Inhibition of HSV-2 infection by pure compounds from Thymus capitatus extract in vitro

Inhibition of HSV-2 infection by pure compounds from *Thymus capitatus* extract *in vitro*

Marwa Mekni Toujani[1], Massimo Rittà[2], Andrea Civra[2], Salvatore Genovese[3], Francesco Epifano[3], Abdeljelil Ghram[1], David Lembo[2], Manuela Donalisio[2]*

1 *University of Tunis El Manar, Laboratory of Epidemiology and Veterinary Microbiology, Institut Pasteur of Tunis, PB 74, 1002 Tunis-Belvedere, Tunisia*

2 *Department of Clinical and Biological Sciences, University of Torino, 10043 Orbassano, Torino, Italy*

3 *Department of Pharmacy, University "G. d'Annunzio" of Chieti-Pescara, Via dei Vestini 31, 66100 Chieti Scalo (CH), Italy*

* Corresponding author: Dr. Manuela Donalisio, Ph.D.
Department of Clinical and Biological Sciences
University of *Torino*, S. Luigi Gonzaga Hospital
Regione Gonzole, 10
10043, Orbassano, *Torino*, Italy
Phone: +39 011 6705427
Fax:+39 011 2365427
E-mail: manuela.donalisio@unito.it

ABSTRACT

Thymus capitatus represents one of the five Tunisian species of the genus *Thymus*, which has long standing use for flavouring and preserving several food products. Its constituents have been reported to endow antimicrobial properties, but little is known about their antiviral activities. The aim of this study was to examine the antiviral activity of pure compounds from the most bioactive inhibitory *Thymus capitatus* extract *in vitro* against HSV-2 infection and to identify their mechanism of action. Either the extracts or the essential oil exert inhibitory activity against HSV-2 infection, with the ethanolic extract showing the lowest EC_{50} value (2.3µg/ml). Three pure compounds were then isolated from the ethanolic extract and investigated for their antiviral activity. β-sitosterol showed the most favorable selectivity index and both cinnamaldehyde and carvacrol exerted moderate antiviral effect. Investigation of the mechanism of action revealed that all three compounds directly inactivated the infectivity of the virus particles. These findings suggest the use of *Thymus capitatus* ethanolic extract as source of anti-HSV-2 pure compounds and warrant further studies to evaluate their therapeutic potential.

Keywords: Thymus capitatus, antiviral activity, HSV-2, β-sitosterol, cinnamaldehyde, carvacrol

Chemical compounds: β-sitosterol (PubChemCID: 222284), cinnamaldehyde (PubChemCID: 6428895), carvacrol (PubChemCID: 10364)

Abbreviations: Thymus capitatus (T. capitatus); herpes simplex virus type 2 (HSV-2); human immunodeficiency virus (HIV); eagle's minimal essential medium (MEM); fetal calf serum (FCS); ethanolic extract (EE); aqueous extracts (AE); essential oil extract (EO); dimethyl sulfoxide (DMSO); milliliter (ml); milligram (mg); grams (g); micrograms (µg); micromolar (µM); [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy- phenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS); 50% cytotoxic concentrations (CC_{50}); multiplicity of infection (MOI); plaque-forming units (PFU); half maximal effective concentration (EC_{50}); selectivity index (SI); 90% effective concentration (EC90); Nuclear magnetic resonance (NMR); thin-layer chromatography (TLC); deoxyribonucleic acid (DNA)

Introduction

The *Thymus* genus is considered to be one of the largest genera within the *Lamiaceae* family that includes approximately 350 species of perennial, aromatic herb and sub shrubs native mainly of Europe, Western Asia and the Mediterranean regions (Stahl-Biskup & Saez, 2002). *Thymus capitatus* (Hoffmanns. & Link, Lamiaceae) represents one of the five Tunisian species of the genus *Thymus*, which has a long standing use for flavouring and preserving several food products. Its essential oil is used in the flavouring cough medicines and oral hygiene products as well as in cosmetic and perfume industry. As a medicinal plant, *T. capitatus* decoction and infusion have traditionally been considered as antispasmodic, carminative, tonic, antiseptic and antitussive drugs (Chiej, 1984). Numerous reports showed antibacterial, antifungal, antioxidant and anti-inflammatory properties of *T. capitatus* extracts and essential oil, rich in carvacrol and thymol (Mkaddem *et al.*, 2010; Achour *et al.*, 2012; Iauk *et al.*, 2014; Maissa *et al.*, 2015). To date, extracts of several species of *Thymus*, as *T. vulgaris* and *T. linearis*, have been investigated for their anti-herpetic effect, even though the constituents responsible of the antiviral activity have not been yet identified (Nolkemper *et al.*, 2006; Schnitzler *et al.*, 2007; Koch *et al.*, 2008; Rajbhandari *et al.*, 2009). Herpes simplex virus type 2 (HSV-2) is a sexually transmitted pathogen that infects about 417 million people aged 15-49 (11%) worldwide, causing genital infections (www.who.int). Most of these infections are asymptomatic but can also cause painful blisters or ulcers at the site of infection. Symptomatic infections are most contagious but sexually transmission can also occur in the absence of symptoms (Roizman, Knipe & Whitley 2007). Of note, genital ulcer disease increases the risk of HIV acquisition since the mucosal damage induces local inflammation, following activation and recruitment of

