



# Inhibition of herpes simplex type 1 and type 2 infections by Oximacro<sup>®</sup>, a cranberry extract with a high content of A-type proanthocyanidins (PACs-A)



Maria Elena Terlizzi<sup>a,1</sup>, Andrea Occhipinti<sup>b,c,1</sup>, Anna Lukanini<sup>a</sup>, Massimo E. Maffei<sup>b,c</sup>,  
Giorgio Gribaudo<sup>a,\*</sup>

<sup>a</sup> Microbiology and Virology Unit, Department of Life Science and Systems Biology, University of Turin, 10123 Turin, Italy

<sup>b</sup> Plant Physiology Unit, Department of Life Science and Systems Biology, University of Turin, 10123 Turin, Italy

<sup>c</sup> Biosfered S.r.l., 10135 Turin, Italy

## ARTICLE INFO

### Article history:

Received 29 February 2016

Received in revised form

7 June 2016

Accepted 13 June 2016

Available online 16 June 2016

### Keywords:

Herpes simplex virus 1

Herpes simplex virus 2

Antiviral activity

Microbicides

*Vaccinium macrocarpon* (cranberry) extract

A-type proanthocyanidins

## ABSTRACT

In the absence of efficient preventive vaccines, topical microbicides offer an attractive alternative in the prevention of Herpes simplex type 1 (HSV-1) and type 2 (HSV-2) infections. Because of their recognized anti-adhesive activity against bacterial pathogens, cranberry (*Vaccinium macrocarpon* Ait.) extracts may represent a natural source of new antiviral microbicides. However, few studies have addressed the applications of cranberry extract as a direct-acting antiviral agent. Here, we report on the ability of the novel cranberry extract Oximacro<sup>®</sup> and its purified A-type proanthocyanidins (PACs-A), to inhibit HSV-1 and HSV-2 replication *in vitro*. Analysis of the mode of action revealed that Oximacro<sup>®</sup> prevents adsorption of HSV-1 and HSV-2 to target cells. Further mechanistic studies confirmed that Oximacro<sup>®</sup> and its PACs-A target the viral envelope glycoproteins gD and gB, thus resulting in the loss of infectivity of HSV particles. Moreover, Oximacro<sup>®</sup> completely retained its anti-HSV activity even at acidic pHs (3.0 and 4.0) and in the presence of 10% human serum proteins; conditions that mimic the physiological properties of the vagina - a potential therapeutic location for Oximacro<sup>®</sup>.

Taken together, these findings indicate Oximacro<sup>®</sup> as an attractive candidate for the development of novel microbicides of natural origin for the prevention of HSV infections.

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## 1. Introduction

Herpes simplex virus (HSV) infection is lifelong and its spectrum of clinical manifestations is wide, ranging from asymptomatic infection or mild mucocutaneous lesions on the lips, cornea, genitals, or skin, up to more severe, and even life-threatening, infections, including encephalitis, neonatal infections, and progressive or visceral disease in immunocompromised hosts (Roizman et al., 2013). Following primary infection, HSV establishes latent infections in the neurons of the sensory ganglia from where they may, or may not, reactivate, causing recurrent lesions at the

site of primary infection. There are two serotypes of HSV, HSV-1 and HSV-2, which can infect either oral or genital sites. HSV-1 is traditionally associated with orofacial lesions and encephalitis, while HSV-2 is associated with genital diseases, although both oral HSV-2 infections and genital herpes caused by HSV-1 are recognized with increasing frequency. Even if HSV infections are often subclinical, their incidence and severity have increased over the past decades due to the increasing number of immunocompromised patients (Roizman et al., 2013); with genital herpes infection becoming one of the world's most prevalent sexually transmitted infections (STIs). Moreover, the impact of genital herpes as a public health threat is amplified because of its association with an increased risk of HIV acquisition (Freeman et al., 2006; Van de Perre et al., 2008; Celum et al., 2010).

Standard treatment of symptomatic HSV infections relies on nucleoside analogues, such as acyclovir (ACV), famciclovir (FAM), and valacyclovir (VCV), which target viral DNA polymerase (Whitley, 2006; Roizman et al., 2013). These drugs can be used to

\* Corresponding author. Department of Life Science and System Biology, University of Turin, Via Accademia Albertina 13, 10123 Turin, Italy.

E-mail addresses: [mariaelena.terlizzi@unito.it](mailto:mariaelena.terlizzi@unito.it) (M.E. Terlizzi), [andrea.occhipinti@unito.it](mailto:andrea.occhipinti@unito.it) (A. Occhipinti), [anna.lukanini@unito.it](mailto:anna.lukanini@unito.it) (A. Lukanini), [massimo.maffei@unito.it](mailto:massimo.maffei@unito.it) (M.E. Maffei), [giorgio.gribaudo@unito.it](mailto:giorgio.gribaudo@unito.it) (G. Gribaudo).

<sup>1</sup> M.E.T. and A.O. contributed equally to this work.

treat primary or recurrent infections. However, to date, none of them can eliminate an established latent infection, and their prolonged clinical use in immunocompromised patients may lead to the incidence of treatment failures due to the development of antiviral-resistant virus strains (Whitley, 2006). Owing to these limitations and the absence of efficacious vaccines, the prevention of HSV infections, in particular genital herpes, thus remains a high priority. These facts highlight need for the development of new anti-HSV agents that may prevent the establishment of infection by inhibiting virus attachment and/or entry (Greco et al., 2007). Molecules with this mode of action could thus provide the starting point for the development of topical microbicides that block transmission at the mucosal surface, thereby providing a realistic method of prophylactic intervention (Keller et al., 2005; Nikolic and Pignet, 2010).

Natural products continue to provide an abundant and successful source of new antimicrobial substances. Among them, products extracted from the fruit of American cranberry (*Vaccinium macrocarpon* Ait., *Ericaceae*), in different formulations, are rich in compounds that have been implicated to exert numerous health benefits, like the prevention of microbial infections and beneficial activity against inflammation (Howell, 2007; Howell et al., 2010; Shmueli et al., 2012; Rane et al., 2014). Indeed, it is well established that cranberry-derived polyphenols are able to prevent urinary tract infections (UTI) by inhibiting the adhesion of P-fimbriated *Escherichia coli* to uroepithelial cells, thus impairing tissue colonization and subsequent infection (Zafiri et al., 1989; Howell et al., 2005; Gupta et al., 2007; Jepson and Craig, 2008; Blumberg et al., 2013; Krueger et al., 2013). This effect appears to be related to the specific phytochemical profile of bioactive compounds from cranberries, that is different from that of other berry fruits; in particular, cranberries are rich in A-type proanthocyanidins (PACs), whereas B-type PACs are present in most of other fruits (Blumberg et al., 2013). Indeed A-type PACs have been observed to mask P-fimbriae during *E. coli* adhesion to uroepithelial cells, and are therefore considered potent antiadhesion agents (Howell et al., 2005; Gupta et al., 2007; Krueger et al., 2013).

To date, very few studies have addressed the suitability of cranberry extracts as antiviral agents (Shmueli et al., 2012), despite the existence of *in vitro* evidence demonstrating their inhibitory activity against influenza virus (Weiss et al., 2005; Oiknine-Djian et al., 2012), reovirus (Lipson et al., 2007a, 2007b), and enterovirus (Su et al., 2010). It is clear that identification of the active antiviral component(s) in cranberry extracts and the determination of their mechanism(s) of action against a specific virus are required in order to propose this fruit extract as a candidate antiviral agent. However, isolation of the cranberry component(s) exerting antiviral activity, in particular on a large scale, is a daunting task, considering the hundreds of compounds found in the fruit. Thus, once the mechanism(s) of action against a virus is determined on an analytical scale and assigned to a specific cranberry component(s), an alternative strategy would be to improve the extraction procedures such that products endowed with high concentrations of the active antiviral component(s) are obtained; this would avoid the cost of further purification procedures, making the large-scale production of extracts more feasible.

