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

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

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27 **ABSTRACT**

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

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44

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

47

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

50

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

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## 75 **Introduction**

76 The *Thymus* genus is considered to be one of the largest genera within the *Lamiaceae*  
77 family that includes approximately 350 species of perennial, aromatic herb and sub  
78 shrubs native mainly of Europe, Western Asia and the Mediterranean regions (Stahl-  
79 Biskup & Saez, 2002). *Thymus capitatus* (Hoffmanns. & Link, Lamiaceae) represents  
80 one of the five Tunisian species of the genus *Thymus*, which has a long standing use for  
81 flavouring and preserving several food products. Its essential oil is used in the  
82 flavouring cough medicines and oral hygiene products as well as in cosmetic and  
83 perfume industry. As a medicinal plant, *T. capitatus* decoction and infusion have  
84 traditionally been considered as antispasmodic, carminative, tonic, antiseptic and  
85 antitussive drugs (Chiej, 1984). Numerous reports showed antibacterial, antifungal,  
86 antioxidant and anti-inflammatory properties of *T. capitatus* extracts and essential oil,  
87 rich in carvacrol and thymol (Mkaddem *et al.*, 2010; Achour *et al.*, 2012; Iauk *et al.*,  
88 2014; Maissa *et al.*, 2015). To date, extracts of several species of *Thymus*, as *T. vulgaris*  
89 and *T. linearis*, have been investigated for their anti-herpetic effect, even though the  
90 constituents responsible of the antiviral activity have not been yet identified (Nolkemper  
91 *et al.*, 2006; Schnitzler *et al.*, 2007; Koch *et al.*, 2008; Rajbhandari *et al.*, 2009). Herpes  
92 simplex virus type 2 (HSV-2) is a sexually transmitted pathogen that infects about 417  
93 million people aged 15-49 (11%) worldwide, causing genital infections ([www.who.int](http://www.who.int)).  
94 Most of these infections are asymptomatic but can also cause painful blisters or ulcers at  
95 the site of infection. Symptomatic infections are most contagious but sexually  
96 transmission can also occur in the absence of symptoms (Roizman, Knipe & Whitley  
97 2007). Of note, genital ulcer disease increases the risk of HIV acquisition since the  
98 mucosal damage induces local inflammation, following activation and recruitment of

99 CD4<sup>+</sup> 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.  $\beta$ -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<sup>-1</sup>) 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  $\beta$ -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.  $\beta$ -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  $\mu$ L loop. Data acquisition was monitored by Waters Empower software (ver.  
173 2.0). Chromatographic separation was accomplished employing a GraceSmart RP<sub>18</sub> (5  
174  $\mu$ m particle size, 250 mm x 4.6 mm, Grace, Deerfield, IL, USA). Column temperature  
175 was maintained at 25  $\pm$  1  $^{\circ}$ C using a cool pocket chiller (ThermoScientific, Waltham,  
176 USA). Elution mixture consisted of H<sub>2</sub>O 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  $\mu$ L. Samples were  
183 filtered through a 0.45  $\mu$ m 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  $^{\circ}$ 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  $\mu$ g/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

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

#### 205 **Viability assay**

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

#### 212 **Anti-HSV inhibition assay**

213 The effect of *T. capitatus* extracts, isolated compounds or acyclovir on HSV infection  
214 was evaluated by plaque reduction assay (Novoa *et al.*, 2016). Vero cells were seeded in  
215 24-well plates at a density of  $10 \times 10^4$  cells/well. Increasing concentrations of extracts or  
216 constituents were added to cells for 2 h; a mixture of different tested substances plus  
217 HSV-1 or HSV-2 or acyclovir resistant HSV-2 at a multiplicity of infection (MOI) of  
218 0.001 plaque-forming units (PFU)/cell (100 PFU), were subsequently added to the cells,

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

#### 230 **Virus yield reduction assay**

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

#### 240 **Virus inactivation assay**

241 Approximately, 10<sup>5</sup> PFU of HSV-2 plus EC<sub>90</sub> of pure compounds were added to MEM  
242 and mixed in a total volume of 110 µl. The virus-compound mixtures were incubated

243 for 0 h or 2 h at 37 °C then diluted serially to the non-inhibitory concentration of  
244 compounds; the residual viral infectivity was determined by titrating virus by plaque  
245 assay on Vero cells, pre-seeded in 96-well plates at a density of  $16 \times 10^4$  cells/well  
246 (Alvarez *et al.*, 2009; Alvarez *et al.*, 2015b).

#### 247 **Pre-treatment assay**

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

#### 251 **Attachment assay**

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

#### 257 **Entry assay**

258 The HSV-2 at 0.004 MOI was adsorbed for 2 h at 4 °C on pre-chilled confluent Vero  
259 cells. Cell layers were then washed three times with cold MEM to remove unbound  
260 virus, treated with different concentrations of  $\beta$ -sitosterol, and incubated for 3 h at 37  
261 °C. The outer virions were inactivated with acidic glycine for 2 min at room  
262 temperature and the cells were washed three times with warm medium and treated as for  
263 plaque reduction assay (Alvarez *et al.*, 2009; Alvarez *et al.*, 2015b).