99 $CD4^+$ HIV target cells (Freeman *et al*., 2006; Corey, 2007; Feng *et al.*, 2013). Although
100 different anti-herpetic drugs are approved and used to treat acute symptomatic
101 infections (acyclovir, penciclovir and other guanine analogues), the development of new
102 antivirals against HSV-2 is needed due to several drawbacks of available drugs. These
103 include the emergence of drug resistant strains, the inability to eradicate latent
104 infections, the poor availability and the incomplete intestinal absorption (Kimberlin &
105 Whitley, 2007; Cavalli *et al.*, 2012). In this context, alternative natural products from
106 plants have been explored, as sources of antiviral molecules endowed with a mechanism
107 of action different from that of acyclovir (Silva-Mares *et al.*, 2016).
108 Herein, we investigated the antiviral activity of aqueous extract, ethanolic extract and
109 essential oil of *T. capitatus* against HSV-2. β-sitosterol, cinnamaldehyde and carvacrol
110 derived from the ethanolic extract, have been identified as inhibitory compounds against
111 acyclovir sensitive and resistant HSV-2 strains and their mechanism of action has been
112 analyzed.
113

114 ## Materials and methods

115 **Plant identification and collection**

116 Fresh *T. capitatus* (Hoffmanns. & Link, Lamiaceae) plants were collected in May 2014
117 from Matmata locality in the South East of Tunisia (33° 32′ North 9°58′ East). Plants
118 was identified by Dr. Marwa Mekni Toujiani. Aerial parts of the plants (leaves, stems
119 and flowers) were separated, thoroughly rinsed in running tap water and air dried at
120 room temperature during 14 days. A voucher specimen (TC-001) has been deposited in
121 the herbarium of the Department of Pharmacy of the University "G. d'Annunzio" of
122 Chieti-Pescara.

123 **Chemicals**

124 Eagle's minimal essential medium (MEM) and fetal calf serum (FCS) were purchased
125 from Gibco/BRL (Gaithersburg, MD, USA) and the antibiotic-antimycotic solution
126 (Zell Shield) from Minerva Biolabs GmbH (Berlin, Germany). Acyclovir and heparin
127 were purchased from Sigma-Aldrich (Milan, Italy).

128 **Cells and culture conditions**

129 African green monkey fibroblastoid kidney cells (Vero, ATCC CCL-81) were grown as
130 monolayers in MEM supplemented with 10% heat inactivated FCS and 1% Zell Shield.

131 **Virus**

132 A clinical isolate of HSV-1 and HSV-2 were kindly provided by Prof. M. Pistello,
133 University of Pisa, Italy. HSV strains were propagated and, when the cytopathic effect
134 involves the whole monolayer, the infected cell suspension is collected and the viral
135 supernatant clarified. The virus stocks were aliquoted, titrated by plaque assay on Vero
136 cells and stored at -80 °C. A HSV-2 strain with phenotypic resistance to acyclovir was
137 generated by serial passage in presence of increasing concentrations of acyclovir, as
138 previously described (Donalisio *et al.*, 2016).

139 **Extracts preparation**

140 The ethanolic and aqueous extracts (EE and AE) were prepared as previously described
141 (Boubaker–Elandalousi *et al.*, 2014). The essential oil (EO) was prepared by dissolving
142 100 g of dried plant material in 1 liter of distilled water and then submitted to
143 microwave-assisted hydro-distillation at 40 °C during 4 h, in a Clevenger type
144 apparatus. Extracts were kept in a dark flask at 4 °C until tested. Working solutions (25
145 mg ml^{-1}) of EO and EE were dissolved in dimethyl sulfoxide (DMSO). Aqueous
146 extracts were prepared in a similar way by 24 h maceration.

147 **Isolation of pure compounds from ethanolic extract of *T. capitatus***

148 *Isolation and identification of β-sitosterol*. The EE (10 g) of the plant was suspended in
149 *n*-hexane (50 ml) and the resulting mixture was centrifuged at 5000 g for 3 h then
150 filtered. The filtrate was evaporated to dryness under vacuum and the resulting waxy
151 solid dissolved in a 1:1 mixture water: ethanol (50 ml) and extracted with n-hexane
152 (5x10 ml) and this latter evaporated to dryness under vacuum. The obtained powder was
153 subjected to purification on a silica gel column chromatography using hexane:
154 dichloromethane 9:1 as the eluent. β-sitosterol (25 mg, purity > 96.3% assayed by
155 HPLC (Figure 1A) following an already reported method (Kakade *et al.*, 2012) was
156 obtained as a white solid and its structure was confirmed by TLC and NMR
157 spectroscopy by comparison with an authentic sample.

158 *Isolation and identification of cinnamaldehyde and carvacrol.* The EE (0.5 g) of the
159 plant was subjected to silica gel column chromatography using dichloromethane and
160 95% dichloromethane / 5% methanol mixture as the eluents. Cinnamaldehyde (10mg,
161 purity > 97.4% assayed by HPLC (Figure 1B)) was eluted first and obtained as
162 yellowish oil and its structure was confirmed by TLC and NMR spectroscopy by
163 comparison with an authentic sample. Carvacrol (12 mg, purity > 96.7% assayed by
164 HPLC (Figure 1C)) was eluted as the second spot and got as a yellowish oil and its
165 structure was confirmed by TLC and NMR spectroscopy by comparison with an
166 authentic sample.

