Antiviral and Immunomodulatory Effect of a Lyophilized Extract of *Capparis spinosa* L. Buds

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Herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2) are common human pathogens that in particular cases can also cause severe problems especially in immunodeficient patients. The present paper reports the antiviral and immunomodulatory properties of a methanolic extract of *C. spinosa* buds (CAP), rich in flavonoids, including several quercetin and kaempferol glycosides. In particular we have investigated whether the in vitro exposure of human peripheral blood mononuclear cells (PBMCs) to CAP might inhibit the replication of HSV-2 and modulate the induction kinetics of IL-12, TNF-α and IFN-γ. Our findings have shown that CAP treatment interferes with HSV-2 replication in PBMCs inhibiting the extracellular virus release upregulating their production of IL-12, IFN-γ and TNF-α. One could speculate that CAP may contribute in improving immune surveillance of PBMCs toward virus infection by up-regulating expression of parallel proinflammatory cytokines; it should thus be successfully employed for treatment of HSV-2 infections in immunocompromised hosts.

**Keywords:** *Capparis spinosa* buds, Herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), antiviral, immunomodulatory, peripheral blood mononuclear cells (PBMCs).

**INTRODUCTION**

Herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2) are common human pathogens that cause localized skin infections of mucosal epithelia in genitals, oral cavity, pharynx, oesophagus and eyes; however HSV infections are latent and can be periodically reactivated (Simmons, 2002). In particular cases HSV can also cause severe problems. In fact the virus can produce serious infections of the central nervous system, and HSV infections may be fatal in immunodeficient patients; furthermore the immediate-early genes of HSV-1 can stimulate the activation of genes belonging to different viruses such as human immunodeficiency virus, varicella-zoster virus and papillomavirus.

Efficient elimination of viruses relies on the ability of the infected host to mount a proinflammatory immune response and develop a Th-1 type immunity (Lucey et al., 1996; Carr and Tomanek, 2006). This response is characterized by activation of monocytes/macrophages, cytotoxic T-lymphocytes and production of proinflammatory cytokines and chemokines, including interferons (IFN), tumor necrosis factor-α (TNF-α) and interleukins (IL). Helper T lymphocytes (Th) may be divided into two functional subclasses, Th-1 and Th-2 cells, based upon the cytokines that they produce and their effects on cell mediated and humoral immunity. Th-1 cells produce IL-2, IFN-γ and IL-12, enhance cell-mediated immunity and inhibit cell-mediated immunological activities. Th-1 cells drive the type-1 pathway (cellular immunity) to fight viruses and other intracellular pathogens and eliminate cancerous cells. Primary biological functions of Th-1 derived cytokines include antiviral and anticancer effects. Th-2 cells produce IL-4, IL-5, IL-6 and IL-10 and upregulate humoral immunity. Th-2 cells drive the type-2 pathway (humoral immunity) and upregulate antibody production to fight extracellular organisms. In addition, Th1 and Th2 derived cytokines cross-regulate each other in various clinical conditions (Kidd, 2003).

Due to the evident need for effective antiviral therapy, in recent years a large number of naturally occurring compounds with antiviral and immunomodulatory components have been investigated in the attempt to develop new safe agent able to inhibit specifically viral functions and/or to influence the response of the host to viruses (Armaka et al., 1999; Arena et al., 2006).

