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# Supercritical fluid extraction as an alternative process to obtain antiviral agents from thyme species

S. Santoyo[a,*], L. Jaime[a], M.R. García-Risco[a], M. Lopez-Hazas[a], G. Reglero[a,b]

[a] Instituto de Investigación en Ciencias de la Alimentación (CIAL), Universidad Autónoma de Madrid, 28049 Madrid, Spain
[b] Imdea-Food Institute, Universidad Autónoma de Madrid, 28049 Madrid, Spain

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ABSTRACT

The antiviral properties of supercritical $CO_2$ extracts obtained from thyme species (*Thymus vulgaris*, *Thymus hyemalis* and *Thymus zygis*) were evaluated against the herpes simplex virus type 1 (HSV-1) at different stages during virus infection. Results indicated that when cells were pre-treated with the thyme extracts, an important reduction of virus infectivity was achieved; being *T. zygis* extract more effective than the other thyme species extracts. Comparing data obtained during the adsorption stage with those found in the pre-treatment step, at the same concentration, the reduction of the virus infectivity was increased by 30%, indicating that extracts were more effective when applied during adsorption period. Moreover, supercritical extracts of thyme species were able to significantly inhibit the in vitro virus replication, showing $IC_{50}$ values among 1.74–2.25 μg/mL. A GC–MS characterization of supercritical extracts was carried out in order to identify the type of compounds responsible for the antiviral activity. Thus, the higher antiviral activity found in *T. zygis* supercritical extract, could be related to the higher percentage of thymol, carvacrol and borneol (79.77%) presented in this extract.

Supercritical fluid extraction represents an alternative method to obtain antiviral extracts from *Thymus* species. These supercritical extracts mainly inhibit HSV-1 intracellular replication, although they were also able to disrupt the virus attachment step.



## 1. Introduction

*Thymus* species have been also used in folk medicine for gastrointestinal complains and as effective therapies for irritating coughs and bronchitis because of their expectorant and antitussive properties (Figueiredo et al., 2008). More recently, various *Thymus* species have been reported to possess a broad spectrum of bioactivities, including antioxidant (Chizzola et al., 2008; Dandlen et al., 2010), anti-inflammatory (Ismaili et al., 2004; Mahmoudi et al., 2008) and antimicrobial activities (Rasooli et al., 2006; Rota et al., 2008). Despite of the antifungal and antibacterial activity exhibited by *Thymus* genus has been demonstrated by several researches (Fadli et al., 2012; Gonçalves et al., 2010; Ruiz-Navajas et al., 2012; Saad et al., 2010), only few studies have evaluated the potential role of thyme as an antiviral agent (Nolkemper et al., 2006; Koch et al., 2008).

Herpes simplex virus type 1 (HSV-1) is a highly prevalent pathogen among children and adults, causing primary infections which are presented clinically as herpes labialis or as primary gingivostomatitis. The virus is also able to establish a latent infection in the nervous system that can be reactivated quite frequently (Withley, 2001). The major therapeutics agents for HSV infections are nucleoside analogues such as acyclovir and vidarabine. However, the increased and prolonged use of these compounds, especially in immunocompromised patients, has led to viral resistance against most of these drugs (Chakrabarti et al., 2000). The drug resistant HSVs retained their pathogenicity and could be associated with progressive and relapsing disease. Thus, it is necessary to explore and discover novel potential antiherpetic approaches.

Thus, in last years, a large number of antiherpes screening experiments on medicinal plants extracts and plats derived secondary metabolites (e.g. polyphenolics, glycosides, terpenes, polysaccharides, polyketides, pheophorbides...) have been reported (Khan et al., 2005). Further, the antiherpes activity of several essential oils of different plant sources as well as of various constituents of essential oils was demonstrated. Among them, aqueous and ethanolic extracts of *Salvia officinalis* and *Salvia coccinia* revealed an important antiviral activity against HSV-1 and HSV-2 (Schnitzler et al., 2008). Also, essential oils of *Melissa officinalis*, *Mentha piperita* and *Rosmarinus officinalis* presented antiviral activity against herpes viruses (Allahverdiyev et al., 2004; Nolkemper et al., 2006; Schuhmacher et al., 2003).

* Corresponding author at: Instituto de Investigación en Ciencias de la Alimentación (CIAL), Campus de la Universidad Autónoma, c/ Nicolas Cabrera, 9, 28049 Madrid, Spain. Tel.: +34 910017926.
*E-mail address:* susana.santoyo@uam.es (S. Santoyo).

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Hydrodistillation is a conventional technique extensively used to extract essential oil from plant material, but this process does not avoid the potential hydrolysis or thermal degradation of the most sensitive compounds (Revenchon and de Marco, 2006). In contrast, supercritical fluid extraction (SFE) enables shorter extraction times, reduces the consumption of hazardous organic solvents and avoids the degradation of active compound, thus preserving the original composition of the extracted volatiles (Al-Marzouqi et al., 2007; Costa et al., 2012). Carbon dioxide ($CO_2$) is widely used as supercritical fluid because is it non-toxic, chemically stable, environmentally acceptable and easily separated from the extract (Herrero et al., 2010).

The goal of the present work was to study the in vitro antiviral activity of supercritical extracts obtained from three different species of thyme (*Thymus vulgaris*, *Thymus hyemalis* and *Thymus zygis*) against HSV-1. Simultaneously, the antiviral activity of the extracts at different steps during the viral infection cycle was also determined. Further, this work analyzed the chemical composition of the extracts and intended to establish a relationship between the extracts' activity and their composition.