167 *GC and HPLC analysis*

168 GC analysis of the essential oil have been accomplished following the same general
169 procedure as recently reported (Ricci *et al.*, 2017). HPLC analysis were carried out
170 using a Waters 600 HPLC system equipped with a Waters 2996 PDA detector, a

171 Rheodyne manual syringe-loading valve injector model 7125 (Cotati, CA., USA) fitted
172 with a 20 μL loop. Data acquisition was monitored by Waters Empower software (ver.
173 2.0). Chromatographic separation was accomplished employing a GraceSmart RP_{18} (5
174 μm particle size, 250 mm x 4.6 mm, Grace, Deerfield, IL, USA). Column temperature
175 was maintained at 25 ± 1 °C using a cool pocket chiller (ThermoScientific, Waltham,
176 USA). Elution mixture consisted of H_2O and acetonitrile both with 0.1% of formic acid
177 (eluent A and eluent B, respectively). Mobile phase was directly on-line degassed by an
178 Infinity Agilent model 1260 (Agilent Technologies, Santa Clara, CA, USA). The flow
179 rate was 1.20 mL/min. The following gradient elution was used: 15% A – 85% B from
180 0.01 min. to 13.0 min., 40% A – 60% from 13.01 min. to 16.0 min., 60% A – 40% B
181 from 16.01 min. to 20.0 min., 80% A – 20% B from 20.01 min. to 23.0 min., 90% A –
182 10% B from 23.01 min. to 26.0 min. The injection volume was 20 μL. Samples were
183 filtered through a 0.45 μm membrane polyamide filter before injection.
184 Method validation was settled according to the "Guidance for Industry-Bioanalytical
185 Method Validation" recommended by Food and Drug Administration (FDA) of the
186 United States. Individual stock solutions for calibration curves of each chemical
187 standard were prepared by dissolution of 10 mg of each reference sample into 10mL of
188 MeOH. The resulting solution was stored in glass-stoppered bottles at 4 °C before each
189 HPLC run. Standards for calibration curves and quality control samples (QC), at
190 concentration of 1.0, 10.0, 20.0, 30.0, 40.0, 50.0, 60.0, 70.0, 80.0, 90.0 and 100.0
191 μg/mL, were daily prepared by appropriate dilution aliquots of the stock solutions in
192 MeOH. Pooled quality control samples of analytes were prepared to determine the limit
193 of quantification (LOQ), the intra-and inter-assay precision and accuracy of the method,
194 and to assess the stability of compounds when stored under different conditions. QC

samples at three different concentration levels (QC_{low}= 5.0, QC_{medium} = 45.0, and QC_{high}=95.0 µg/mL) were used to validate or reject the analytical run. On five separate days, six calibration curves were plotted against the corresponding concentrations. Correlation coefficients, slopes, and intercepts of each calibaration curve were also evaluated. The limit of detection (LOD), defined as 3 times the standard deviation of a blank samples divided by the analytical sensitivity, was calculated from the calibration graphic following the the guidelines provided by IUPAC s. The LOQ was defined as the lowest concentration on the calibration curve, which could measured (n=5) with a precision (RSD %) not exceeding 20% and with an accuracy between 80% and 120% (Fiorito *et al.*, 2017; Taddeo *et al.*, 2017).

Viability assay

Cell viability was measured using the MTS assay as described by Pauwels *et al.* (1988). The effect on cell viability of the different concentrations was expressed as a percentage, by comparing absorbance of treated cells with that of cells incubated with culture medium supplemented with equal volume of DMSO. The 50% cytotoxic concentrations (CC_{50}) and the 95% confidence intervals (CIs) were determined using Prism software (Graph-Pad Software, San Diego, CA).

Anti-HSV inhibition assay

The effect of *T. capitatus* extracts, isolated compounds or acyclovir on HSV infection was evaluated by plaque reduction assay (Novoa *et al*., 2016). Vero cells were seeded in 24-well plates at a density of $10x10^4$ cells/well. Increasing concentrations of extracts or constituents were added to cells for 2 h; a mixture of different tested substances plus HSV-1 or HSV-2 or acyclovir resistant HSV-2 at a multiplicity of infection (MOI) of 0.001 plaque-forming units (PFU)/cell (100 PFU), were subsequently added to the cells,

which were then incubated at 37 °C for 2 h. The virus inoculum was then removed and the cells washed and overlaid with a medium containing 1.2% methylcellulose (Sigma-Aldrich) and serial dilutions of extracts or constituents. For acyclovir antiviral assay, the compound was added only after infection, diluted in the medium containing methylcellulose as described before. After 24 h (HSV-2) or 48 h (HSV-1) of incubation at 37°C, cells were fixed and stained by using 20% ethanol and 0.1% crystal violet and viral plaques counted. A longer incubation time was used for HSV-1 since this clinical strain generated viral plaques with smaller size than those of HSV-2 at 24 hpi, not suitable for the count. The concentration that reduced the plaque formation by 50% (EC_{50}) was determined by comparing treated and untreated wells using Prism software. Three independent experiments were performed in duplicate.

Virus yield reduction assay

Vero cells seeded in 24-well plates at a density of $10x10^4$ cells/well were pre-treated with serial dilutions of pure compounds for 2 h at 37 °C and infected in duplicate with HSV-2 at a MOI of 0.01 PFU/cell in the presence of the compounds. Following adsorption at 37 °C for 2 h, the virus inocula was removed and cultures were grown in the presence of compounds until control cultures displayed extensive cytopathology. Supernatants were harvested and pooled 48 h after infection and cell-free virus infectivity titers were determined. The aim of the assay was to obtain, if possible, the effective concentration of compound that reduced virus yield by 50% (EC_{50}) as compared to untreated virus controls.