Oximacro<sup>®</sup>, a cranberry extract produced by Biosfered (Turin, Italy), possesses a high content of PACs and a high percentage of A-type PAC dimers and trimers. In a recent pre-clinical double-blind controlled study, Oximacro<sup>®</sup> was effective in preventing UTIs when administered as capsules (Occhipinti et al., 2016).

The aim of this study was to investigate the anti-HSV activity of Oximacro<sup>®</sup>. Here we show the ability of Oximacro<sup>®</sup> to prevent *in vitro* HSV-1 and HSV-2 replication via a mechanism that involves inhibition of virion attachment due to functional alteration of

envelope glycoproteins. The A-type PACs purified from Oximacro<sup>®</sup> were identified as the active anti-HSV constituents of the extract. These results indicate Oximacro<sup>®</sup> as a promising natural candidate for the development of novel topical microbicides for the prevention of HSV-1 and HSV-2 infections.

## 2. Materials and methods

### 2.1. Characterization of the cranberry extract and fractionation of PACs-A

Oximacro<sup>®</sup>, a cranberry (*Vaccinium macrocarpon* Aiton) extract, produced by Biosfered S.r.l. (Turin, Italy), is a reddish powder with a total PAC content > 360 mg/g (Lot # CR0105-PD04). The CoA of the lot can be provided upon request. The PAC-A2 standard was obtained from Extrasynthese (France) and dissolved in 96% v/v ethanol (Sigma-Aldrich, USA) to generate a final concentration of 100 µg/ml. Aliquots of stock solutions were stored in 1.5 ml HPLC vials at –80 °C until use. The chemical purity and integrity of standard compound was assessed prior to use (see below).

To determine the total PAC content, the BL-DMAC assay was performed according to the method described by Prior et al. (2010). Total proanthocyanidins were quantified using an external calibration curve generated using the pure PAC-A2 standard. The quantification was performed in triplicate within the linear range of the calibration curve (5–30 µg/ml). Oximacro<sup>®</sup> was then assayed exactly at 20 min, which was demonstrated to be the optimal timing for PAC-A quantification (Occhipinti et al., 2016).

PAC-A and PAC-B content of Oximacro<sup>®</sup> was determined by HPLC-ESI-MS/MS as previously described (Occhipinti et al., 2016). The identification of PACs (dimers and trimers) was performed via multiple reaction monitoring (MRM) mass spectrometry of the following molecular ions [M-H]<sup>-</sup>: 575 *m/z* for A-type dimers and 577 *m/z* for B-type dimers; 861 and 863 *m/z* for A-type trimers; and 865 *m/z* for B-type trimers.

To fractionate Oximacro<sup>®</sup> by gel filtration chromatography, 1 ml of Oximacro<sup>®</sup> solution in 70% v/v ethanol (0.2 g/ml) was loaded onto a Sephadex LH-20 (25 g) glass column (2.0 cm I.D. x 31 cm length) and fractionation was performed as described by Prior et al. (2001, 2010). The collected fractions were concentrated at 30 °C using a Centrifugal Vacuum Concentrator combined with the CentriVap Cold Trap (Labconco, USA) and freeze-dried before further analyses. Total recoveries were calculated based on the weight of the extract applied onto the column and the total weight in each of the freeze-dried fractions from the Sephadex LH-20 column. Evaluation of PAC-A content of purified fractions was performed as above.

### 2.2. Cells, culture conditions and viruses

African green monkey kidney cells (Vero) (ATCC CCL-81) and low-passage primary human foreskin fibroblasts (HFFs; passages 10 to 15) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Biowest) supplemented with 10% fetal bovine serum (FBS; Biowest), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 µg/ml streptomycin sulfate. Clinical isolates of HSV-1 and HSV-2 sensitive to ACV, and a clinical isolate of Adenovirus (ADV) were kindly provided by Dr. V. Ghisetti, from Amedeo di Savoia Hospital, Turin, Italy. HSV-1 and HSV-2 were propagated and titrated by plaque reduction assay on Vero cells. ADV was propagated and titrated on HFFs as previously described (Luganini et al., 2010). To obtain highly concentrated virus suspensions, extracellular HSV particles were partially purified by ultracentrifugation through a sorbitol cushion as previously described (Bronzini et al., 2012).

### 2.3. Antiviral assays

To determine cell viability, Vero or HFF cells were exposed to increasing concentrations of Oximacro<sup>®</sup> or its purified fractions (1–5; obtained as described in 2.1). After 3 days of incubation, the number of viable cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method, as previously described (Pauwels et al., 1988).

To evaluate the anti-HSV activity of Oximacro<sup>®</sup> and its purified fractions, Vero cells were seeded in 96-well plates at a density of  $25 \times 10^3$  cells. After 24 h, cells were treated with different concentrations of either Oximacro<sup>®</sup> or its fractions 1 h prior to infection, and then infected with HSV-1 or HSV-2 (30 PFU/well). Following virus adsorption (2 h at 37 °C), cultures were maintained in medium-containing 0.8% methylcellulose (Sigma) plus Oximacro<sup>®</sup> or its fractions. At 48 h postinfection (h.p.i.), cells were fixed and stained by using 10% methanol and 1% crystal violet. Plaques were microscopically counted, and the mean plaque counts for each concentration expressed as a percentage of the mean plaque count for the control virus. The number of plaques was plotted as a function of drug concentration; concentrations producing 50% and 90% reductions in plaque formation (IC<sub>50</sub> and IC<sub>90</sub>) were determined.

ADV viral yield assay was performed as described previously (Luganini et al., 2010).

Viral attachment and entry assays were performed as previously described (Shogan et al., 2006; Luganini et al., 2011). The effect of Oximacro<sup>®</sup> on viral infectivity, its stability at different pHs and the effect of human serum proteins on Oximacro<sup>®</sup> antiviral activity were performed according to the procedures described in Luganini et al. (2011).

### 2.4. Immunoblotting

Whole-cell extracts were prepared as previously described (Luganini et al., 2008, 2011). Proteins were separated by 8% SDS-PAGE and then transferred to PVDF membranes (BioRad). Filters were blocked for 2 h at 37 °C in 5% non-fat dry milk in 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 0.05% Tween 20 and then immunostained with the mouse anti-HSV-1/2 ICP27 mAb (clone H1113; Virusys), anti-HSV-2 ICP8 mAb (clone 4E6; Virusys), anti-HSV-1 ICP8 mAb (clone 10A3; Abcam), anti-HSV-1/2 gD mAb (clone 2C10; Virusys), anti-HSV-1/2 gB mAb (clone 10B7; Virusys Corporation), or with anti-tubulin mAb (Chemicon International) as a control for protein loading. Immunocomplexes were detected with a goat anti-mouse Ig Ab conjugated to horseradish peroxidase (Life Technologies) and visualized by enhanced chemiluminescence (Western Blotting Luminol Reagent, Santa Cruz).

To study the interaction of Oximacro<sup>®</sup> with HSV envelope proteins, aliquots of partially purified HSV-1 and HSV-2 particles or purified recombinant HSV-1 and HSV-2 gD ectodomain (1–306 aa) produced in the insect cell-baculovirus expression system (a generous gift of G. Cohen and R. Eisenberg) (Sisk et al., 1994) were incubated at 37 °C with Oximacro<sup>®</sup>. Then, mixtures were suspended in SDS sample buffer, separated by SDS-PAGE, and analyzed by Coomassie blue staining or immunoblotting using the mouse anti-HSV-1/2 gB and anti-HSV-1/2 gD mAbs, or the goat anti-VP16 antibody (sc-17547, Santa Cruz).

### 2.5. Immunofluorescence

Immunofluorescence analysis of viral antigens was performed as previously described (Cavaletto et al., 2015) using the mouse mAbs raised against the ectodomains of both HSV-1/2 gD and HSV-1/2 gB. The binding of primary antibodies was detected with

CF594-conjugated rabbit anti-mouse IgG antibodies (Sigma) Samples were examined using an Olympus IX70 inverted laser scanning confocal microscope, and images were captured using FluoView 300 software (Olympus Biosystems).

