Antiviral Components from Cleome droserifolia and Lotus creticus

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Abstract

Objectives: Searching for new antiviral compounds is important due to continuous appearance of new viral strains that resist the commonly used drugs and due to toxic side effects of some of these drugs. This current study was designed to investigate some herbs to discover new natural antiviral agents. Methods: Eight herbal extracts were screened for antiviral activities against Hepatitis A, Herpes Simplex-1 and Coxsackie viruses. Bio guided fractionation of the active extracts using different solvents and further investigation of the bioactive one using various chromatographic techniques including normal phase column chromatography, poly amide column and preparative TLC led to isolation and identification of twelve compounds. Structural elucidation of these compounds was done using different spectroscopic techniques including IR, HR-MS, 1D and 2D-NMR. The antiviral activities of the isolated compounds were assessed by MTT assay that depends on mixed treatment assay which test inactivation of virus particles in the virus suspension by samples. The concentration of samples which inactivate virus particles in virus suspension by 50% compared with untreated control was determined for each sample and was compared with IC50 of positive control, Acyclovir. Findings: Twelve known compounds were isolated from the bioactive fractions of Cleome droserifolia and Lotus creticus. Nine compounds (1-9) from methylene chloride and ethyl acetate fractions of Cleome droserifolia and four compounds (10-13) from ethyl acetate and butanol fractions of Lotus creticus. Teuclatriol (4) was the most potent, with IC50 value of 4.89 ±0.061 μM against HSV-1 and a selective index (SI) value of 4.8. Moreover, the pentamethoxyflavone (3) was the most active against HAV with IC50 value of 9.52±0.329 μM and SI of 3.4. The potent activity against Cox. B4 virus with IC50 of 13.054 ±0.348 μM and SI of 3.4 exhibited by β-sitosterol 3-O-β-D-glucoside (6, 12). Novelty: The results highlight that these compounds are candidates for the development of new lead antiviral compounds. This is the first report for proving antiviral activities of these compounds.

Keywords: Antiviral activity; MTT; pentamethoxyflavone; teuclatriol
1 Introduction

Viruses are serious pathogens for humans. Hepatitis A virus acute infection in young children is frequently a symptomatic but fulminant hepatic failure is the common manifestation in adult patients. There is no available drug to cure HAV disease, but immune prophylaxis, using Immune Serum Globulin, is the available way to attenuate or prevent disease with no long-term protection in exposed individuals. Herpes simplex-1 is a serious pathogen that causes clinical manifestations that range from flu-like syndrome followed by local symptoms and serious systemic illness in neonates and immunocompromised patients. Since 1970, Acyclovir is the most used remedy for HSV-1 but, there is a need for more new effective antiviral agents. Type B Coxackie viruses cause epidemic pleurodynia which includes headache, abdominal pain and fever. In this study, seven plants were selected; including Cyperus rotundus (aerial part and rhizomes), C. alopecuroides, C. helferi, Fagonia mollis, Francoeuria crispa, Cleome droserifolia and Lotus creticus and evaluated for their antiviral activity against HAV, HSV-1 and Cox. B4. Two plant extracts, Cleome droserifolia and Lotus creticus showed the highest antiviral activity against these viruses. Cleome droserifolia is an herb that belongs to the family Capparidaceae and known in Egypt as Samwah, Afein or Reeh-El-Bard. It stems from Egyptian traditional medicine and used for treating diabetes mellitus. Anti-diabetic, cytotoxic, anti-schistosomiasis and antibacterial activities of this plant have been reported. Singh et al. has reported the isolation of dollylvanone diterpene, sesquiterpenes, flavonoid, and benzyl isothiocyanate from C. droserifolia. Lotus creticus is an herb that belongs to the family Fabaceae and grows in Egypt in the coastal sandy plains. No biological activity was reported for L. creticus and only few reports on its chemistry, where cyanogenic glycosides, steroids, triterpenes, flavonoids, and coumestan derivatives were isolated. Herein, our aim is to investigate the chemical composition of the active fractions of C. droserifolia and L. creticus, and to isolate the compounds that might contribute to the antiviral activity.