*Capparis spinosa* L. (family Capparidaceae) is a plant from the dry regions in west or central Asia and widely grown particularly in the Mediterranean basin. From ancient times, the floral buttons of *C. spinosa* (capers) were employed as a flavouring in cooking and are also used in traditional medicine for their diuretic, anti-hypertensive, poultice and tonic properties (Baytop, 1984; Çalış et al., 1999). Previous chemical studies on *C. spinosa* have shown the presence of alkaloids, lipids, polyphenols, flavonoids, indole and aliphatic...
glucosinolates (Rodrigo et al., 1992; Sharaf et al., 2000). Recently, Çalış and coworkers (Çalıṣ et al., 1999, 2002) have isolated from mature fruits of C. spinosa two glucose-containing 1H-indole-3-acetonitrile compounds, capparillosides A and B and two (6S)-hydroxy-3-oxo-a-ionol glucosides, corchoionoside C and a phenyl glucoside. Glucoepparine has been shown to be the main glucosinolate in C. spinosa buds (Matthäus and Özcan, 2002). Finally, the presence of both flavonoids and hydroxycinnamic acids, has also been demonstrated in capers (Bonina et al., 2002). In a recent study a methanol extract of C. spinosa buds (CAP), rich in flavonoids, including several quercetin and kaempferol glycosides, was demonstrated to possess strong antioxidant/free radical scavenging effectiveness in different in vitro tests; in vivo this extract showed a noteworthy antiallergic effectiveness against bronchospasm in guinea-pigs (Trombetta et al., 2005), and, when topically applied, it afforded significant in vivo protection against UV-light-induced skin erythema in healthy human volunteers (Bonina et al., 2002). Finally this extract was able to counteract the inflammatory process induced in vitro by IL-1β in human chondrocyte cultures (Panico et al., 2005).

Since it is well known that food-derived flavonoids can modulate a variety of immune functions (Middleton, 1998; Middleton and Kandaswami, 1992) and exhibit antiviral properties and they have been shown to possess antiviral effects, and chemokines with pleiotropic effects orchestrate inflammatory and autoimmune diseases, it is clear that the investigation whether the in vitro and in vivo modes of action of flavonoids could modulate the induction kinetics of IL-12, TNF-α, IFN-γ and (2) might inhibit the replication of HSV-2.

**MATERIALS AND METHODS**

**Plant material.** Flower buds of *Capparis spinosa*, collected in October 1999, were obtained from ‘Consorzio dei Produttori di Capperi Pantelleria’ (Trapani, Italy) and authenticated by the botanists of the School of Pharmacy, University of Catania, Catania, Italy; a voucher specimen has been deposited in the herbarium of the School of Pharmacy, University of Catania, Catania, Italy. The extract was reported as prepared in previous papers (Panico et al., 2005; Bonina et al., 2002).

**Virus.** Herpes simplex virus type 2 (HSV-2), Nahamias strain, was used throughout the study. The virus infection was propagated on WISH cell line. Viral stocks were prepared by pelleting infected cells exhibiting a cytopathic effect, and freezing aliquots at −80 °C. The virus titer was assessed on WISH cells and expressed as plaque forming unit (PFU)/mL. The multiplicity of infection (MOI), used in all experiments, was 0.1 PFU/cell.

**Isolation and treatment of PBMCs.** PBMCs were obtained from healthy, HIV-, HBV- and HCV-seronegative donors, after centrifugation of heparinized venous blood over Ficol-Hypaque gradient (Boyum, 1968) and were then washed twice in RPMI 1640 medium, seeded in 24-well plates (Corning, Bibby srl, Milan) at a concentration of 2 × 10⁶ cells/well in RPMI 1640 medium supplemented with 50 μg/mL gentamicin and 10% fetal calf serum and cultured at 37 °C in 5% CO₂. The PBMCs were then treated with CAP (400 and 600 μg/mL). Lipopolysaccharide (LPS) from *E. coli* strain 055:B5 was used as the positive control (Pulendran et al., 2001). At different times (24 and 48 h) after the treatment, the supernatants were harvested, aliquoted and stored at −80 °C until cytokine analysis. The cells were then infected with HSV-2 at a MOI 0.1 PFU/cell for a further incubation of 24 h at 37 °C in 5% CO₂, after which the plates were frozen and thawed three times in order to release the virus. Cell lysates and supernatants were kept at −80 °C until virus titration.

The culture media and reagents were previously tested for the presence of endotoxin in the Limulus test by means of the E-Toxate kit (Sigma, Milan, Italy) and were found to contain ≤10 pg endotoxin/mL.

**Cytotoxicity test.** The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (MTT assay) was employed to determine the effect of CAP on cell viability (Mosmann, 1983). Briefly, PBMCs (2 × 10⁶ cells/well) were seeded in 96-microwell plates, then treated with CAP (400, 500 and 600 μg/mL) for 24 h. An appropriate amount of MTT was added to the cells and incubated for 4 h, after which the formed crystals of formazan were solubilized by DMSO. The plates were read at a wavelength of 570 nm. The percentage of viability was calculated as follows:

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\frac{[\text{OD}_{\text{exp}} - \text{OD}_{\text{blank}}]}{[\text{OD}_{\text{veh}} - \text{OD}_{\text{blank}}]} \times 100.
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The trypan blue exclusion test was performed to confirm cytotoxicity data obtained with the MTT test.