## 2. Materials and methods

### 2.1. Samples and chemicals

The thyme samples (*T. vulgaris*, *T. hyemalis* and *T. zygis*) consisted of dried leaves obtained from a herbalist' shop (Murcia, Spain). Cryogenic grinding of the samples was performed under carbon dioxide. The size of the particle (between 999 and 500 μm) was determined by passing the ground plant material through sieves of appropriate size. The whole sample was stored at −20 °C until use.

1,8-Cineole, camphor, borneol, linalool, carvacrol and thymol standards were purchased from Sigma (Madrid, Spain). $CO_2$ (N38 quality) was supplied from Air Liquid (Madrid, Spain).

### 2.2. Extraction method

Extractions were carried out using a 30 MPa pilot-plant supercritical fluid extractor (Thar Technology, Pittsburgh, PA, USA, model SF2000) comprising a 2 L cylinder extraction cell and two different separators (S1 and S2), each of 0.5 L capacity, with independent control of temperature and pressure. This plant also included a $CO_2$ recirculation system, where $CO_2$ is condensed and pumped up to the desired extraction pressure. The temperature of extraction cell and separators was maintained constant at 313 K. In each assay, the extraction vessel was packed with 0.55 kg of thymus. Fractionation of the extracted material was accomplished by setting the pressure in the first separator (S1) to 10 MPa, while the second separator (S2) was maintained at the recirculation system pressure (5 MPa). This fractionation system produced two different extracts with different composition which were collected in separator 1 (S1) and separator 2 (S2). According to previous kinetic studies, the extraction time was set to be 8 h (Fornari et al., 2012). The extracts produced were kept at −20 °C under $N_2$ until analysis.

### 2.3. Antiviral assays

#### 2.3.1. Cells and viruses

Vero cells (African green monkey kidney cell line) were obtained from the American Type Culture Collections (ATCC), Rockville, MD. They were used as host for HSV-1. The cells were grown using Eagle's Minimum Essential Medium (MEM) supplemented with 5% foetal bovine serum (FBS), 1% penicillin–streptomycin, 1% hepes buffer 1 M, 1% non essential aminoacids and 1% L-glutamine. Maintenance medium for Vero cells was as described above but with 2% FBS.

Herpes virus simplex type 1 (HSV-1) (KOS) was obtained from the American Type Culture Collections (ATCC), Rockville, MD, prepared in aliquots and stored at −80 °C until use. Virus titre was determined by plaque reduction assay in Vero cells and expressed as plaque forming units (pfu) per mL.

#### 2.3.2. Cytotoxicity assays

The cytotoxic effect of the different extracts and standards on Vero cells was tested using MTT assay, according to a published method (Mosmann, 1983). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma, Spain) is a yellow water soluble tetrazolium dye that is reduced by live cells, but not dead to a purple formazan product that is insoluble in aqueous solutions. Monolayers of Vero cells in 24-multiwell plates were incubated with MEM containing different concentrations of the extracts for 48 h at 37 °C. Cells were then washed with PBS and 0.5 mg/ml of MTT were added to each well and incubated 4 h at 37 °C. Supernatants were discarded and formazan crystals dissolved in a extraction solution (10% sodium dodecyl sulphate in a mixture of dimethyl formamide and water 1:1 (v/v), adjusted to pH 4.7 with acetic acid) overnight at 37 °C. Formazan quantification was performed by measuring the optical density at 570 nm using a multiscanner autoreader (Sunrise, Tecan) with the extraction solution as a blank. The data were plotted as dose–response curves, from which the concentration required to reduce 50% the number of viable Vero cells ($CC_{50}$) after 48 h of incubation with the different extracts were obtained.

#### 2.3.3. Evaluation of virucidal activity

Virus samples containing $10^5$ pfu/ml were mixed and incubated at 37 °C for 1 h with MEM containing different extracts or pure compounds concentrations or MEM alone (control). Samples were then diluted and used to infect confluent Vero cells for 1 h at 37 °C. After incubation, the virus inocula was removed, the cells washed with PBS and then overlaid with maintenance medium (with 0.5% agarose) for 48 h at 37 °C. The infected cells were fixed acetone:methanol (50:50) at 4 °C, stained with a 1% solution of crystal violet and the number of the plaques counted. The percentage of inhibition of plaque formation was calculated as follows: [(mean number of plaques in control) − (mean number of plaques in test)]/(mean number of plaques in control) × 100.

#### 2.3.4. Influence of various treatment periods on the anti-HSV-1activity of the extracts

Vero cells and viruses were incubated with the extracts at different stages during the viral infection cycle in order to determine the mode of antiviral action. (1) Cells pretreatment: monolayers of Vero cells in 24-multiwell plates were pretreated with MEM containing different concentrations of the extract or pure compounds for 3 h at 37 °C. Cells were then washed with PBS and infected with 120 pfu of HSV-1. After incubation for 1 h at 37 °C, the virus inocula was removed, the cells washed with PBS and then overlaid with maintenance medium (with 0.5% agarose) for 48 h at 37 °C. The infected cells were fixed, stained and the number of the plaques counted. Control consisted on untreated cells infected with HSV-1. (2) Adsorption period: cells were infected with 120 pfu of HSV-1 in presence of different concentrations of the extracts for 1 h at 37 °C. Then, the virus inocula and the extract were removed, the cells washed with PBS and then overlaid with maintenance medium (with 0.5% agarose) for 48 h at 37 °C. The infected cells were fixed, stained and the number of the plaques counted. Control consisted on cells infected without the extracts or the pure components. (3) Intracellular replication: cells were infected with 120 pfu of HSV-1. After incubation for 1 h at 37 °C, the virus inocula was removed, the cells washed with PBS and then overlaid with maintenance medium (with 0.5% agarose) containing different concentrations