2 Material and Methods

2.1 Plant material

Francoeuria crispa (Forssk.) Cass. (Syn. Pulicaria undulata (L.) C.A.Mey.), Cleome droserifolia (Forssk.) Delile, and Fagonia mollis Delile were collected from wadi Hagul, North Eastern desert, Egypt; Lotus creticus L. from Coastal desert, Egypt; C. alopecuroides Rottb. from Dikerness, Dakahlia, Egypt; C. rotundus L. and C. Helferi Boeckeler from Bahtim, Al Qalyubiyah, Egypt. The freshly collected plant materials were air dried in shade at room temperature. The plants were authenticated by Prof. Dr. Ihsan El Habashy, Department of Ecology and Taxonomy, Faculty of Sciences, Mansoura University, Egypt. Voucher specimens have been deposited in Pharmacognosy department, Faculty of Pharmacy, Mansoura University.

2.2 General Experimental procedures

The UV spectra were determined with UV-visible spectrophotometer, Mattson 5000 FTIR (England), Infra-red spectra were recorded on spectrophotometer (Mattson 5000 FTIR, England), NMR spectroscopic measurements were conducted using Bruker Ascend spectrometer at 400 and 600 MHz for proton, and 100 and 150 MHz for carbon, respectively. Samples were dissolved in CDCl$_3$, CD$_3$OD or mixture of both solvents, and DMSO -d$_6$. Chemical shifts were given in ppm with a TMS as internal standard and the obtained data were processed using ACD-NMR processor software. HRESI-MS was carried out on UPLC-Quadrupole Orbitrap MS (Thermo Scientific Company). TLC was performed on pre-coated TLC plates with silica gel 60 GF$_{254}$ (20x20 cm x 0.2 mm thick) on aluminum sheet (Merck, Germany). Normal phase column chromatography was carried out using silica gel G 60-200 microns (Merck, Germany) and polyamide 50-160 microns (Woelm, Germany). Chromatographic detection of compounds was carried out using ammonia vapors and vanillin-sulfuric acid spray reagent.