**Cytokine evaluations.** The supernatants from PBMCs were analysed for the presence of IL-12, IFN-γ and TNF-α by an immunoenzymatic method (Bender Medsystems, Milan, Italy); the detection limit of the assay was 19.1 pg/mL for IL-12, 1.5 pg/mL for IFN-γ and 5.8 pg/mL for TNF-α.

**Statistical evaluation.** The results are expressed as mean ± SD of five experiments. Data were analysed by one-way analysis of variance (ANOVA) and the Student-Newman-Keuls test.

**RESULTS AND DISCUSSION**

It is now well documented that a cell-mediated immune response plays a crucial role in the control of HSV infection. In fact a complex network of cytokines and chemokines with pleiotropic effects orchestrate the immune response of the host to viruses (Villar and Dongari-Bagtzoglou, 2006; Salazar-Mather and Hokeness, 2006). Thus, also due to the emergence of resistant viral mutants, there is an imperative need for discovering new efficient antiviral drugs able to inhibit specifically viral functions, endowed with immunomodulatory properties and non-toxic to eukaryotic cells.

In recent years various plant drugs, such as flavonoids, limonoids and monoterpenes, have been subjected to intense scientific investigations for their biological properties and they have been shown to possess antiviral activities against some RNA and DNA viruses (Martin et al., 2005). Since it is well known that food-derived flavonoids and hydroxycinnamic acids, exhibit antiviral activity, and, when topically applied, it afforded significant in vivo protection against UV-light-induced skin erythema in healthy human volunteers (Bonina et al., 2002). Finally this extract was able to counteract the inflammatory process induced in vitro by IL-1β in human chondrocyte cultures (Panico et al., 2005). Thus, also due to the emergence of resistant viral mutants, there is an imperative need for discovering new efficient antiviral drugs able to inhibit specifically viral functions, endowed with immunomodulatory properties and non-toxic to eukaryotic cells.

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Figure 1. Antiviral activity of PBMCs treated with different concentrations of CAP. Data are expressed as mean ± SD of five experiments. * p < 0.05 vs control (untreated cells).

Figure 2. Production of IL-12 (pg/mL) by PBMCs at 24 and 48 h following treatment with different concentrations of CAP. Data are expressed as mean ± SD of five experiments. n.d. = not determinable. * p < 0.05 vs the respective control (untreated cells). § p < 0.05 vs 400 μg/mL.

Figure 3. Production of TNF-α (pg/mL) by PBMCs at 24 and 48 h following treatment with different concentrations of CAP. Data are expressed as mean ± SD of five experiments. Data are not shown. * p < 0.05 vs the respective control (untreated cells).

Figure 4. Production of IFN-γ (pg/mL) by PBMCs at 24 and 48 h following treatment with different concentrations of CAP. Data are expressed as mean ± SD of five experiments. n.d. = not determinable. * p < 0.05 vs control (untreated cells). § p < 0.05 vs 400 μg/mL.

and Ernst, 2003). Furthermore, several studies support the hypothesis that some nutrients and hormones significantly influence the Th-1/Th-2 balance. In particular flavonoids, such as quercetin, exert significant antiviral and antitumor effects by modulating the production of Th-1 and Th-2 derived cytokines (Nair et al., 2002).

However, treatment with CAP does not influence virus replication in WISH cells (data not shown).

In order to clarify whether the antiviral activity of CAP in PBMCs can be related to an immunomodulatory mechanism, the study evaluated the production of IFN-γ, TNF-α, and IL-12, cytokines involved in the immune surveillance against virus infection.

The levels of all cytokines measured in supernatants obtained from control tubes (unstimulated cells not treated with CAP) were below the detection limits of the assay, but the cytokine release was maximal following LPS stimulation (data not shown).