Virus inactivation assay

Approximately, 10^5 PFU of HSV-2 plus EC_{90} of pure compounds were added to MEM and mixed in a total volume of 110 µl. The virus-compound mixtures were incubated

for 0 h or 2 h at 37 °C then diluted serially to the non-inhibitory concentration of compounds*;* the residual viral infectivity was determined by titrating virus by plaque assay on Vero cells, pre-seeded in 96-well plates at a density of $16x10^4$ cells/well (Alvarez *et al*., 2009; Alvarez *et al*., 2015b).

Pre-treatment assay

Cells were exposed to serial dilutions of pure compounds in a 24-well plate at 37 °C for two hours. After washing, cells were infected with HSV-2 at 0.001 MOI for 2 h, washed and treated as for plaque reduction assay (Donalisio *et al*., 2016).

Attachment assay

Pre-chilled Vero cells were treated with β-sitosterol or heparin for 30 min at 4 °C and then infected with HSV-2 at 0.004 MOI for 2 h at 4 °C in presence of the pure compound. After three washes with cold MEM to remove unbound virus, cells were overlaid with 1.2% methylcellulose and shifted to 37 °C. After 24 h incubation, cells were stained and viral plaques counted (Alvarez *et al*., 2009; Alvarez *et al*., 2015b).

Entry assay

The HSV-2 at 0.004 MOI was adsorbed for 2 h at 4 °C on pre-chilled confluent Vero cells. Cell layers were then washed three times with cold MEM to remove unbound virus, treated with different concentrations of β-sitosterol, and incubated for 3 h at 37 °C. The outer virions were inactivated with acidic glycine for 2 min at room temperature and the cells were washed three times with warm medium and treated as for plaque reduction assay (Alvarez *et al*., 2009; Alvarez *et al*., 2015b).

Post-treatment assay

Vero cells monolayers in 24-well plate were infected with HSV-2 at 0.001 MOI for 2 h at 37 °C, followed by two gentle washes to remove unbound virus. Increasing

concentrations of pure compounds were then added to cultures in 1.2% methylcellulose medium. Cells were treated as for plaque reduction assay (Donalisio *et al*., 2013).

Data analysis

All results are presented as the mean values from three independent experiments performed in duplicate. The EC_{50} and CC_{50} values were calculated by regression analysis using the software GraphPad Prism version 4.0 (GraphPad Software, San Diego, California, U.S.A.), by fitting a variable slope-sigmoid dose–response curve. The selectivity index (SI) was calculated by dividing the CC_{50} by the EC_{50} value. For virus inactivation and virus yield reduction assays, the viral infectivity in presence and absence of pure compounds was compared using a one-way analysis of variance (ANOVA) followed by Bonferroni test, if P values showed significant differences in virus titers. Significance was set at the 95% level.

Results and Discussion

Inhibitory activity of *T. capitatus* extracts against HSV-1 and HSV-2 infections

Within a project aiming at evaluating the antiviral potential of Tunisian endemic plants, we investigated the antiviral activity of *Thymus capitatus* aqueous (AE) and ethanolic extracts (EE) and essential oil (EO) against HSV-2 infection. To generate dose response curves, assays were performed by treating cells in presence of decreasing concentrations of extracts (ranging from 100 µg/ml to 0.13 µg/ml) before, during, and after viral infection. 24 h post infection, the EC_{50} was determined by comparing the number of viral plaques in treated and untreated wells, as described in Materials and Methods. As reported in Table 1, the AE, EE and EO exerted an antiviral activity, although to a different extent, with EC_{50} values of 23.6 µg/ml, 2.3 µg/ml and 18.6 µg/ml, respectively.

In all cases, the antiviral effect was not a consequence of cytotoxicity since the CC_{50} values on Vero cells were much higher than the EC_{50} values. Of note, an inhibitory effect of these extracts from *T. capitatus* was also observed against HSV-1, another member of *Alphaherpesvirinae* subfamily, showing EC_{50} values of 23.4 μg/ml, 16.6 μg/ml and 17.6 μg/ml, respectively. Our data evidenced a minor antiviral activity of EE against HSV-1 rather than that against HSV-2. Although HSV-1 and HSV-2 are structurally and genetically similar, their sensitivity to antiviral compounds may vary depending on the strains and cell type used in antiviral assays as reported for instance in Leary *et al.* (2002). Similar antiviral results were previously observed against Bovine Herpesvirus type-1 infection with EC_{50} values of 164 μg/ml, 47.8 μg/ml and 3.3 μg/ml, respectively (Boubaker-Elandalousi *et al.*, 2014). These data indicate a broad spectrum of action of *T. capitatus* extracts against Herpes viruses. As reported in Table 1, the EE of *T. capitatus* showed the most favorable selectivity index (SI) value (26.8) against HSV-2 infection and, therefore, it was selected as a source of bioactive compounds.

Chemical characterization of extracts and essential oil

All the obtained phytopreparations (EO, EE, and AE) were screened in order to obtain the respective chemical fingerprint. The essential oil of *T. capitatus* have been analyzed by GC-MS using a well established procedure by our group and employed several times in recent years for the analysis of essential oil (Ricci *et al.*, 2017) (Figure 2).