2.3 Extraction and isolation

The dried powdered Cleome droserifolia (1 kg) and Lotus creticus (0.8 kg) were extracted by maceration with methanol at room temperature. The collected methanolic extracts were concentrated to a syrupy consistency under reduced pressure and dried until constant weight of 115 g and 100 g, respectively. Each crude extract was suspended in distilled water then partitioned with petroleum ether, methylene chloride, ethyl acetate and finally with n-butanol. All fractions were tested against viruses using MTT assay. The methylene chloride and the ethyl acetate fractions of C. droserifolia showed the highest antiviral activity, while the ethyl acetate and the butanol fractions of L. creticus showed moderate antiviral activity against the tested viruses. The methylene chloride fraction of C. droserifolia (33.9 g) was subjected to column chromatography on silica gel (77 x 5 cm, 480 g) and eluted with petroleum ether-ethyl acetate (95-5) and increasing eluent polarity by 10 % until to 100% ethyl acetate. Fifteen fractions were collected (CDM1-15) and monitored by TLC. Fraction CDM1 (20 mg) was eluted with petroleum ether-
ethyl acetate (90:10) over silica gel column and purified by crystallization from acetone to afford compound 1 (5 mg). Fraction CDM2 (40 mg) was re-chromatographed onto the top of silica gel column and eluted with 100% methylene chloride, then methylene chloride ethyl acetate (99-1) and yielded 5 mg of compound 2 and 16.5 mg of compound 3. Fraction CDM3 (100 mg) was subjected to further chromatographic separation on the top of silica gel column and isocratic elution is adopted with methylene chloride-methanol (99-1) to afford compound 4 (16 mg). Fraction CDM4 (300 mg) was re-chromatographed on normal silica gel column and eluted with methylene chloride-methanol (98-2) to yield compound 5 (5 mg). Compound 6 (15 mg) was obtained by crystallization from methylene chloride solution of fraction CDM5. The ethyl acetate fraction of C. drosenrifolia (13 g) was applied onto the top of silica gel column (73 x 4 cm, 340 g) and gradiently eluted with ethyl acetate (100%) then ethyl acetate containing increasing proportion of ethyl acetate-methanol-water mixture (100:16.5:13.5) to afford 5 fractions (CDE1-5). Fraction CDE1 (50 mg) was further purified by applying onto a top of polyamide glass column and eluted with methanol-water (5 - 95, 0.5L and 10 - 90, 1.5L) to yield compound 7 (2 mg). Fraction CDE2 (25 mg) was crystallized with methanol resulting in yellow needle crystals of compound 8 (15 mg). Fraction CDE3 (261 mg) was re-chromatographed on silica gel column and eluted with ethyl acetate-methanol (95 - 5, 0.5L and 93 - 7, 2L) to get compound 9 (22 mg). The ethyl acetate fraction of L. creticus (5.7 g) was applied to silica gel normal phase column chromatography (60 x 2.5 cm, 100 g) and eluted with petroleum ether - ethyl acetate (2 - 8; 1L, 1 - 9; 1L, 0 - 10; 1L), to yield 3 sub- fractions (LCE 1-3). Fraction LCE 1 (500 mg) was subjected to further chromatographic separation by applying onto the top of silica gel column and eluted with petroleum ether - ethyl acetate (9 - 1) to afford compounds 10 (10 mg) and 11 (10 mg). Compound 12 (20 mg) was obtained by re-crystallization of fraction LCE2 from methylene chloride. Compound 13 was obtained as a yellowish white powder (24 mg) by crystallization from methanol solution of the butanol fraction of L. creticus (10.5 g).

2.4 Cells and viruses

Vero cell line ATCC CCL-81 (Cercopithecus aethiops African green monkey kidney cells) was obtained from VACSERA, Agouza, Egypt. Hepatitis A, Herpes simplex-I and Coxsackie B4 viruses were kindly provided by Dr. Mohammed Ali, Virology Lab., Science way for viral and microbiological studies, Faculty of Medicine, Al-Azhar University, Egypt. Cells were grown in DMEM supplemented with 10% FBS, 100 µg/mL of streptomycin, 100 units/mL of penicillin, 0.07% NaHCO₃ and 2 mM L-glutamine and maintained at 37°C in humidified 5% CO₂ atmosphere.

2.5 Antiviral assay

The antiviral activity was conducted following the procedure reported(13).

2.5.1 Determination of samples cytotoxicity on Vero cells

The 96-well culture plate was inoculated with 1 x 10⁵ cells / mL of Vero cells and incubated at 37°C for 24 hours until complete monolayer sheet is developed. The culture media was removed from wells and washed twice with washing media. Different concentrations of each sample (starting from 10000 g/mL for extracts and 10000 g/mL for fractions and 100 µM for compounds) were prepared by double fold dilution. Each dilution (0.1 mL) was tested in different wells leaving 3 wells as control, receiving only maintenance medium, then the plate was incubated at 37°C and examined frequently for signs of cytotoxicity of Vero cells. The MTT solution (5 mg/ mL) was added (20 µL) to each well and mixed. After 4 hours of incubation, the formed formazan crystals were dissolved in 200 µL DMSO and the optical density (OD) was measured at 560 nm. The cytotoxic concentration (CC₅₀) as well as the maximum nontoxic concentration (MNTC) were determined. Sun et al.(14) has defined the maximum non-cytotoxic concentration as the concentration of compound needed to keep viability of cell with no significant difference in comparison with the cell control (p < 0.05).