High levels of all these cytokines were measured in supernatants from CAP-treated PBMCs. Figure 2 shows that CAP treatment triggered PBMCs in releasing IFN-γ in a dose- and time-dependent manner. In fact, the highest dose of CAP (600 μg/mL) resulted in an increased release of IFN-γ from PBMCs in comparison with 400 and 500 μg/mL (p < 0.05); furthermore, CAP treatment induced a higher IFN-γ production at 48 h than at 24 h.

As to TNF-α (Fig. 3), CAP induced a higher cytokine production at 48 h when compared with that obtained at 24 h after the treatment; at both times the effect was dose-dependent.

Furthermore, as shown in Fig. 4, CAP treatment triggered PBMCs in releasing IL-12 in a dose-dependent manner. However, IL-12 release was higher at 24 h than at 48 h following CAP treatment.

It is well demonstrated that the release of antiviral cytokines, such as IFN-γ, IL-12 and TNF-α, from PBMCs may contribute to improving immune surveillance towards virus infection (Villar and Dongari-Bagtzoglou, 2006; Salazar-Mather and Hokeness, 2006). The course of infection is thought to be regulated by two distinct T-cell cytokine patterns: Th1 cytokines are generally associated with resistance to infection, whereas Th2 cytokines are associated with progressive diseases. The factors that may influence the nature of cytokine response can greatly influence antiviral defence.

In this study, CAP treatment interfered with HSV-2 replication in PBMCs inhibiting the extracellular virus release; furthermore, CAP upregulated PBMCs production of IL-12, IFN-γ and TNF-α. On the contrary, no increase in the production of IL-4 (a strong hallmark of Th-2 responses) was observed (data not shown). Although this hypothesis is speculative at the present time, the present data lead us to hypothesize that the
Caper extract tested in this research is able to inhibit HSV-2 replication in PBMCs, by triggering polarization in favour of the Th-1 subset. The effect exhibited by CAP under these experimental conditions could be at least partially due to its polyphenolic active components, in particular flavonoids, which are known to possess antiviral and immunomodulatory properties (Martin and Ernst, 2003; Spellman et al., 2006).

A preliminary series of experiments has demonstrated that treatment with CAP shows the ability to stimulate the production of IFN-γ, TNF-α and IL-12 also in PBMCs infected with HSV-2, although to a lower degree in comparison with non-infected cells (data not shown). This issue needs further studies, since HSV is able to suppress expression of proinflammatory cytokines by decreasing the stability of mRNAs, thereby potentially impeding the antiviral host response to infection (Alcami and Efstathiou, 2000; Alcami, 2004).

It is interesting to mention that ethanol and water extracts of Capparis zeylanica Linn. (Indian caper) leaves have demonstrated, by in vitro and in vivo experimental models, to stimulate both cellular and humoral immune responses (Ghule et al., 2006).

The effect exhibited by CAP under our experimental conditions could be due to its polyphenolic active components, in particular flavonoids, which are known to possess antiviral and immunomodulatory properties. According to our hypothesis, Lyu and coworkers (2005) have recently demonstrated the antiviral activity of quercetin and kaempferol (that are two of the main components present as glycoside derivatives in CAP) against HSV-1 and HSV-2 infecting Vero cells. However, quercetin was shown to induce the production of IFN-γ (Nair et al., 2002), but to inhibit the production of TNF-α in normal PBMCs (Nair et al., 2006); similarly kaempferol was proved to inhibit TNF-α expression in J774.2 macrophages (Kowalski et al., 2005). Thus, with the present status of our research, the immunomodulatory properties of CAP cannot be attributed to a component in particular, but to the phytocomplex as a whole. In the recent past, the biological properties of phytocomplexes, which are constituted by several and different ingredients, have been justified by the presence of a particular molecule having that specific biological activity. Nowadays, many scientific works underline that the pharmacological effectiveness of a phytocomplex is due not to one or a few of its active principles, but rather is determined by a combined effect of some, or all, the components of the phytocomplex.

In conclusion, the data suggest that the CAP may contribute to improving immune surveillance of PBMCs toward virus infection by up-regulating the expression of peculiar proinflammatory cytokines.

REFERENCES


ANTIVIRAL EFFECT OF CAPPARIS SPINOSA