Inhibitory activity of isolated compounds from *T. capitatus* ethanolic extract and their mechanism of action

Data reported in Table 1 indicate that the EE was the most active one against both viruses under investigation. So we decided to define its phytochemical composition by isolation and structural characterization of its main components. Three compounds were

315 isolated from *T. capitatus* EE, β-sitosterol, cinnamaldehyde, and carvacrol, and they
316 were tested for their ability to inhibit the replication of the main cause of genital herpes,
317 HSV-2. All of them were active against HSV-2 infection in a dose-response manner,
318 with EC_{50} values of 2.7 μM, 39.7 μM and 51.9 μM, respectively (Table 2); acyclovir
319 was tested in parallel as a reference drug. Among these compounds, β-sitosterol showed
320 the strongest inhibitory activity with a SI value of 76.2. This is an interesting result
321 since, in a previous study, the same compound isolated from *Euphorbia segetalis*
322 exerted very low HSV-2 plaque reduction (Madureira *et al.*, 2003). Our finding is in
323 agreement with previous data that also showed a strong anti HSV-2 activity for β-
324 sitosterol (Alvarez *et al*., 2015a). A novel finding is the anti-HSV-2 activity of
325 cinnamaldehyde (SI: 28.3) reported here for the first time. Finally, the anti-HSV-2
326 activity of carvacrol (SI: 12.1) confirmed previous findings by Pilau *et al.* (2011) and
327 Lai *et al.* (2012). As shown in Figure 2, carvacrol and cinnamaldehyde, were also found
328 as components of the EO, however this was not the most active phytopreparation. Such
329 a discrepancy can be explained by a decrease in bioavailability of both phytochemicals
330 when part of a complex mixture like an essential oil, that in a certain way can modify
331 key parameters relevant to the biological activity like solubility in the medium
332 employed for the antiviral assays. Such an effect has been already described for other
333 biologically active secondary metabolites (Bakkali *et al.*, 2008).
334 To evaluate whether the antiviral activity of isolated compounds was correlated to virus
335 sensitivity to acyclovir, similar experiments were performed using an acyclovir-resistant
336 HSV-2 (EC_{50} value of 336.9 μM for acyclovir). As reported in Table 2, the resistant
337 strain was susceptible to β-sitosterol, cinnamaldehyde and carvacrol, with EC_{50} values
338 equal to 2.3 μM, 73.5 μM and 82.2 μM, respectively. These data suggest a different

mode of action of the pure compounds from that of acyclovir, a known inhibitor of the HSV-2 DNA polymerase, making these molecules a potential starting point for research and development of new antiviral therapies against HSV-2 infection. Therefore, further experiments were performed to investigate their major mechanism of action.

Firstly, we investigated the ability of pure compounds to directly inactivate HSV-2 viral particles. The assay was performed by mixing a virus aliquot containing 10^5 PFU with a concentration of each compound that reduced almost completely the virus growth (>EC_{90}) in the plaque reduction assay. The reduction of the viral titers of treated versus untreated samples was analyzed at high dilutions at which extracts were no more active. As reported in Figure 3, experiments were performed by incubating the mixtures for 0 h or 2 h at 37 °C. Both β-sitosterol and cinnamaldehyde did not show any significant inhibition when the mixtures were promptly added on the cells without incubation (Figure 3A and 3B). By contrast, after 2 h incubation, both compounds reduced significantly the HSV-2 titer by 99% and 91%, respectively. The ability of β-sitosterol and cinnamaldehyde to directly inactivate HSV-2 infectivity is a novel finding, corroborating the results of Jarikasem *et al.* (2013) who have reported a virucidal activity of a fraction containing β-sitosterol and stigmasterol against both HSV-1 and HSV-2. Furthermore, we have also evidenced the significant ability of carvacrol to inactivate extracellular HSV-2 particles either at 0 h or 2 h of incubation (Figure 3C). This data confirm the results of Jarikasem *et al.* (2013) who have previously shown the virucidal activity of carvacrol against HSV-1 particles. Since the anti-herpetic activity of carvacrol was previously reported in literature, we decided to gain further insights into β-sitosterol and cinnamaldehyde mode of antiviral action. To explore whether they target additional steps of the HSV-2 replicative cycle, specific cell assays we carried

363 out. As shown in Figure 4A, pretreatment of cells with both β-sitosterol and
364 cinnamaldehyde did not produce any inhibitory effect indicating that upon treatment,
365 the cells remained susceptible to viral infection (pre-treatment assay). This finding
366 exclude that the compounds could act by stably interacting with a cellular component(s)
367 thereby preventing its/their interaction with viral glycoproteins. To evaluate the
368 possibility that the inhibitory activity is due, not only to a virucidal effect, but also to
369 the ability of β-sitosterol to inhibit early steps of the virus replicative cycle, attachment
370 and entry assays were performed. As reported in Figure 4B and 4C, a weak inhibitory
371 activity, around 20-30%, was only observed when the cells were treated with a high
372 dose of compounds (80 μM) in both assays, whereas a dose response curve was
373 obtained treating cells with heparin, a known inhibitor of attachment.
374 These findings ruled out any effect β-sitosterol and cinnamaldehyde on virus attachment
375 and entry. Then, we wished to investigate whether the compounds were able to block
376 the cell-to-cell transmission of HSV-2 (post-treatment assay). When β-sitosterol and
377 cinnamaldehyde were added to the cells after virus infection they strongly reduced the
378 number of viral plaques as compared to the untreated cells with EC_{50} values of 6.9 μM
379 and 7.5 μM, respectively (Figure 4D). This finding indicate their ability to prevent cell-
380 to-cell spread of HSV-2 in a dose-dependent manner at non toxic concentrations.
381 Finally, both β-sitosterol and cinnamaldehyde also reduced the viral titer, in a dose-
382 response manner, when they were tested in a virus yield reduction assay, with EC_{50}
383 value of 3.64 μM and 8.41 μM, respectively (Figure 5). These data indicate their ability
384 to inhibit multiple cycles of viral replication at high doses thus limiting the production
385 of viral progenies. Acyclovir was used as standard positive drug of this assay.