#### 264 **Post-treatment assay**

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

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

## 269 **Data analysis**

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

279

## 280 **Results and Discussion**

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

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

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

### 305 **Chemical characterization of extracts and essential oil**

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

### 310 **Inhibitory activity of isolated compounds from *T. capitatus* ethanolic extract and** 311 **their mechanism of action**

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

315 isolated from *T. capitatus* EE,  $\beta$ -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<sub>50</sub> values of 2.7  $\mu$ M, 39.7  $\mu$ M and 51.9  $\mu$ M, respectively (Table 2); acyclovir  
319 was tested in parallel as a reference drug. Among these compounds,  $\beta$ -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  $\beta$ -  
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<sub>50</sub> value of 336.9  $\mu$ M for acyclovir). As reported in Table 2, the resistant  
337 strain was susceptible to  $\beta$ -sitosterol, cinnamaldehyde and carvacrol, with EC<sub>50</sub> values  
338 equal to 2.3  $\mu$ M, 73.5  $\mu$ M and 82.2  $\mu$ M, respectively. These data suggest a different



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

343 Firstly, we investigated the ability of pure compounds to directly inactivate HSV-2 viral  
344 particles. The assay was performed by mixing a virus aliquot containing  $10^5$  PFU with a  
345 concentration of each compound that reduced almost completely the virus growth  
346 ( $>EC_{90}$ ) in the plaque reduction assay. The reduction of the viral titers of treated versus  
347 untreated samples was analyzed at high dilutions at which extracts were no more active.

348 As reported in Figure 3, experiments were performed by incubating the mixtures for 0 h  
349 or 2 h at 37 °C. Both  $\beta$ -sitosterol and cinnamaldehyde did not show any significant  
350 inhibition when the mixtures were promptly added on the cells without incubation  
351 (Figure 3A and 3B). By contrast, after 2 h incubation, both compounds reduced  
352 significantly the HSV-2 titer by 99% and 91%, respectively. The ability of  $\beta$ -sitosterol  
353 and cinnamaldehyde to directly inactivate HSV-2 infectivity is a novel finding,  
354 corroborating the results of Jarikasem *et al.* (2013) who have reported a virucidal  
355 activity of a fraction containing  $\beta$ -sitosterol and stigmasterol against both HSV-1 and  
356 HSV-2. Furthermore, we have also evidenced the significant ability of carvacrol to  
357 inactivate extracellular HSV-2 particles either at 0 h or 2 h of incubation (Figure 3C).

358 This data confirm the results of Jarikasem *et al.* (2013) who have previously shown the  
359 virucidal activity of carvacrol against HSV-1 particles. Since the anti-herpetic activity  
360 of carvacrol was previously reported in literature, we decided to gain further insights  
361 into  $\beta$ -sitosterol and cinnamaldehyde mode of antiviral action. To explore whether they  
362 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  $\beta$ -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  $\beta$ -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  $\mu$ M) 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  $\beta$ -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  $\beta$ -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  $\mu$ M  
379 and 7.5  $\mu$ M, 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  $\beta$ -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  $\mu$ M and 8.41  $\mu$ M, 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.

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

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

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404

#### 405 **Conflict of interest**

406 The authors declare no conflict of interest.

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529

530 **Figure captions**

531 **Figure 1.** HPLC chromatograms of  $\beta$ -sitosterol (A), cinnamaldehyde (B) and carvacrol  
532 (C)

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

534 **Figure 3.** Evaluation of virus inactivation by  $\beta$ -sitosterol (panel A), cinnamaldehyde  
535 (panel B) and carvacrol (panel C) on infectious HSV-2 particles at 37 °C for 0 h or 2 h.

536 On the y axis, the infectious titers are expressed as plaque-forming units per ml  
537 (PFU/ml). Error bars represent standard error of the mean (SEM) of 3 independent  
538 experiments. \* $p < 0.001$

539 **Figure 4.** Effect of  $\beta$ -sitosterol and cinnamaldehyde on viral replicative cycle. Pre-  
540 treatment assay (A), attachment assay (B), entry assay (C) and post-treatment assay (D).

541 Heparin was used as a known inhibitor of attachment. Acyclovir were used as standard  
542 positive drugs when it was added after infection. Error bars represent the SD of the  
543 mean of three independent experiments.

544 **Figure 5.** Effect of  $\beta$ -sitosterol (panel A), cinnamaldehyde (panel B) or acyclovir (panel  
545 C) on multiple cycles of HSV-2 replication. Viral titers (expressed as PFU/ml) are

546 shown as means plus standard error of the mean (SEM) for three independent  
547 experiments. \* $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\* $p < 0.001$

**Table 1. Antiviral activity of *Thymus capitatus* extracts**

	<b>Virus</b>	<b>EC50<sup>a</sup> (µg/ml) – 95% C.I. <sup>b</sup></b>	<b>CC50<sup>c</sup> (µg/ml)</b>	<b>SI<sup>d</sup></b>
Aqueous extract	HSV-2	23.6 (15.5 - 30.2)	> 300	> 12.6
	HSV-1	23.4 (14.3 - 32.4)	> 300	> 12.7
Ethanollic 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

<sup>a</sup> half maximal effective concentration<sup>b</sup> confidence interval<sup>c</sup> half maximal cytotoxic concentration<sup>d</sup> selectivity index

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

	<b>Virus</b>	<b>EC50<sup>a</sup> (µg/ml) – 95% C.I. <sup>b</sup></b>	<b>CC50<sup>c</sup> (µg/ml)</b>	<b>SI<sup>d</sup></b>
β-sitosterol	HSV-2	2.7 (1.8 - 4.1)	212.8	76.2
	HSV-2 ACV-r <sup>e</sup>	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

<sup>a</sup> half maximal effective concentration<sup>b</sup> confidence interval<sup>c</sup> half maximal cytotoxic concentration<sup>d</sup> selectivity index<sup>e</sup> HSV-2 acyclovir resistant strain

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