### 2.6. Statistical analysis

Chemical analyses were performed in triplicate. All other data were generated in duplicate in at least three independent experiments. PRISM software version 5.0 (GraphPad Software, Inc.) was used for statistical analysis (one-way ANOVA test) and to calculate IC<sub>50</sub>, IC<sub>90</sub>, and CC<sub>50</sub> parameters.

## 3. Results

### 3.1. Chemical analysis of Oximacro<sup>®</sup> confirms a high content of A-type proanthocyanidins

The presence of A-type PACs in cranberry extract is central to its bioactivity. Analysis using the BL-DMAC method by showed a total PAC content of 366.06 mg/g ( $\pm 4.96$ ) PACs. Although the BL-DMAC method is generally recognized as the most accurate (Prior et al., 2010), it detects both PACs-A and PACs-B. Thus, we also subjected Oximacro<sup>®</sup> to HPLC followed by electrospray ionization and tandem mass spectrometry (ESI-MS/MS) to assess the relative content of the two PAC types. The percentage content of PAC-A in Oximacro<sup>®</sup> was 86.72% ( $\pm 1.65$ ); mainly composed of PAC-A dimers and small amounts of PAC-A trimers. The percentage of PAC-B dimers was 13.99% ( $\pm 1.03$ ), whereas no PAC-B trimers were detected (Supplementary Fig. S1). The percentage content of PAC polymers was below the threshold of detection.

Gel filtration chromatography allowed us to fractionate Oximacro<sup>®</sup> into five major fractions which were chemically characterized and showed to contain anthocyanins, flavonoids, and PACs dimers and trimers. In particular, fraction 1 was mainly composed of delphinidin-3-sambubioside, cyanidin.3.sambubioside, and rutin; fraction 2 contained quercetin and isorhamnetin; fractions 3 and 4 were dominated by several isomers of PAC-A dimers and trimers, whereas fraction 5 did not contain any detectable compound (see Supplementary Fig. S1 and Table S1). The Oximacro<sup>®</sup> whole extract and its purified fractions were then used to investigate their antiviral activity.

### 3.2. Inhibition of HSV-1 and HSV-2 replication by Oximacro<sup>®</sup>

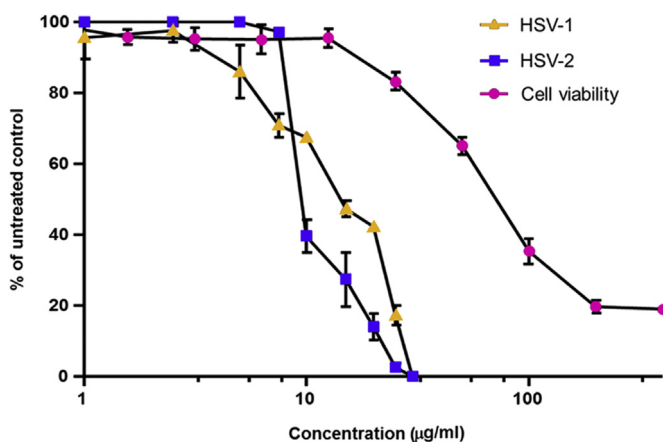
Pretreatment of Vero cells with Oximacro<sup>®</sup> 1 h before infection produced a significant concentration-dependent inhibition of both clinical isolates, HSV-1 and HSV-2 (Fig. 1). The IC<sub>50</sub> and IC<sub>90</sub> of Oximacro<sup>®</sup> against HSV-1 replication were  $14.2 \pm 0.5$   $\mu\text{g/ml}$  and  $27.1 \pm 1.0$   $\mu\text{g/ml}$ , respectively, whereas against HSV-2, they were  $9.6 \pm 0.2$   $\mu\text{g/ml}$  and  $21.7 \pm 0.1$   $\mu\text{g/ml}$ . For comparison, in the same assay, the IC<sub>50</sub> of the reference drug ACV were 0.01  $\mu\text{g/ml}$  against HSV-1 and 0.02 against HSV-2, respectively. Then, to exclude the possibility that the antiviral activity of Oximacro<sup>®</sup> might be due to cytotoxicity, its effects on the viability of uninfected Vero cells were assessed using MTT assays. As seen in Fig. 1, its antiviral activity was not due to cytotoxicity of the target cells themselves since a significant toxic effect was only observed at concentrations higher than 50  $\mu\text{g/ml}$  (CC<sub>50</sub>  $92.3 \pm 2.2$   $\mu\text{g/ml}$ ). The Selectivity Index (SI) of Oximacro<sup>®</sup> was thus 6.5 for HSV-1 and 9.6 for HSV-2. In contrast, the replication of a clinical isolate of adenovirus in HFFs cells was not significantly affected by Oximacro<sup>®</sup>, with its IC<sub>50</sub> > 75  $\mu\text{g/ml}$ , thus supporting its specificity against herpesviruses.

Analysis of the anti-HSV activity of Oximacro<sup>®</sup>-derived purified fractions identified fractions 3 and 4 as responsible for the

inhibitory activity of the whole extract (Table 1). The IC<sub>50</sub> for both fractions against HSV-2 were 2.5-fold lower than those against HSV-1, thus suggesting a higher sensitivity of HSV-2 to the antiviral activity of Oximacro<sup>®</sup> compared with that of HSV-1. Considering that A-type PACs were separated in fractions 3 and 4 of Oximacro<sup>®</sup> (fraction 3 = 400 mg/g; fraction 4 = 420 mg/g) (also see Supplementary Fig. S1 and Table S1), these results indicate that the anti-HSV activity of Oximacro<sup>®</sup> indeed is due to its A-type PAC content.

### 3.3. Oximacro<sup>®</sup> inhibits an immediate-early event in the HSV replication cycle

To obtain more insight into the nature of the anti-HSV activity of Oximacro<sup>®</sup>, we investigated its effects on the gene expression program in both HSV-1 and HSV-2. To this end, total protein cell extracts were prepared from HSV-1- and HSV-2-infected Vero cells treated with Oximacro<sup>®</sup> for various lengths of time post-infection. The expression levels of ICP27, ICP8, and gD were then examined by immunoblotting with specific antibodies to assess the levels of immediate-early, early and late HSV protein expression, respectively. As depicted in Fig. 2, Oximacro<sup>®</sup> inhibited the expression of all representative HSV proteins at all of the time points analyzed, thus indicating that it affects a very early stage in the HSV replication cycle, i.e. a stage prior to the onset of IE gene expression. Consistent with this observation, the addition of Oximacro<sup>®</sup> after 2 h of virus adsorption did not significantly reduce HSV-1 and HSV-2 replication, in stark contrast with the antiviral activity observed when it was applied up to 1 h prior to or at the time of infection (data not shown). These results support the view that Oximacro<sup>®</sup> targets a very early phase of the HSV cycle, such as virus adsorption and/or entry.



**Fig. 1.** Antiviral activity of Oximacro<sup>®</sup> on HSV-1 and HSV-2 replication. Vero cell monolayers were infected with clinical isolates of either HSV-1 or HSV-2 (30 PFU/well), and, where indicated, the cells were treated with increasing concentrations of Oximacro<sup>®</sup> 1 h before as well as during virus adsorption, and which remained in the culture media throughout the experiment. At 48 h p.i., viral plaques were microscopically counted and the mean plaque counts for each drug concentration were expressed as a percent of the mean count of the control. The number of plaques was plotted as a function of Oximacro<sup>®</sup> concentration, and the concentrations producing 50 and 90% reductions in plaque formation (IC<sub>50</sub> and IC<sub>90</sub>, respectively) were determined. The data shown represent means ± SD (error bars) of three independent experiments performed in duplicate. To determine cell viability, Vero cells were exposed to increasing concentrations of Oximacro<sup>®</sup>. After 3 days of incubation, the number of viable cells was determined by means of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method.