2.5.2 MTT assay protocol

The antiviral assay was performed by MTT method and depends on mixed treatment assay that tests inactivation of virus particles in the virus suspension by samples(14). A virus suspension was incubated with non-lethal concentrations of the tested samples (1:1 v/v) at room-temperature for 1 h. Vero cells (1 x 10⁴ cells / 200 µL media per well) were seeded in a 96-well plate leaving 3 wells empty for blank controls and incubated overnight at 37°C to allow the cells to attach to the wells. A 100 µL from viral/ sample suspension were added to the wells and mixed in a shaker at 150 rpm for 5 minutes. After one day incubation, 20 µL of MTT solution (5mg/ mL in PBS) was added to each well. The mixture was incubated for 5 hours to allow metabolism of MTT to formazan crystals which is then dissolved into 200 µL of DMSO, and the OD was determined at 560 nm which is directly correlated with the cell quantity. All tests were compared with the standard antiviral drug Acyclovir. The percent protection is calculated as [(A-B)/(C-B)] x100, where A, B, and C are the OD₅₆₀ of treated infected, untreated infected, and
un-treated uninfected cells, respectively\textsuperscript{(15)}. The IC\textsubscript{50} which is the concentration of samples that inactivate virus particles in virus suspension by 50% compared with untreated control and was determined from dose-response curves.

2.6 Statistical Analysis

Statistical analysis was performed with GraphPad Prism version 7 to calculate IC\textsubscript{50} and CC\textsubscript{50}. The level of significance was set at (p>0.05). Quantitative data were described as mean ± standard deviation (SD). Microsoft office excel 2010 was used to plot multiple bar chart of the antiviral activity.

3 Results

3.1 Structure determinations of the isolated compounds

Twelve known compounds were isolated from the active fractions of \textit{C. droserifolia} and \textit{L. creticus} (Figure 1). The structures of the isolated compounds were confirmed by comparing their spectral data with those in literature. Compound 1 was isolated as colorless needles. The physical, chemical and IR spectral data of 1 were identical to those published for β-sitosterol\textsuperscript{(16)}. Compound 2 was isolated as yellow amorphous substance. Its molecular weight was determined from HRESIMS showing a molecular ion peak at m/z [M + H]\textsuperscript{+} 419.1330 consistent with the molecular formula C\textsubscript{21}H\textsubscript{23}O\textsubscript{9}. The APT and \textsuperscript{1}H-NMR spectral data of 2 are in full agreement with reported data\textsuperscript{(17)} and identified as 5-hydroxy-3,6,7,3',4',5'-hexamethoxyflavone. Compound 3 was isolated as yellow crystals and its structure was determined as 5,3'-dihydroxy-3,6,7,4',5'-pentamethoxyflavone. Compounds 4 and 5 were isolated as colorless oily substances. Their \textsuperscript{1}H-NMR and APT spectra were identical with those reported for guaiane-4β,6β,10α-triol (also known as teuclatriol) and guaiane-4β,6β,10β-triol (also known as epiteuclatriol), respectively\textsuperscript{(18)}. This is the first report for isolation of these compounds from the family Capparidaceae. Compound 6 was obtained as white powder and was identified as β-sitosterol-3-O-β-D-glucoside. Compound 7 spectral data were matched with those reported for kaempferol-4'-methoxy-3,7-O-dirhamnoside\textsuperscript{(6)}. Compounds 8 and 9 were isolated as yellow needles and their \textsuperscript{13}C and \textsuperscript{1}H-NMR spectra are in full accordance with kaempferol-3,7-di-O-α-L-rhamnoside (also known as Kaempferitrin) and kaempferol-3-O-β-D-glucopyranoside-7-O-α-L-rhamnoside\textsuperscript{(19)} and \textsuperscript{(20,21)} respectively, this is the first report to isolate these compounds from \textit{C. droserifolia}. Compound 10 was obtained as white powder and its structure was determined to be Lupeol (Mahato and Kundu, 1994)\textsuperscript{(22)}. Compounds 11 and 13 spectral data were consistent with those published for oleanolic acid\textsuperscript{(22)} and 3-O-[(α-L-rhamnopyranosyl-1”-2”]-β-D-galactopyranosyl-(1”-2”)-β-D-glucuronopyranosyl] soyasapogenol B (also known as

![Fig. 1. Chemical structures of compounds (1-13), (1-9) isolated from Cleome droserifolia and (10-13) from Lotus creticus.](https://www.indjst.org/)

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soyasaponin I)\(^{23}\), respectively. This is the first report for isolation of these compounds from \(L.\ creticus\). Compound 12 was identical to compound 6 and was identified as \(\beta\)-sitosterol -3-O-\(\beta\)-D-glucoside.