Taken together, such antiviral properties might be useful in the setting of a productive infection *in vivo*, where *T. capitatus* extracts or its antiviral compounds might be able to prevent both cell-to-cell spread, which represents a major route of transmission for HSV-2 *in vivo*, and the transmission of extracellular free virus, which is often present in the site of infection at high titers (Dingwell *et al.*, 1994).

In conclusion, this study reports on the anti-herpetic activity of *T. capitatus* extracts and shows, for the first time, the ability of its isolated compounds β-sitosterol, cinnamaldehyde and carvacrol to inhibit infection by acyclovir-responsive and acyclovir-resistant HSV-2 strains. The mechanism of action of pure compounds mainly consists in direct inactivation of HSV-2 extracellular particles along with a reduced cell-to-cell virus spread. Findings identified pure compounds from *Thymus capitatus* ethanolic extract as inhibitors of HSV-2 infection suggesting their potential for treatment of herpetic lesions.

Acknowledgements

This work was supported by the grant from the Ministry of Higher Education and Research of Tunisia (LR11IP03) and by a donation by Silvana Legnani to DL.

Conflict of interest

The authors declare no conflict of interest.

410 **REFERENCES**

411 Achour S., Khelifi E., Attia Y., Ferjani E., & Noureddine Hellal A. (2012).
412 Concentration of antioxidant polyphenols from *Thymus capitatus* extracts by membrane
413 process technology. *Journal of food science,* 77(6), C703-709.

414 Alvarez A.L., Diñeiro Y., Del Barrio G., Picinelli A., Suarez B., Valdes S., Acosta M.,
415 Roque A., Parra F. (2009). Bioactivity-guided separation of anti HSV-2 and antioxidant
416 metabolites from the plant *Phyllanthus orbicularis*. *Planta Medica*, 75 (9), 990.

417 Alvarez AL, Habtemariam S. and Parra F. (2015a). Inhibitory effects of lupene-derived
418 pentacyclic triterpenoids from *Bursera simaruba* on HSV-1 and HSV-2 *in vitro*
419 replication. *Natural Product Research*, 29(24), 2322-2327.

420 Alvarez A.L., Habtemariam S., Moneim A.E.A., Melon S., Dalton K.P., Parra F.
421 (2015b). A spiroketal-enol ether derivative from *Tanacetum vulgare* selectively inhibits
422 HSV-1 and HSV-2 glycoprotein accumulation in Vero cells. *Antiviral research* 119, 8-
423 18.

424 Bakkali F., Averbeck S., Averbeck D., & Idaomar M. (2008). Biological effects of
425 essential oils: a review. *Food and Chemical Toxicology,* 46(2), 446-475.

426 Boubaker–Elandalousi R., Mekni–Toujani M., Kaabi B., Larbi I., Diouani M.-F.,
427 Gharbi M., Akkari H., B'chir F., & Ghram A. (2014). Non-cytotoxic *Thymus capitata*
428 extracts prevent Bovine herpesvirus-1 infection in cell cultures. *BMC veterinary*
429 *research,* 10, 231.

430 Cavalli R., Donalisio M., Bisazza A., Civra A., Ranucci E., Ferruti P., & Lembo D.
431 (2012). Enhanced Antiviral Activity of Acyclovir Loaded into Nanoparticles. *Methods*
432 *in enzymology,* 509, 1-19.

433 Chiej R. (1984). *The Macdonald encyclopedia of medicinal plants*, Macdonald & Co
434 (Publishers) Ltd.
435 Corey L. (2007). Herpes simplex virus type 2 and HIV-1: the dialogue between the 2
436 organisms continues. *Journal of Infectious Diseases,* 195(9), 1242-1244.
437 Dingwell K.S., Brunetti C.R., Hendricks R.L., Tang Q., Tang M., Rainbow A.J., &
438 Johnson D.C. (1994). Herpes simplex virus glycoproteins E and I facilitate cell-to-cell
439 spread in vivo and across junctions of cultured cells. *Journal of virology,* 68(2), 834-
440 845.
441 Donalisio M., Nana H.M., Ngane R.A., Gatsing D., Tchinda A.T., Rovito R., Cagno V.,
442 Cagliero C., Boyom F.F., Rubiolo P., Bicchi C., Lembo D. (2013). In vitro anti-Herpes
443 simplex virus activity of crude extract of the roots of Nauclea latifolia Smith
444 (Rubiaceae). *BMC Complement Altern Med.* 13, 266.
445 Donalisio M., Quaranta P., Chiuppesi F., Pistello M., Cagno V., Cavalli R., Volante M.,
446 Bugatti A., Rusnati M., Ranucci E., Ferruti P., & Lembo D. (2016). The AGMA1 poly
447 (amidoamine) inhibits the infectivity of herpes simplex virus in cell lines, in human
448 cervicovaginal histocultures, and in vaginally infected mice. *Biomaterials,* 85, 40-53.
449 Feng Z., Qiu Z., Sang Z., Lorenzo C., & Glasser J. (2013). Modeling the synergy
450 between HSV-2 and HIV and potential impact of HSV-2 therapy. *Mathematical*
451 *biosciences,* 245(2), 171-187.
452 Fiorito S., Epifano F., Palmisano R., Genovese S., & Taddeo V.A. (2017). A Re-
453 investigation of the Phytochemical Composition of the Edible Herb *Amaranthus*
454 *retroflexus L. Journal of Pharmaceutical and Biomedical Analysis,*143, 183-187.