### 3.4. Oximacro<sup>®</sup> inhibits HSV attachment to target cells

To investigate whether the inhibitory activity of Oximacro<sup>®</sup> is due to interference with HSV entry into cells, prechilled Vero monolayers were infected with the clinical isolates of HSV-1 or of HSV-2 for 3 h at 4 °C. Oximacro<sup>®</sup> or heparin (as a positive control for inhibition of virus attachment) was then added and the cells were incubated at 37 °C to allow viral entry. These experimental conditions allow for the synchronization of virus penetration following attachment at low temperature (Shogan et al., 2006; Luginini et al., 2011). After 3 h at 37 °C, any HSV virion still attached to the cell surface was inactivated by acidic glycine treatment. The cells were then overlaid with 0.8% methylcellulose to measure the infectivity of HSV that had successfully entered into cells. As shown in Fig. 3A, Oximacro<sup>®</sup> did not affect HSV-1 or HSV-2 entry of already adsorbed virions at any of examined concentrations.

Then, to test the effects of Oximacro<sup>®</sup> on HSV attachment, prechilled Vero cell monolayers were infected with HSV-1 or HSV-2 in the presence of Oximacro<sup>®</sup> or heparin for 2 h at 4 °C (a condition that is known to allow virus adsorption only). Cells were washed to remove the compounds and any unattached virus and covered with a solution of 0.8% methylcellulose in order to measure the infectivity of HSV that had successfully attached onto the cells. As shown in Fig. 3B, Oximacro<sup>®</sup> clearly impaired the attachment of HSV in a concentration-dependent manner and to a similar degree as observed in the virus yield reduction assay (Fig. 1). As expected, heparin blocked the ability of HSV-1 to attach to Vero cells by preventing the virus from interacting with cell surface heparan sulfate proteoglycans (Shieh et al., 1992).

Altogether, these findings indicate that Oximacro<sup>®</sup> was able to inhibit the initial step of HSV attachment to target cells.

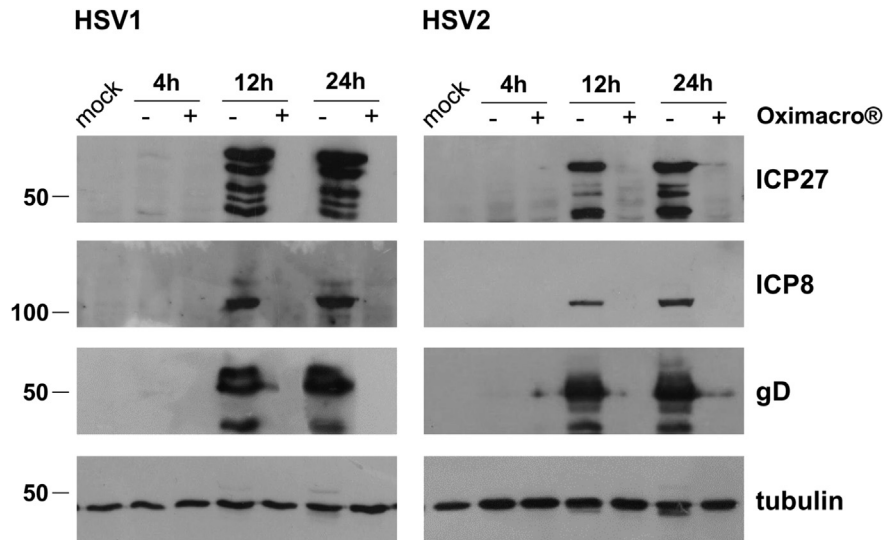
### 3.5. Virucidal activity of Oximacro<sup>®</sup> against HSV

Since inhibition of HSV attachment might result from an irreversible Oximacro<sup>®</sup>-induced inactivation of the virions, we investigated whether Oximacro<sup>®</sup> was able to interact with HSV particles and thus to inactivate their infectivity prior to their adsorption onto target cells. To this end, HSV-1 and HSV-2 aliquots were incubated with Oximacro<sup>®</sup> at 37 °C for various lengths of time. After incubation, the samples were diluted to reduce the Oximacro<sup>®</sup> concentrations well below those that inhibit HSV replication (0.025 µg/ml), and the residual infectivity of pre-incubated virions was titrated on Vero cells. As seen in Fig. 4, the pre-incubation of virions with Oximacro<sup>®</sup> brought about a complete loss of HSV infectivity within 120 min for HSV-2, and 180 min for HSV-1, respectively. Similar results were also obtained with fraction 4, the richest in A-type PACs (data not shown). Thus, these results demonstrate that Oximacro<sup>®</sup> inhibits HSV infection by preventing the ability of viral particles to attach to target cells.

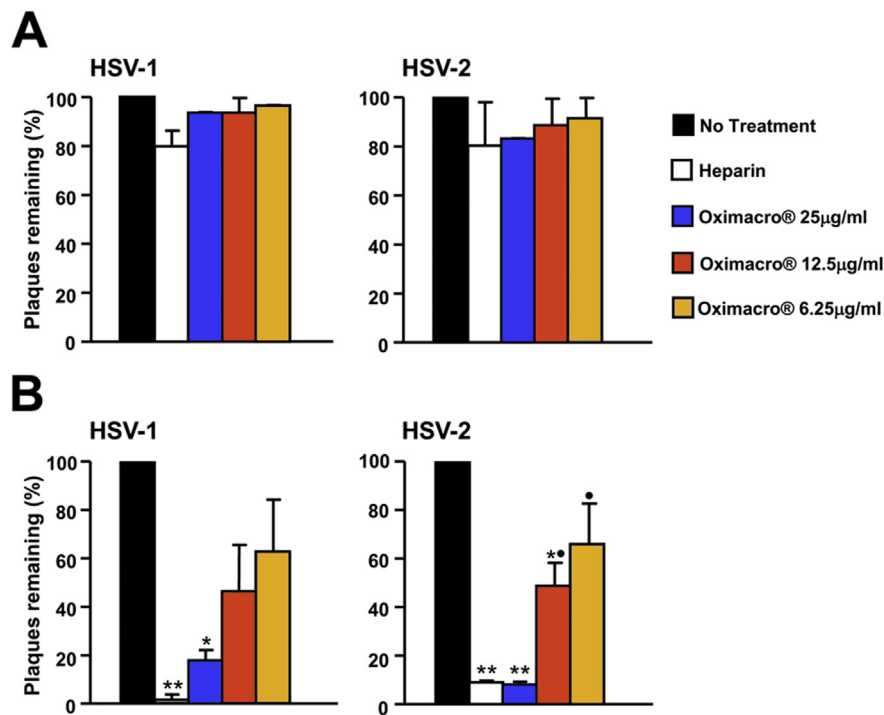
**Table 1**  
Antiviral activity of Oximacro<sup>®</sup> fractions.

Fraction	Cytotoxicity activity (CC <sub>50</sub> µg/ml)	Antiviral activity <sup>a</sup> (IC <sub>50</sub> µg/ml)	
		HSV-1	HSV-2
1	>200	>75	>75
2	>200	>75	>75
3	188.2 ± 2.3	19.8 ± 3.1	6.8 ± 3.2
4	106.9 ± 1.1	19.2 ± 3.7	7.6 ± 2.2
5	>200	>75	>75

<sup>a</sup> Oximacro<sup>®</sup> concentration that inhibits 50% HSV replication as determined by a plaque reduction assay. The values are means ± SD of three independent experiments performed in duplicate.



**Fig. 2. Oximacro® inhibits accumulation of representative HSV proteins.** Vero cells were infected with HSV-1 or HSV-2 at an MOI of 1, or mock infected and, where indicated, the cells were pretreated and treated with 25  $\mu\text{g/ml}$  Oximacro® 1 h prior to and during infection. Total cell extracts were prepared at different times p.i., fractionated by 8% SDS-PAGE (50  $\mu\text{g}$  protein/lane), and analyzed by immunoblotting with anti-HSV-1/2 ICP27, anti-HSV-1/2 ICP8, and anti-HSV-1/2 gD. Tubulin immunodetection served as internal control for protein loading.