### 3.2 Antiviral activity

#### 3.2.1 Cytotoxicity of tested samples

The cytotoxicity of the tested samples was assessed on VERO cells using MTT assay to ensure that they are nontoxic. The maximum nontoxic concentration and the cytotoxic concentration of each sample were shown in Tables 1, 2 and 3. The CC\(_{50}\) of the tested extracts varied from 292.1±26.64 to 890.45 \(\mu\)g/mL and their MNTC from 78.12 to 1000 \(\mu\)g/mL. In addition, the fractions of \(C.\ droserifolia\) and \(L.\ creticus\) showed CC\(_{50}\) ranged from 128.6 ± 8.87 to 6288.24 ± 225.04 \(\mu\)g/mL except the aequous fractions of both plants that were nontoxic. The MNTC of these fractions varies from 78.12 to 10000 \(\mu\)g/mL. Teuclatriol showed CC\(_{50}\) value of 23.62 ± 4.908 \(\mu\)M, thus the MNTC was the lowest among the other compounds and equal to 6.25 \(\mu\)M. The other compounds exhibited cytotoxicity range from 32.61 ± 4.913 to 88.179 \(\mu\)M and their MNTC from 12.5 to 25 \(\mu\)M.

#### 3.2.2 Antiviral Activity of Tested Samples

The antiviral activity of eight plant extracts against HAV, HSV-1 and Cox. B4 virus has been studied adopting the MTT assay protocol. The methanolic extracts of \(C.\ droserifolia\) and \(L.\ creticus\) were able to protect HAV-infected cells from the virus-induced destruction with percent protection 93.2 % and 68.5 %, respectively. The protection of 90.2 % and 50.2 % against HSV-1 was observed for \(C.\ droserifolia\) and \(L.\ creticus\) methanol extracts, respectively. They also showed a moderate antiviral activity against Cox. B4 virus with protection of 28.2 % and 21.7 % for \(C.\ droserifolia\) and \(L.\ creticus\), respectively. The protection percent of the extracts in comparison with the positive control Acyclovir is cited in Table 1.

| Tested extracts              | CC50±SD (\(\mu\)g/ml) | MNTC (\(\mu\)g/ml) | Percent protection against |
|-----------------------------|------------------------|--------------------|---------------------------|
| Media/DMSO (-Ve Control)    | NT                     | NA                 | NA                        |
| Acyclovir (+Ve Control)     | NT                     | 78.12              | 21.6 %                    |
| Cyperus rotundus (rhizomes) | 292.1±26.64            | 125                | NA                        |
| Cyperus rotundus (aerial part)| NT                    | 1000               | NA                        |
| Cyperus helferi             | 890.45*                | 500                | NA                        |
| Cyperus alopecuroides       | 510.6*                 | 250                | 8.97 %                    |
| Francoeuria crispa          | 389.90±35.56           | 125                | 27.18 %                   |
| Cleome droserifolia         | 690.31*                | 250                | 93.2 %                    |
| Lotus creticus             | 855.36*                | 500                | 68.57 %                   |
| Fagonia mollis             | 634.72±14.23           | 250                | 12.96 %                   |

* SD can’t be calculated (infinity), NA: Not active, NT: Nontoxic.

The methanolic extracts of \(C.\ droserifolia\) and \(L.\ creticus\) were fractionated using different organic solvents and yielded five fractions for each plant (petroleum ether, methylene chloride, ethyl acetate, butanol, and aqueous fractions). The antiviral activities at nontoxic concentration together with IC\(_{50}\) for each fraction were determined and represented in Table 2.