455 Freeman E. E., Weiss H. A., Glynn J. R., Cross P. L., Whitworth J. A. & Hayes R. J.
456 (2006). Herpes simplex virus 2 infection increases HIV acquisition in men and women:
457 systematic review and meta-analysis of longitudinal studies. *AIDS*, 20(1), 73-83.

458 Iauk L., Acquaviva R., Mastrojeni S., Amodeo A., Pugliese M., Ragusa M., Loizzo
459 M.R., Menichini F., & Tundis R. (2015). Antibacterial, antioxidant and hypoglycaemic
460 effects of *Thymus capitatus (L.) Hoffmanns. et Link* leaves' fractions. *Journal of enzyme*
461 *inhibition and medicinal chemistry,* 30(3), 360-365.

462 Jarikasem S., Charuwichitratana S., Siritantikorn S., Chantratita W., Iskander M.,
463 Frahm A.W., & Jiratchariyakul W. (2013). Antiherpetic effects of *Gynura procumbens*.
464 *Evidence-Based Complementary and Alternative Medicine,* 2013:394865.

465 Kakade A.N., & Magdum C.S. (2012). HPLC analysis of β-sitosterol in herbal
466 medicines and vegetable oils. *International Journal of Pharmacy and Life Sciences,*
467 3(5), 1666-1669.

468 Kimberlin D.W., & Whitley R.J. (2007). Antiviral therapy of HSV-1 and -2. In: *Human*
469 *Herpesviruses: Biology, Therapy, and Immunoprophylaxis,* Arvin, A., Campadelli-
470 Fiume G., Mocarski E., Moore P.S., Roizman B., Whitley R., & Yamanishi K. (eds).
471 Cambridge University Press: Cambridge; chapter 6.

472 Koch C., Reichling J., Schneele J., & Schnitzler P. (2008). Inhibitory effect of essential
473 oils against herpes simplex virus type 2. *Phytomedicine,* 15(1-2), 71-78.

474 Lai W.L., Chuang H.S., Lee M.H., Wei C.L., Lin C.F., & Tsai Y.C. (2012). Inhibition
475 of herpes simplex virus type 1 by thymol-related monoterpenoids. *Planta medica,*
476 78(15), 1636-1638.

Leary J.J., Wittrock R., Sarisky R.T., Weinberg A., Levin M.J. (2002). Susceptibilities of herpes simplex viruses to penciclovir and acyclovir in eight cell lines *Antimicrobial Agents Chemotherapy* 46(3), 762-768.

Madureira A., Ascenso J., Valdeira L., Duarte A., Frade J., Freitas G., & Ferreira M. (2003). Evaluation of the antiviral and antimicrobial activities of triterpenes isolated from *Euphorbia segetalis*. *Natural product research,* 17(5), 375-380.

Maissa B.J., & Walid H. (2015). Antifungal activity of chemically different essential oils from wild Tunisian *Thymus spp*. *Natural product research,* 29(9), 869-873.

Mkaddem M.G., Romdhane M., Ibrahim H., Ennajar M., Lebrihi A., Mathieu F., & Bouajila J. (2010). Essential oil of *Thymus capitatus Hoff. et Link.* from Matmata, Tunisia: gas chromatography-mass spectrometry analysis and antimicrobial and antioxidant activities. *Journal of medicinal food,* 13(6), 1500-1504.

Nolkemper S., Reichling J., Stintzing F.C., Carle R., & Schnitzler P. (2006). Antiviral effect of aqueous extracts from species of the *Lamiaceae* family against Herpes simplex virus type 1 and type 2 in vitro. *Planta medica,* 72(15), 1378-1382.

Novoa B., Romero A., Álvarez Á.L., Moreira R., Pereiro P., Costa M.M., Dios S., Estepa A., Parra F., Figueras A. (2016). Antiviral Activity of Myticin C Peptide from Mussel: an Ancient Defense against Herpesviruses. *Journal of virology* 90(17), 7692-7702.

Pauwels R., Balzarini J., Baba M., Snoeck R., Schols D., Hederwijin P., Desmyter J., and De Clerq E. (1988). Rapid and automated tetrazolium-based colorimetric assay for the detection of anti HIV compounds. *J. Virol. Methods,* 20(4), 309-321.