**Fig. 3. HSV attachment to target cells is prevented by Oximacro®.** (A) Oximacro® does not affect viral entry after virus adsorption. Prechilled Vero cells were infected with precooled HSV-1 or HSV-2 at an MOI of 0.002 for 3 h at 4 °C to allow virion attachment to cells. Unattached virus was removed by washing and cells were treated with various concentrations of Oximacro® or heparin for 3 h at 37 °C prior to inactivation of extracellular virus with acidic glycine for 2 min at RT. After further washing, cells were covered with 0.8% methylcellulose containing medium. At 48 h p.i., viral plaques were stained and counted. The results shown are means  $\pm$  SD (error bars) from three independent experiments performed in duplicate. (B) Oximacro® inhibits HSV adsorption to target cells. Prechilled Vero cells were treated with various concentrations of Oximacro®, or heparin at 4 °C for 30 min and then infection was carried out with precooled HSV-1 or HSV-2 at a MOI of 0.002 for 3 h at 4 °C in the presence of the compounds indicated. After virus adsorption, cells were overlaid with 0.8% methylcellulose and incubated at 37 °C. At 48 h p.i., viral plaques were stained and counted. The results shown are means  $\pm$  SD (error bars) of three independent experiments performed in duplicate. \*\* ( $p < 0.01$ ) and \* ( $p < 0.05$ ) compared to the 100% infection of untreated cells; ● ( $p < 0.05$ ) compared to the higher dose of Oximacro® (25  $\mu\text{g/ml}$ ).

### 3.6. Oximacro® affects HSV glycoproteins required for binding to cell receptors and entry

The entry process of HSV begins with the binding of gD

glycoprotein to specific cell surface receptors and concludes with the fusion of the viral envelope with cell membranes and delivery of nucleocapsids into target cells upon gB activation and fusion execution. gD and gB, together with gH and gL, thus constitute

essential components of the multipartite system that mediates HSV entry (Campadelli-Fiume et al., 2012; Einsenberg et al., 2012). Since we observed an inhibition of HSV virions infectivity after their incubation with Oximacro<sup>®</sup> (Fig. 4), we proceeded by investigating whether Oximacro<sup>®</sup> affects gD and gB, thereby inhibiting their functions in attachment and entry. As shown in Fig. 5, the gD and gB of untreated HSV virions migrated with molecular weights of about 55–60 and 130 kDa, respectively. However, incubation at 37 °C of both HSV-1 and HSV-2 with Oximacro<sup>®</sup> for different times determined alterations of the electrophoretic mobility of both gD and gB (Fig. 5). In particular, after 30 min incubation with Oximacro<sup>®</sup>, HSV-1 gB was detected as a smear consisting of gB molecules with a lower electrophoretic mobility compared with the untreated control, while at longer incubation times the gB band completely disappeared. The smearing pattern of gB was even more visible when HSV-2 virions were incubated with Oximacro<sup>®</sup> for up to 2 h, followed by the complete disappearance of the gB band at 3 h (Fig. 5). A similar pattern was observed for gD of both HSV types. Alteration of the electrophoretic mobility and the subsequent disappearance of HSV gD and gB bands were also observed following incubation of purified virions with fraction 4 (data not shown). Moreover, immunodetection of the tegument protein VP16 used as a control for an inner virion protein was not significantly affected by the exposure of viral particles to Oximacro<sup>®</sup>.

The reduction of the major gD and gB bands and their disappearance at longer incubation times likely resulted from virion-bound Oximacro<sup>®</sup> interfering with the recognition of their ectodomains by mAbs during immunoblotting. A similar masking effect has been observed for pomegranate extracts rich in polyphenols that reduced the binding of HA- and NA-specific mAbs to influenza virus particles incubated with the extracts prior to the addition of antibodies (Sundararajana et al., 2010).

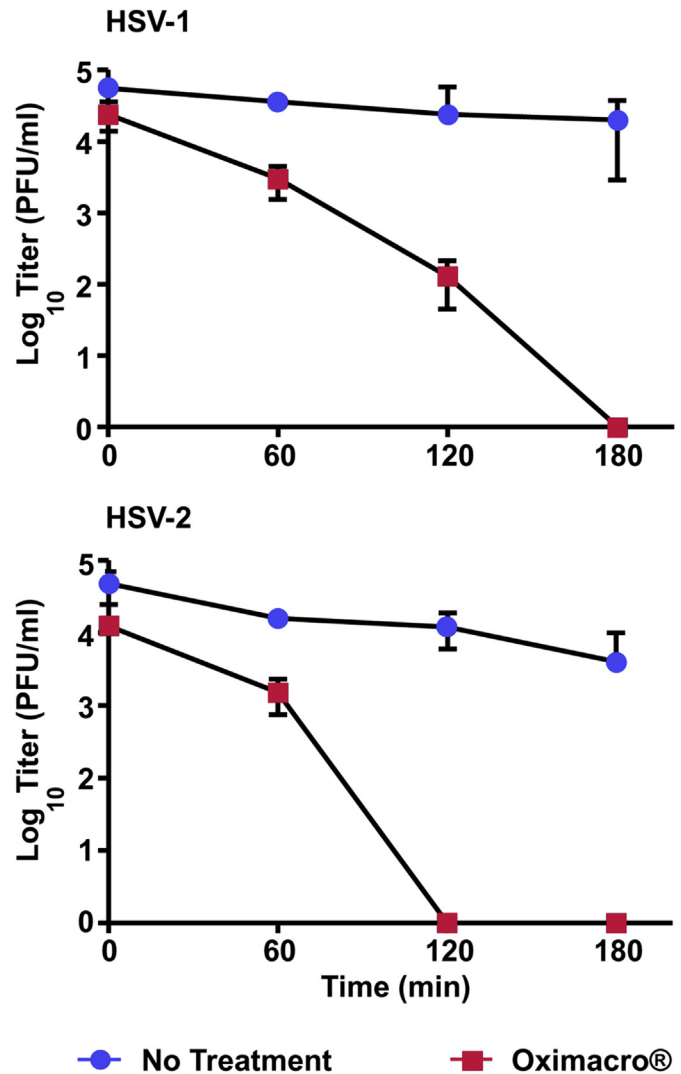
Immunofluorescence experiments were thus performed to verify that Oximacro<sup>®</sup> interferes with the immunodetection of gD and gB ectodomains expressed on the surface of infected cells. Fig. 6 shows that incubation of HSV-infected Vero cells with either Oximacro<sup>®</sup> or fraction 4 almost completely inhibited the immunostaining of gD and gB on their surface.

Then, to further sustain the ability of Oximacro<sup>®</sup> to interact with the ectodomain of HSV envelope glycoproteins, aliquots of purified recombinant HSV-1 and HSV-2 gD protein's ectodomains (1–306 aa) produced in baculovirus expression system (Sisk et al., 1994) (Fig. 7A) were incubated at 37 °C with increasing concentration of Oximacro<sup>®</sup>. As shown in Fig. 7B, exposure of purified gD to the extract led to a reduction of the major protein band and to its disappearance at the highest Oximacro<sup>®</sup> concentrations. Moreover, as seen in Fig. 7C, incubation of recombinant gD with Oximacro<sup>®</sup> for different times produced the disappearance of the gD band at longer incubation times similar to that observed with HSV viral particles (Fig. 5), thus indicating that Oximacro<sup>®</sup> interacts with the ectodomain of HSV gD in a concentration- and time-dependent manner.

Together the results of this section indicate the ability of Oximacro<sup>®</sup> to interact with HSV envelope gD and gB glycoproteins, thus inhibiting their functions in virus attachment and entry.