The methylene chloride fraction of \(C.\ droserifolia\) was the most promising fraction against HAV with IC\(_{50}\) value of 185.8±2.82 \(\mu\)g/mL. The ethyl acetate fraction of both extracts showed antiviral activity against HAV with IC\(_{50}\) values of 218.06±2.77 and 566.7 \(\mu\)g/ml for \(L.\ creticus\) and \(C.\ droserifolia\), respectively. On the other hand, the ethyl acetate fraction of \(L.\ creticus\) was the most potent against the HSV-1 followed by ethyl acetate of Cleome and butanol of Lotus with IC\(_{50}\) values of 152.7±1.68, 569.8±2.96 and 572.8±3.98 \(\mu\)g/ml, respectively. Moreover, the methylene chloride fraction of \(C.\ droserifolia\) showed the highest anti-Cox. B4 virus in comparison with other fractions with IC\(_{50}\) value of 202.5±2.85 \(\mu\)g/ml. The active fractions of \(C.\ droserifolia\) and \(L.\ creticus\) were selected for chromatographic isolation of the active compounds.

Multiple bar chart showing IC\(_{50}\) (\(\mu\)g/mL) values for different fractions of \(C.\ droserifolia\) and \(L.\ creticus\) were represented in Figure 2.
**Table 2.** Results of antiviral assay of different fractions showing MNTC, CC_{50}, IC_{50} and antiviral activity % against HAV, HSV-I and Cox. B4 viruses

| Tested fractions | Petroleum ether | Methylene chloride | Ethyl acetate | Butanol | Aqueous | Petroleum ether | Methylene chloride | Ethyl acetate | Butanol | Aqueous |
|------------------|----------------|--------------------|---------------|---------|---------|----------------|--------------------|---------------|---------|---------|---------|
| **C. droserifolia** | 128.6 ± 8.87 | 78.12 | 185.81 ± 2.82 | 212.5 ± 2.26 | 10000 | 665.1 ± 44.61 | 357.6 ± 31.41 | 3200 ± 310.2 | 4358 ± 316.68 | NT |
| **L. creticus** | 282.1 ± 24.56 | 156.25 | 566.78* | 629.51 ± 2.26 | 6288.24 | 665.1 ± 44.61 | 357.6 ± 31.41 | 3200 ± 310.2 | 4358 ± 316.68 | NT |

* SD can't be calculated (infinity), NA: Not active, NT: Nontoxic.

**Fig 2.** IC_{50} values for different fractions of C.droserifolia and L. creticus, where A2, A3 are petroleum ether fractions; B2, B3 are methylene chloride fractions; C2, C3 are ethyl acetate fractions; D2, D3 are butanol fractions; E2, E3 are aqueous fractions.

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Activity-guided isolation of the antiviral compounds from *L. creticus* and *C. droserifolia* resulted in the identification of twelve metabolites. These compounds were evaluated for their antiviral activity by MTT assay (Table 3). Out of the purified compounds, 5,3’-dihydroxy-3,6,7,4’,5’-pentamethoxyflavone and epiteuclatriol were more likely to be responsible for the observed anti-HAV activity of the methylene chloride fraction of *C. droserifolia* with IC₅₀ values of 9.52±0.329 and 10.41±0.252 μM, respectively and with a wide safety margin. These two compounds are slightly better than kaempferitrin with the IC₅₀ value of 11.05±0.072 μM and to which the anti-HAV activity of ethyl acetate fraction of *C. droserifolia* may be attributed.

