particles prior to attachment to host cell or post-infection of the cell. Flavonoids exhibit antiviral effects via inhibiting the RNA synthesis of viruses and polyphenols act principally by binding to the protein coat, thus preventing absorption of the virus (Esimone *et al.*, 2007; Behravan *et al.*, 2011). In the study conducted by Amoros *et al.* (1987), saponin isolated from the *Anagallis arvensis* plant was reported to inhibit adhesion and penetration of HSV-1 to Vero cell. In another study, conducted by Andrighetti-Frohner *et al.* (2005) plants from Brazilian tropical forest demonstrate anti-herpetic effect possibly due to their flavonoids content, especially biflavones and glycosylflavones.

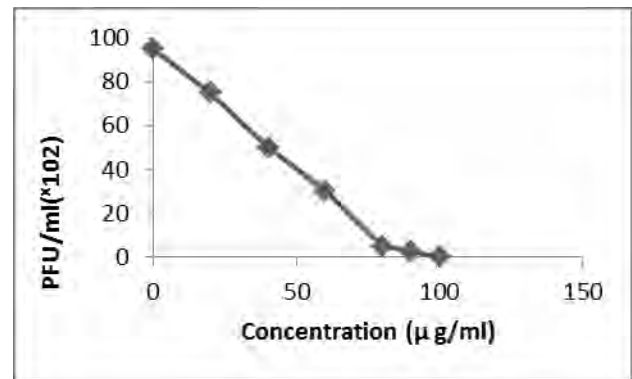


Figure 2: The yield reduction assay with hexane fraction. The inhibition in the yield of the virus significantly ($p < 0.05$) at 100, 90, and 80 µg/mL.

Hsiang and Ho (2008) investigated the action of emodin on HSV-1, the result indicated that the emodin possesses antiviral activities via the inhibition both UL12 and CK2 and disruption of the phospholipid bilayer of the envelope. A study carried out by Zandi *et al.* (2007) revealed that hot glycerine *Aloe vera* extract which contains anthraquinones could be a good choice for preventing of virus adsorption, attachment or entry to the host cell. Cohen *et al.* (1996) reported that the most active anthraquinone was 5,7-dichloroemodin against HSV-1 compared to (2,6-8,13) anthraquinones. Further confirmation on the active compounds available in *M. vulgare* however, needs to be done in the future.

In summary, our findings suggest that *M. vulgare* contains antiviral active compounds especially in the crude extract and hexane fraction. This set the stage for future studies on the plant for the development of topical agents against HSV-1. In doing so, the mechanism of

action of the active compound from this plant is worthy to be determined. Furthermore, the compound which is responsible to work singly or synergistically in their contribution to disintegrate the envelope of the virus must be determined.

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