### 3.7. Activity of Oximacro<sup>®</sup> at different pH or in the presence of human serum proteins

The results of the above experiments addressing the mechanism of action of Oximacro<sup>®</sup> pointed to its ability to prevent HSV adsorption. However, to evaluate Oximacro<sup>®</sup> as possible candidate for the development of topical microbicides for preventing the sexual transmission of HSV-1 and HSV-2, it is fundamental that the influence of specific physiological properties of the vagina, such as

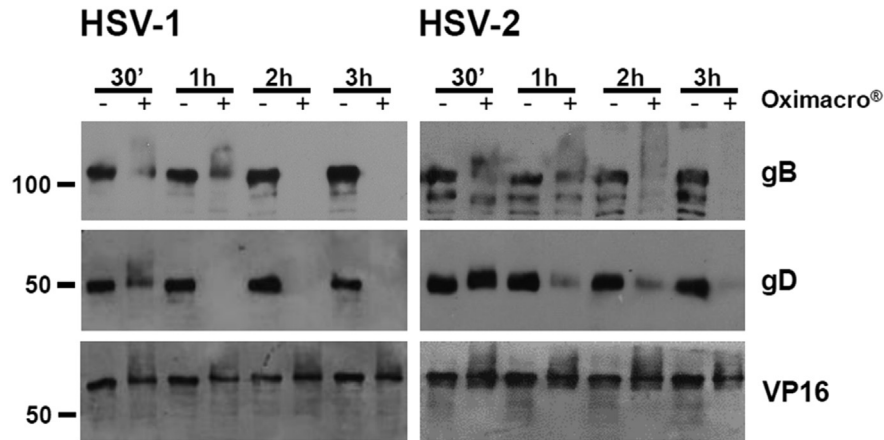


**Fig. 4. Preincubation of HSV with Oximacro<sup>®</sup> inhibits virus infectivity.** HSV-1 and HSV-2 aliquots ( $10^5$  PFU) were incubated at 37 °C for various lengths of time with no Oximacro<sup>®</sup> (closed circle) or 25  $\mu$ g/ml Oximacro<sup>®</sup> (closed squares). After incubation, the samples were diluted to reduce Oximacro<sup>®</sup> concentration below that which inhibits HSV replication (0.025  $\mu$ g/ml) and the resulting infectivity was evaluated by titration on Vero cells. Plaques were microscopically counted, and the mean plaque counts for each Oximacro<sup>®</sup> concentration expressed as PFU/ml on a log<sub>10</sub> scale. The data shown represent means  $\pm$  SD (error bars) of three independent experiments performed in duplicate.

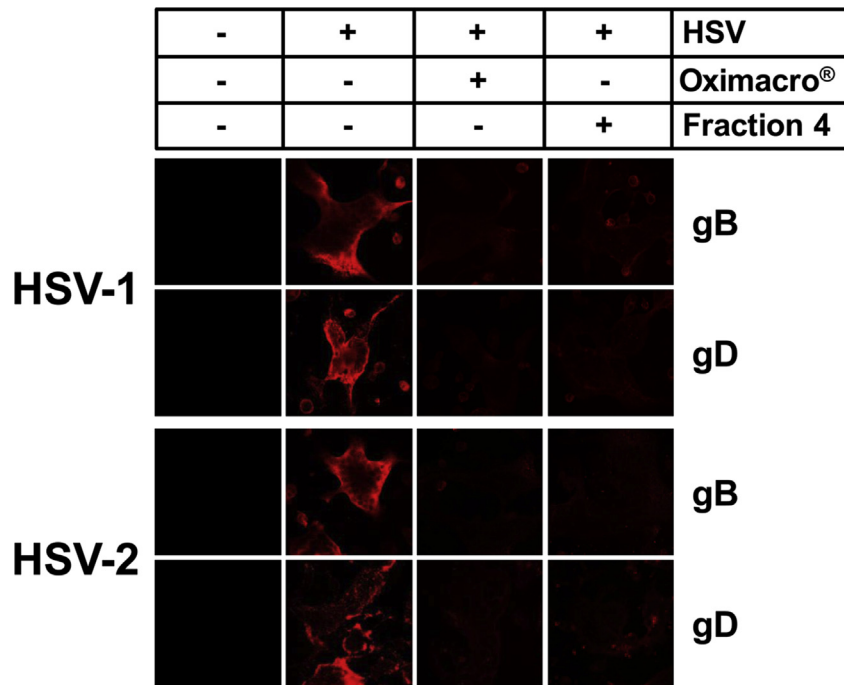
the pH and the presence of proteins, on their antiviral efficacy is also considered.

To examine the stability of Oximacro<sup>®</sup> at different pHs (ranging between 3.0 and 9.0), the extract was incubated in different pH buffers for 2 h at 37 °C and its IC<sub>50</sub> was then determined using plaque reduction assays at neutral pH. As reported in Table 2, acidic treatment (pH 3.0 and 4.0) for 2 h did not affect the stability of Oximacro<sup>®</sup> since its activity against HSV-1 or HSV-2 was similar to that observed for the extract incubated at neutral pH. This confirms that the low pH of the vagina (normal values range from 3.5 to 4.5) would not interfere with the anti-HSV action of Oximacro<sup>®</sup>.

To evaluate the influence of the presence of human proteins on Oximacro<sup>®</sup> antiviral activity, the extract was incubated at 37 °C with 10% human serum from HSV negative donors for 1 or 18 h prior to using it to pretreat and treat cells in the virus yield reduction assay in the presence of 10% human serum protein. As



**Fig. 5.** Oximacro<sup>®</sup> affects the electrophoretic mobility of HSV glycoproteins gD and gB.  $10^7$  purified HSV-1 or HSV-2 PFU were incubated at 37 °C for various lengths of time with medium or 25 µg/ml Oximacro<sup>®</sup>. At 30 min, 1, 2, and 3 h of incubation, virion suspensions were lysed and their protein content analyzed by immunoblotting, using anti-HSV-1/2 gB and anti-HSV-1/2 gD mAbs, or the anti-VP16 antibody. Sizes are indicated in kilodaltons.



**Fig. 6.** Oximacro<sup>®</sup> and its partially purified A-type PACs block antibody recognition of the gD and gB ectodomains exposed on the surface of HSV-infected cells. Vero cells were infected with HSV-1 or HSV-2 at a MOI of 0.5, and at 15 h p.i. cells were left untreated or treated with either Oximacro<sup>®</sup> (25 µg/ml) or fraction 4 (50 µg/ml) for 3 h. Cells were then fixed, left not permeabilized, and then immunostained with mAbs against ectodomains of gD and gB. Results are representative of three independent experiments.

seen in Table 3, Oximacro<sup>®</sup> retained its activity against HSV-1 and HSV-2 in the presence of 10% human serum even after an overnight incubation at 37 °C (Table 2).

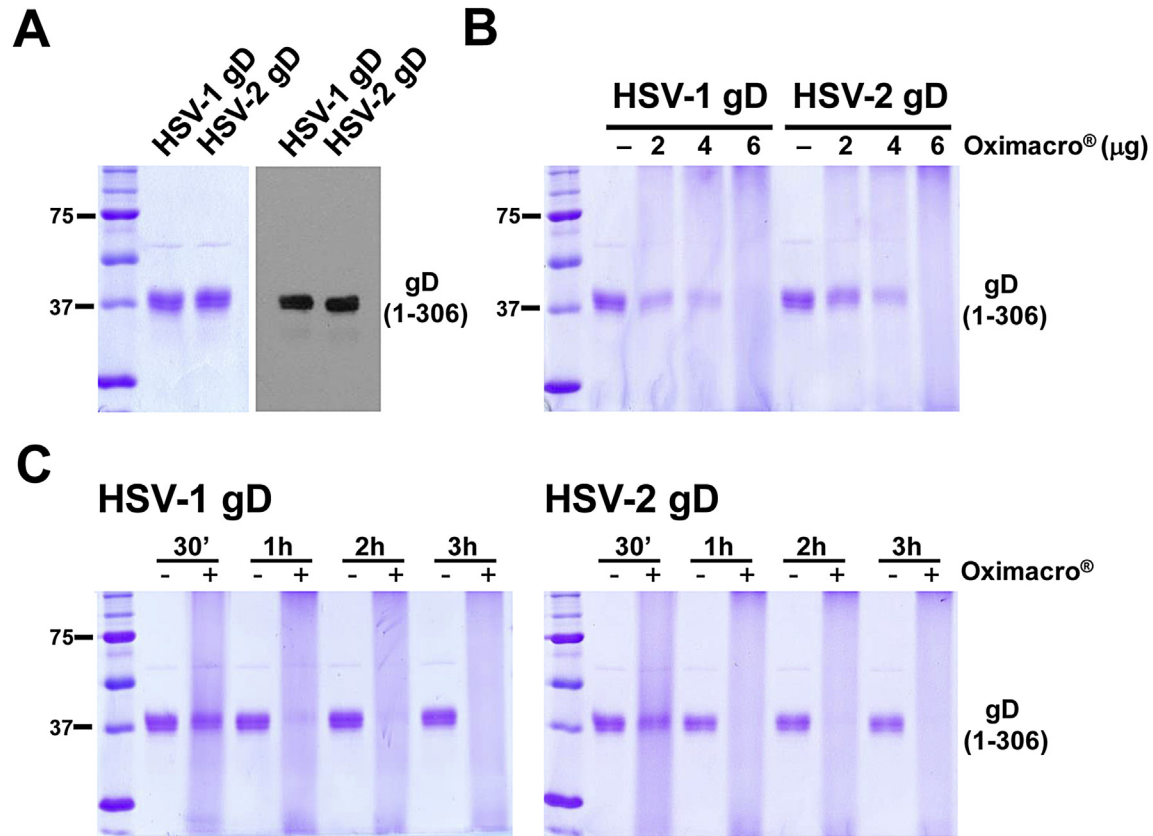
Altogether, these findings indicate that the stability of Oximacro<sup>®</sup> was not affected by parameters which characterize the vaginal environment.