Table 3. Results of antiviral assay of the compounds isolated from the active fractions showing CC₅₀, MNTC, IC₅₀, selective indices (SI) and antiviral activity % against HAV, HSV-I and Cox. B4 viruses

| Tested Cpd | CC₅₀ ±SD (μM) | MNTC (μM) | IC₅₀ (μM) ±SD | % antiviral | SI | CC₅₀/IC₅₀ |
|------------|---------------|------------|----------------|-------------|----|-----------|
| 1          | 50.199±2.74   | 25         | 29.937±0.276   | 42.8        | 1.6 | 11.469±3.12 | 71.1 |
| 2          | 44.92±2.658   | 25         | 10.592±2.22    | 86.4        | 4.2 | 16.963±0.114 | 84.1 |
| 3          | 32.61±4.913   | 12.5       | 9.52±0.329     | 68.4        | 3.4 | 5.899±0.36   | 92.1 |
| 4          | 23.62±4.908   | 6.25       | 4.89±0.061     | 89.09       | 4.8 | 14.654±0.061 | 44.9 |
| 5          | 37.74±2.695   | 12.5       | 10.415±0.252   | 61.02       | 3.6 | 8.929±0.263  | 76.3 |
| 6          | 44.49±1.773   | 12.5       | 16.365±0.312   | 36.1        | 2.7 | 11.379±0.261 | 51.9 |
| 7          | 52.47±1.431   | 25         | 21.746±0.194   | 60.5        | 2.4 | 12.073±2.24  | 76.3 |
| 8          | 47.78±5.762   | 12.5       | 11.05±0.072    | 58.7        | 4.3 | 11.408±1.52  | 63.2 |
| 9          | 83.757*       | 25         | 16.871±0.224   | 77.6        | 4.9 | 17.855±0.276 | 70.7 |
| 10         | 82.855*       | 25         | NA             | 51.2        | 16.172±0.119 | 94.5 |
| 11         | 88.179*       | 25         | 15.268±0.256   | 41.5        | 5.7 | 17.526±0.239 | 83.0 |
| 13         | 83.089*       | 25         | 14.086±0.51    | 92.3        | 5.8 | 8.748±0.315  | 98.3 |
| ACV        | NT*           | 100        | 31.704±0.284   | 39.7        | 9.108±0.289 | 97.3 |

*SD can't be calculated (infinity), ACV: Acyclovir, NA: Not active, NT: Nontoxic.

Interestingly, a remarkable anti-HSV-1 was established for three compounds, namely, teuclatriol, 5,3’-dihydroxy-3,6,7,4’,5’-pentamethoxyflavone and epiteuclatriol with IC₅₀ values 4.89±0.06, 5.89±0.36 and 8.92±0.263 μM, respectively; despite of inactivity of the methylene chloride fraction containing these compounds. This may be partly attributed to the antagonistic effect among the different components of the total fraction.

Soyasaponin I also showed anti-HSV-1 with IC₅₀ equal to 8.74±0.315μM when compared with Acyclovir whose IC₅₀ values was 9.108±0.289 μM. Out of the tested compounds, β-sitosterol 3-O-β-D-glucoside and epiteuclatriol suppressed Coxsackie virus infectivity with IC₅₀ equal to 13.05±0.348 and 14.65±0.061 μM, respectively and may be responsible for the anti-Cox. B4 activity of the methylene chloride fraction of *Cleome*. Selective index is an important parameter to select samples for developing drugs and fortunately all the tested compounds showed a wide safety margin with higher toxicity to the tested viruses than normal Vero cells. Multiple bar chart showing IC₅₀ values for different compounds of *C. droserifolia* and *L. creticus* in compared with Acyclovir were shown in Figure 3.

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4 Discussion

4.1 Mechanism of Antiviral Activity

Li and Peng\(^{(24)}\) have established that each step-in virus life cycle including virus entry, replication and release could be a crucial target by antiviral drugs. The antiviral mechanism could be assayed by testing inactivation of virus suspension, blocking of virus entry or inhibition of the replication of virus\(^{(14)}\). Herein, the isolated compounds were evaluated for their antiviral activity using the protocol which test inactivation of viruses in suspension. Using mixed treatment assay, at MNTC, viral activity of HSV-1 was significantly reduced by 89.09 % for teuclatriol. Also, 5,3'-dihydroxy-3,6,7,4',5'-pentamethoxyflavone and epiteuclatriol were able to suppress the viral activity of HSV-I by 92.1, 76.3 % and HAV by 68.4, 61.02 %, respectively. Additionally, \(\beta\)-sitosterol 3-O-\(\beta\)-D-glucoside and epiteuclatriol reduce the viral activity of Coxsackie virus by 49.2 % and 44.9 %, respectively.

