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 anti-edematogenic (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 al., 2008.

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al. (2008) with minor modifications. 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 glycosides, steroids, terpenes and anthraquinones 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/mL) 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 4 °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 were 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 cultured 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:

\[
\text{Percentage inhibition} = \frac{1 - \left(\frac{\text{Average number of plaques}}{\text{control}}\right)_{\text{test}}}{\left(\frac{\text{Average number of plaques}}{\text{control}}\right)_{\text{control}}} \times 100\%
\]
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. vulgar* essential oils contains major components including tetramethyl heptadecan-4-ol, germacrene d-4-ol, α-pinene, phytol, dehydro-sabina ketone, pipertone, 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. vulgar* 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 anthraquinons. 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 meroderpenoid atomaric acid from the *Sydiskis 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. Sydiskis et al. (2013) identified 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    |               |           |          |         |                   |         |               |         |
| Ethyl acetate fraction |       |           | +        | +       | +                 | +       | _             | _       |
| n-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).

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Table 2: Cytotoxicity (CC_{50}), inhibition concentration (IC_{50}) and selective index (SI) of crude and fractions of Marrubium vulgare.

| Test sample        | CC_{50} (μg/mL) | IC_{50} (μg/mL) | SI  |
|--------------------|-----------------|-----------------|-----|
| Crude extract      | 280             | 90              | 3.11|
| Hexane extract     | 140             | 50              | 2.8 |
| Chloroform extract | 180             | 140             | 1.28|
| Ethyl acetate      | 400             | ND              | ND  |
| n-butanol          | 350             | ND              | ND  |

NF: No inhibition in plaque reduction assay
ND: Not determined

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 Anagalis 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.

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.
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