#### 4. Discussion

This report adds to the growing body of knowledge on the antiviral activity of polyphenol-enriched extracts derived from plants. We show for the first time that a cranberry extract highly enriched in A-type PACs exerts potent dose-dependent antiviral activity against clinical isolates of HSV-1 and HSV-2, the

mechanism for which involves the inhibition of the initial virus attachment to the surface of target cells.

Many small molecule phytochemicals, like phenolics, polyphenols, terpenes, and flavonoids from a range of plant species have been reported to exert inhibitory activities on HSV replication (Khan et al., 2005; Son et al., 2013; Hassan et al., 2015). In particular, polyphenols have been found to interfere with the early phases of the HSV replicative cycle and/or with viral particles directly (Khan et al., 2005; Yang et al., 2007; Schnitzer et al., 2008; Xiang et al., 2011). To this regard, it has been reported that polyphenol-enriched extracts from *Rumex acetosa* and *Myrothamnus flabellifolia* exert antiviral activity against HSV-1 through the inhibition of virus attachment (Gescher et al., 2011a, 2011b). The anti-adhesive effect of the *R. acetosa* extract was mainly due to its B-type PAC,



**Fig. 7.** Oximacro<sup>®</sup> interacts with the purified ectodomain of HSV-1 and HSV-2 gD. (A) SDS-PAGE and immunoblot analysis of recombinant HSV-1 and HSV-2 gD ectodomains. Purified proteins were analyzed on 12% SDS-polyacrylamide gels. Gels were stained with Coomassie blue, or the proteins analyzed by immunoblotting with an anti-HSV-1/2 gD mAb. Left panel: Coomassie blue-stained gel of 2  $\mu$ g of purified recombinant HSV gD. Right panel: Immunoblot analysis with of 200 ng of the same samples as in the left panel. (B) Oximacro<sup>®</sup> interacts with recombinant HSV-1 and HSV-2 gD proteins in a concentration- (B) and time-dependent (C) manner. (B) Aliquots of purified recombinant HSV-1 and HSV-2 gD (2  $\mu$ g) were incubated at 37 °C with medium or increasing amounts of Oximacro<sup>®</sup> for 3 h, and then mixtures were analyzed by SDS-PAGE. (C) Aliquots of recombinant gD (2  $\mu$ g) were incubated at 37 °C for various lengths of time with medium or 6  $\mu$ g of Oximacro<sup>®</sup>. At 30 min, 1, 2, and 3 h of incubation, mixtures were analyzed by SDS-PAGE. Gels were then stained with Coomassie blue. Sizes are indicated in kilodaltons. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

epicatechin-3-O-gallate-(4 $\beta$   $\rightarrow$  8)-epicatechin-3-O-gallate, which was shown to produce gD oligodimerization; thus demonstrating

an association between the loss of HSV-1 infectivity and envelope glycoprotein alterations, consistent with B-type PAC interaction with the virion surface (Gescher et al., 2011a). It was thus concluded that the *R. acetosa*-derived PAC-B dimer blocks HSV-1 adsorption by directly interacting with viral particles and causing alterations of the envelope glycoproteins, such as gD, that mediate binding of viral particles to cell receptors.

This view is further sustained by the observation that epigallocatechin-3-gallate (EGCG), a major polyphenol component of green tea extract, is able to inhibit the entry of hepatitis C virus (HCV) by altering the viral particle structure in a way that impairs

**Table 2**  
Stability of Oximacro<sup>®</sup> at different pHs.

Virus and pHs	Antiviral activity <sup>a</sup> (IC <sub>50</sub> $\mu$ g/ml)
<b>HSV-1</b>	
3.0	12.4 $\pm$ 0.2
4.0	18.4 $\pm$ 1.5
5.0	8.8 $\pm$ 1.2
6.0	18.9 $\pm$ 1.1
7.0	11.4 $\pm$ 1.4
8.0	20.5 $\pm$ 1.5
9.0	22.9 $\pm$ 1.1
<b>HSV-2</b>	
3.0	9.7 $\pm$ 0.5
4.0	5.9 $\pm$ 1.2
5.0	7.7 $\pm$ 0.7
6.0	6.7 $\pm$ 1.3
7.0	5.1 $\pm$ 1.5
8.0	5.1 $\pm$ 2.0
9.0	7.1 $\pm$ 1.2

<sup>a</sup> Oximacro<sup>®</sup> concentration able to inhibit 50% HSV replication as determined by a plaque reduction assay in Vero cells. The values are means  $\pm$  SD of data derived from three independent experiments performed in duplicate.

**Table 3**  
Stability of Oximacro<sup>®</sup> in the presence of human serum.

Incubation time <sup>a</sup> and virus	Antiviral activity <sup>b</sup> (IC <sub>50</sub> $\mu$ g/ml)
<b>1h</b>	
HSV-1	15.9 $\pm$ 2.8
HSV-2	9.2 $\pm$ 0.9
<b>18h</b>	
HSV-1	14.7 $\pm$ 1.2
HSV-2	13.8 $\pm$ 3.2

<sup>a</sup> Treatment with 10% human serum at different incubation time.

<sup>b</sup> Oximacro<sup>®</sup> concentration able to inhibit 50% HSV replication as determined by a plaque reduction assay. The values are means  $\pm$  SD of data derived from three independent experiments performed in duplicate.



its attachment to the cell surface (Calland et al., 2015); while its derivative palmitoyl-EGCG (p-EGCG) prevents HSV-1 adsorption to Vero cells, most likely by binding to virion glycoproteins (de Oliveira et al., 2013).

Similarly, in the present study, we have observed that the A-type PACs-enriched Oximacro<sup>®</sup> extract prevents HSV-1 and HSV-2 infection via a mechanism associated to alterations of envelope glycoproteins required for entry, such as gB and gD. The anti-adhesive effect of Oximacro<sup>®</sup> on HSV is thus likely due to direct interactions with the virion surface as suggested by the results of interaction experiments between Oximacro<sup>®</sup> and either HSV particles (Fig. 5) or purified gD protein's ectodomain (Fig. 7).