Pilau M.R., Alves S.H., Weiblen R., Arenhart S., Cueto A.P., & Lovato L.T. (2011). Antiviral activity of the *Lippia graveolens* (Mexican oregano) essential oil and its main

501 compound carvacrol against human and animal viruses. *Brazilian Journal of*
502 *Microbiology,* 42(4), 1616-1624.
503 Rajbhandari M., Mentel R., Jha P., Chaudhary R., Bhattarai S., Gewali M.,
504 Karmacharya N., Hipper M., & Lindequist U. (2009). Antiviral activity of some plants
505 used in Nepalese traditional medicine. *Evidence-Based Complementary and Alternative*
506 *Medicine,* 6(4), 517-522.
507 Ricci D., Epifano F., & Fraternale D. (2017). The Essential Oil of *Monarda dydima L.*
508 (*Lamiaceae*) Exerts Phytotoxic Activities in Vitro Against Various Weed Seeds.
509 *Molecules,* 22(2), 222.
510 Roizman B., Knipe D., & Whitley R. (2007). Herpes simplex viruses. In *Fields virology*
511 (5^{th} edn), Knipe, D.M., *et al*. (eds). Lippincott, Williams & Wilkins: Philadelphia; 2501-
512 2601.
513 Schnitzler P., Koch C., & Reichling J. (2007). Susceptibility of drug-resistant clinical
514 herpes simplex virus type 1 strains to essential oils of ginger, thyme, hyssop, and
515 sandalwood. *Antimicrobial agents and chemotherapy,* 51(5), 1859-1862.
516 Silva-Mares D., Torres-López E., & Rivas-Galindo V.M. (2016). Antiherpetic Plants:
517 A Review of Active Extracts, Isolated Compounds, and Bioassays. *Natural Product*
518 *Communications,*11(4), 557-66.
519 Stahl-Biskup E., & Saez F. (2002). *Thyme: The genus Thymus. Medicinal and aromatic*
520 *plants-Industrial profiles* (vol 17). Taylor & Francis: London.
521 Taddeo V.A., Genovese S., de Medina P., Palmisano R., Epifano F., & Fiorito S.
522 (2017). Quantification of Biologically Active O-prenylated and Unprenylated
523 Phenylpropanoids in Dill (*Anethum graveolens*), anise (*Pimpinella anisum*), and Wild

Celery (*Angelica archangelica*). *Journal of Pharmaceutical and Biomedical Analysis,* 134, 319-324.

WHO. Herpes simplex virus. (2017). http://www.who.int/mediacentre/factsheets/fs400/en/ Accessed 02.10.2017

Figure captions

Figure 1. HPLC chromatograms of β-sitosterol (A), cinnamaldehyde (B) and carvacrol (C)

Figure 2. GC chromatogram of *T. capitatus* essential oil

Figure 3. Evaluation of virus inactivation by β-sitosterol (panel A), cinnamaldehyde (panel B) and carvacrol (panel C) on infectious HSV-2 particles at 37 °C for 0 h or 2 h. On the *y* axis, the infectious titers are expressed as plaque-forming units per ml (PFU/ml). Error bars represent standard error of the mean (SEM) of 3 independent experiments. *p< 0.001

Figure 4. Effect of β-sitosterol and cinnamaldehyde on viral replicative cycle. Pre-treatment assay (A), attachment assay (B), entry assay (C) and post-treatment assay (D). Heparin was used as a known inhibitor of attachment. Acyclovir were used as standard positive drugs when it was added after infection. Error bars represent the SD of the mean of three independent experiments.

Figure 5. Effect of β-sitosterol (panel A), cinnamaldehyde (panel B) or acyclovir (panel C) on multiple cycles of HSV-2 replication. Viral titers (expressed as PFU/ml) are shown as means plus standard error of the mean (SEM) for three independent experiments. *p< 0.05; ** p <0.01; ***p < 0.001

Table 1. Antiviral activity of *Thymus capitatus* extracts

	Virus	EC50[a] (µg/ml) – 95% C.I.[b]	CC50[c] (µg/ml)	SI[d]
Aqueous extract	HSV-2	23.6 (15.5 - 30.2)	> 300	> 12.6
	HSV-1	23.4 (14.3 - 32.4)	> 300	> 12.7
Ethanolic extract	HSV-2	2.3 (1.4 - 3.8)	62.5	26.8
	HSV-1	16.6 (11.2 - 24.6)	58.5	3.5
Essential oil	HSV-2	18.6 (13.4 - 25.9)	129.1	6.9
	HSV-1	17.6 (6.5 - 27.5)	107.0	6.0

[a] half maximal effective concentration
[b] confidence interval
[c] half maximal cytotoxic concentration
[d] selectivity index

548

549

550

Table 2. Antiviral activity of purified compounds derived from *Thymus capitatus* ethanolic extract

	Virus	EC50[a] (µg/ml) – 95% C.I.[b]	CC50[c] (µg/ml)	SI[d]
β-sitosterol	HSV-2	2.7 (1.8 - 4.1)	212.8	76.2
	HSV-2 ACV-r[e]	2.3 (2.1- 2.5)	212.8	90.9
Cinnamaldehyde	HSV-2	39.7 (30.4 - 51.8)	1126	28.3
	HSV-2 ACV-r	73.5 (47.3- 94.3)	1126	15.3
Carvacrol	HSV-2	51.9 (36.3-74.2)	632.9	12.1
	HSV-2 ACV-r	82.2 (62.3-108.5)	632.9	7.6
Acyclovir	HSV-2	0.7 (0.4-1.0)	754	1062
	HSV-2 ACV-r	336.9 (263.3-395.3)	754	2.2

[a] half maximal effective concentration
[b] confidence interval
[c] half maximal cytotoxic concentration
[d] selectivity index
[e] HSV-2 acyclovir resistant strain

551