4.2 Correlation between Antiviral Activity and Structure

Although teuclatriol and epiteuclatriol are epimers that differ only in one stereogenic center, they exhibited different antiviral activities against the tested viruses. Teuclatriol showed no activity against HAV and Cox. B4 virus despite the highest anti-HAV and anti-Cox. B4 activities of its epimer among the tested compounds. Besides, teuclatriol exhibited nearly a double fold anti-HSV-1 activity in comparison to epiteuclatriol. The reason that those enantiomers have different biological activities may be explained by difference between the interactions of the two enantiomers with their virion receptor. Thus, the fitting interaction produces an active biological effect. In contrast, the inactive enantiomer cannot bind in the same way with the receptor. This is the first report for proving antiviral activity of these sesquiterpenes. Tsuchiya et al.\(^{(25)}\) has proved that a hydroxyl group at C-5 as well as a methoxy group at C-3 play crucial role for the antiviral activity of flavonoids. A comparison of 5-hydroxy-3,6,7,3(c),4',5'-hexamethoxyflavone with, 5,3'-dihydroxy-3,6,7,4',5'-pentamethoxyflavone showed that the anti-HSV-1 activity was greatly reduced and the anti-HAV was diminished when the substituent at C-3' is a methoxy group. This indicated that the hydroxyl group at C-3' may be also important for antiviral activity of the flavonoid. The antiviral activity has not been previously reported for these methoxylated flavonoids. Furthermore, the presence of electron donating group such as a methoxy group on C-4' of the flavonoid resulted in potent inhibitory activity against Cox. B4 virus\(^{(26)}\). This evidence is consistent with our results for the anti-Cox. B4 activity of compounds 7 and 8. Kaempferol-4'-methoxy-3,7-O-dirhamnoside exhibited anti-Cox. B4 activity at a lower IC\(_{50}\) value compared with kaempferol-3, 7-di-O-\(\alpha\)-L-rhamnoside. In contrast, the anti-HAV activity of kaempferol-3,7-di-O-\(\alpha\)-L-rhamnoside is higher than that of Kaempferol-4'-methoxy-3,7-O-dirhamnoside. Therefore, C-4' methoxy substituent is important for the antiviral activity against Coxsackie viruses but will decrease the antiviral activity against HAV. \(\beta\)-sitosterol 3-O-\(\beta\)-D-glucoside showed a better anti-Cox. B4 activity when compared to its genin part alone.
\(\beta\)-sitosterol. This may be explained by the fact that glycosylation can enhance biological activity and clinical efficacy (27).

### 5 Conclusion

It has been established that the MTT assay can be used for evaluating anti-HIV and anti-HSV-1 activities and sometimes preferred over methods of counting the number of plaques which are difficult in the case of very small plaque sizes (24). In this study, the bioassay-guided fractionation of \(C.\) droserifolia and \(L.\) c配送us led to the isolation of twelve compounds. The isolated compounds (1-12) were evaluated for their antiviral activities against HAV, HSV-1 and Cox. B4 virus. The study introduces 5,3'-dihydroxy-3,6,7,4',5'-pentamethoxyflavone and epiteuclatriol as valuable lead compounds possessing in vitro antiviral activities against HAV and HSV-1. Similarly, teucatrol and \(\beta\)-sitosterol 3-O-\(\beta\)-D-glucoside showed promising activity and can be considered as candidates for the development of new anti-HSV-1 and anti-Cox. B4 drugs.

**Conflict of interest:** None

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