The reported ability of polyphenols to bind and aggregate proteins (Charlton et al., 2002; Ebrahimejad et al., 2014) may explain the effect of PACs isolated from *R. acetosa* (Gescher et al., 2011a) or *V. macrocarpon* (this study) to bind and alter HSV envelope proteins. The formation of protein-PACs complexes is thought to be mainly due to hydrogen bonding, van der Waals and electrostatic interactions, as well as covalent bond formation (Ebrahimejad et al., 2014). To this regard, we observed that the alterations issued by Oximacro<sup>®</sup> on virion gD and gB and as well as on purified gD ectodomain were resistant to boiling of protein samples in SDS sample buffer (Figs. 5 and 7). Therefore, it is likely that exposure of virions and purified gD ectodomain to Oximacro<sup>®</sup> results in the formation of covalent linkages between the A-type PACs and viral proteins. These covalent interactions may then ultimately result in protein-protein crosslinking as most PACs have two or more reactive quinone moieties (Ebrahimejad et al., 2014), thus explaining the smearing and disappearance of glycoprotein bands observed in interactions experiments with virions or purified gD (Figs. 5 and 7). However, at present, it remains still unclear whether the

interactions between A-type PACs of Oximacro<sup>®</sup> and HSV envelope glycoproteins result in binding to specific protein domains, or whether the A-type PACs simply “coat” the whole glycoproteins (Fig. 8), thus preventing access to their normal binding partners on target cells.

The specificity of Oximacro<sup>®</sup> against HSV and its inactivity against an adenovirus strain, as shown here, is consistent with the observation that extracts from both *R. acetosa* and *M. flabellifolia* have no effect on adenovirus replication (Gescher et al., 2011a, 2011b). The differential activity of these plants extracts against the two different viruses might be ascribed to the different amino acid sequences of adenoviral capsid proteins versus those of HSV envelope glycoproteins. Indeed, glycosylation may also affect the binding, affinity, and specificity of PACs to proteins (Ebrahimejad et al., 2014). To this regard, the reported ability of pomegranate polyphenols to damage the integrity of influenza virions by interacting with surface HA and NA glycoproteins (Sundararajana et al., 2010), may suggest a certain degree of preference for PACs to bind to glycoproteins that are abundant on HSV particles, as well as on the influenza virus envelope.

Considering the significant global incidence, morbidity, and mortality rates of viral sexually transmitted infections (STIs), the development of new, safe, topically applied microbicides for their prevention is of high priority (Obiero et al., 2012). Therefore, attachment/entry inhibitors that block virus shedding and transmission by close personal contact may provide a realistic method of microbicide intervention. Furthermore, given the ability of HSV to establish latency and frequently reactivate, to prevent its transmission the ideal microbicide should prevent the establishment of infection. From this perspective, natural extracts like Oximacro<sup>®</sup> that impede HSV attachment to target cells may be most

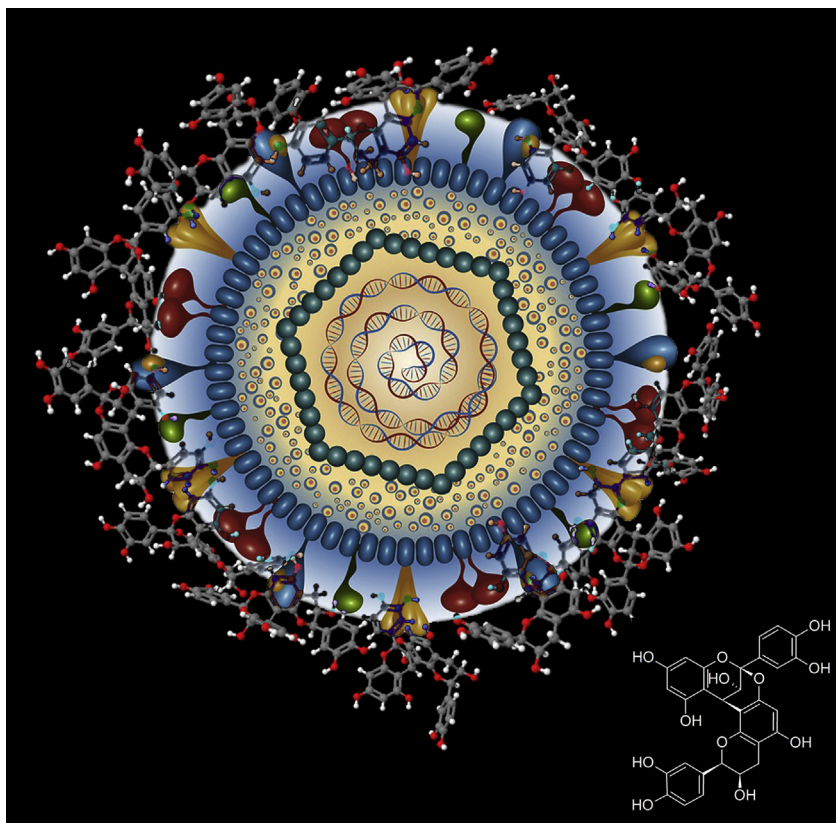


Fig. 8. Schematic representation of the interaction between the A-type PACs present in Oximacro<sup>®</sup> and HSV envelope glycoproteins. A typical Oximacro<sup>®</sup> PAC-A dimer is shown in the lower right corner.

advantageous. Moreover, several negatively-charged polyanions and dendrimers that, in recent years, have been selected for development as candidate microbicides due to their ability to block virion attachment and entry into target cells provides testimony to the suitability of this strategy for tackling HSV infections (Rupp et al., 2007; Nikolic and Piguet, 2010; Luginini et al., 2011).

Based on their mechanism of action, microbicides against HSV can be categorized into three groups. The first group includes compounds such as surfactants and detergents that directly inactivate the virus, while the second group consists of compounds that enhance the natural defense mechanisms of mucosal surfaces; compounds of both these groups are quite nonspecific and may exert a broad spectrum of antiviral activity. The third group contains molecules that impair viral attachment and/or entry into host cells and that may display a certain degree of specificity (Keller et al., 2005; Nikolic and Piguet, 2010). Oximacro<sup>®</sup>, as a candidate microbicide, clearly belongs to this last category. Nevertheless, regardless of their mechanism of action, microbicides directed against HSV need to address fundamental requirements, such as safety, efficacy, and low cost (Friend, 2010). Thus, while the widespread use of cranberry juice, juice concentrate, and different formulations of dried extracts to prevent UTIs bears witness to the high safety profile of these products, it also calls for an in-depth assessment of their efficacy in both *in vitro* and *in vivo* models of HSV infection in order that such products can be proposed for the prevention of herpesvirus infections in the near future.

Topical microbicides against genital herpes infection should be applied directly to the genital tract in order to protect against the acquisition of STIs; thus it remains possible that the unique physiological properties of the vagina could affect their activity. For this reason, we tested the stability of Oximacro<sup>®</sup> at various pH and in the presence of human serum proteins. The results showed that these treatments did not reduce the stability of Oximacro<sup>®</sup> to any significant degree, thus suggesting that Oximacro<sup>®</sup> is suitable for vaginal application without incurring any significant loss of antiviral activity. Finally, besides its anti-HSV activity, Oximacro<sup>®</sup> was also recently observed to be active also against HIV-1 infections (C. Parolin, M.E. Maffei, G. Gribaudo, unpublished results). Therefore, this extract has the potential for further development as the active ingredient of broad-spectrum microbicides with the goal of preventing transmission of the major viral sexually transmitted infections.

## 5. Conclusion

In conclusion, the results of this study indicate that the ability of the cranberry extract Oximacro<sup>®</sup> to target HSV-1 and HSV-2 attachment could be exploited to prevent the establishment of herpesvirus infections. The *in vitro* anti-HSV activity of Oximacro<sup>®</sup> thus calls for further studies to be performed to evaluate its efficacy and safety in murine models of acute infection, in order to validate its development as a novel candidate microbicide of natural origin for the prevention of HSV infections.

## Acknowledgments

We gratefully thank Gary Cohen and Roselyn Eisenberg (University of Pennsylvania) for providing the purified recombinant HSV-1 and HSV-2 gD (1-306 aa) proteins.

This work was supported by grants from the Italian Ministry for Universities and Scientific Research (Research Programs of Significant National Interest, PRIN 2010-11, grant no. 2010PHT9NF) to G.G.; Ex-60% to A.L., M.E.T., and G.G.; and partly by Biosfered S.r.l. The authors are grateful to C. Campobenedetto for technical assistance during Oximacro<sup>®</sup> fractionation.

## Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.antiviral.2016.06.006>.

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