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1 **The traditional use of *Vachellia nilotica* for sexually transmitted diseases is substantiated by**
2 **the antiviral activity of its bark extract against sexually transmitted viruses.**

3 Manuela Donalisio^{1a}, Valeria Cagno^{1a}, Andrea Civra¹, Davide Gibellini², Giuseppina Musumeci³,
4 Massimo Rittà¹, Manik Ghosh⁴, David Lembo^{1*}

5 ¹Department of Clinical and Biological Sciences, University of Torino, 10043 Orbassano, Torino,
6 Italy

7 ²Department of Pathology and Diagnostic, University of Verona, 37134 Verona, Italy

8 ³Department of Experimental, Diagnostics and Specialty Medicine, University of Bologna, 40138
9 Bologna, Italy.

10 ⁴Department of Pharmaceutical Sciences & Technology, Birla Institute of Technology, Mesra,
11 Ranchi 83215, India

12 ^aequal contribution

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15 medicine Asia & Oceania

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18 * Corresponding authors: Prof. David Lembo
19 Department of Clinical and Biological Sciences
20 University of Torino, S. Luigi Gonzaga Hospital
21 Regione Gonzole, 10
22 10043, Orbassano, Torino, Italy
23 Phone: +39 011 6705484
24 Fax: +39 011 2365484
25 E-mail: david.lembo@unito.it

26 **ABSTRACT**

27 *Ethnopharmacological relevance:* *Vachellia (Acacia) nilotica* and other plants of this genus have
28 been used in traditional medicine of Asian and African countries to treat many disorders, including
29 sexually transmitted diseases, but few studies were performed to validate their anti-microbial and
30 anti-viral activity against sexually transmitted infections.

31 *Aim of the study:* The present study was undertaken to explore whether the ethnomedical use of
32 *V.nilotica* to treat genital lesions is substantiated by its antiviral activity against the human
33 immunodeficiency virus (HIV), the herpes simplex virus (HSV) and the human papillomavirus
34 (HPV).

35 *Materials and methods:* The antiviral activity of *V.nilotica* was tested *in vitro* by virus-specific
36 inhibition assays using HSV-2 strains, sensible or resistant to acyclovir, HIV-1IIIb strain and HPV-
37 16 pseudovirion (PsV). The potential mode of action of extract against HSV-2 and HPV-16 was
38 further investigated by virus inactivation and time-of-addition assays on cell cultures.

39 *Results:* *V.nilotica* chloroform, methanolic and water bark extracts exerted antiviral activity against
40 HSV-2 and HPV-16 PsV infections; among these, methanolic extract showed the best EC50s with
41 values of 4.71 and 1.80 µg/ml against HSV-2 and HPV-16, respectively, and it was also active
42 against an acyclovir-resistant HSV-2 strain with an EC50 of 6.71 µg/ml. By contrast, no
43 suppression of HIV infection was observed. Investigation of the mechanism of action revealed that
44 the methanolic extract directly inactivated the infectivity of the HPV-16 particles, whereas a partial
45 virus inactivation and interference with virus attachment (EC50 of 2.74 µg/ml) were both found to
46 contribute to the anti-HSV-2 activity.

47 *Conclusions:* These results support the traditional use of *V.nilotica* applied externally for the
48 treatment of genital lesions. Further work remains to be done in order to identify the bioactive
49 components.

50

51 **1. Introduction**

52 *Vachellia nilotica*, widely known by the taxonomic synonym *Acacia nilotica* (L.), belongs to family
53 *Fabaceae* of genus *Acacia* containing an excess of 1350 species (Seigler et al., 2003). It is an
54 ornamental and medicinal plant, that grows to 14-17 meters in height and 2-3 meters in diameter. It
55 is widespread, distributed throughout tropical and sub-tropical regions of Africa, Middle East and
56 Indian subcontinent (Bargali and Bargali, 2009; Malviya et al., 2011; Ali et al., 2012). *A. nilotica* is
57 a rich source of many secondary metabolites, mainly condensed tannins, flavonoids, alkaloids. All
58 parts of the plant (pods, bark, leaves, roots, flower, gum, branches and seeds) have been used in
59 traditional medicine of India, Pakistan and African countries, as Kenya, Zimbabwe and Sudan, for
60 the treatment of enteric and respiratory ailments, children's fevers, toothache and eye complaints
61 (Kaur et al., 2005; Ali et al., 2012; Rather et al., 2015). Interestingly, *A. nilotica* and other plants of
62 *Acacia* genus have been used by traditional healers to treat sexually transmitted infections (STI) and
63 HIV/AIDS-related diseases (Kambizi et al., 2001; Chinsembu et al., 2016). For instance, in
64 Guruvedistrict, Zimbabwe, the *A. nilotica* fruits are grounded into powder and applied of penile sores
65 a kind of lesions that can be caused either by a bacterial infection like syphilis or by a viral infection
66 like herpes simplex virus (Kambizi et al., 2001). In Livingstone, Zambia, herbalists use *A. nilotica*,
67 *A. albida*, *A. polyacantha*, *A. ataxacantha* and *A. Schweinfurthii* to treat STI like syphilis, gonorrhoea
68 and other AIDS-related infections (Chinsembu et al., 2016). Recently, various extracts of *A. nilotica*
69 have been investigated for their antibacterial (Vijayasanthiet al., 2011; Amin et al., 2013; Oladosu et
70 al., 2013; Baiet al., 2015; Shekaret al., 2015), antiprotozoal (Jigam et al., 2010; Mann et al., 2011;
71 Bapna et al., 2014; Alli et al., 2016), antifungal (Satish et al., 2007; Mbatchou et al., 2012; Rai et
72 al., 2014), and antiviral activities (Hussein et al., 1999; Hussein et al., 2000; Asres et al., 2005;
73 Rehman et al., 2011; Raheel et al., 2013; Sharma et al., 2014). The traditional use against sexually
74 transmitted diseases and its antiviral potential prompted us to investigate on the *V. nilotica* antiviral
75 activity against three sexually transmitted viruses, namely the human immunodeficiency virus

76 (HIV), the herpes simplex virus (HSV) and the human papillomavirus (HPV).
77 STIs caused by bacterial and protozoan pathogens, including syphilis, gonorrhoea, Chlamydia, and
78 trichomoniasis, are generally curable. By contrast, HIV, HSV and HPV infections are currently
79 incurable through drug treatment (<http://www.who.int/en/>).

80 According to WHO, around 36.7 million people were living with HIV at the end of 2015. The “high
81 activity antiretroviral therapy” or HAART has transformed a terminal illness into a chronically
82 managed disease where patients can have a near-healthy quality of life. However, to date, there is
83 not an effective vaccine or a cure to eradicate the established infection, firstly for the high mutation
84 rate of viral genome and its ability to escape the host immune response, secondly for the integration
85 of provirus in cellular DNA and, thirdly for the presence of cellular and anatomical HIV reservoirs
86 where virus maintains low level of viral replication, despite treatment (Svicher et al., 2014; Sarmati
87 et al. 2015). Genital herpes infection, mainly caused by HSV type 2, affects more than 500 million
88 people worldwide. These infections are mostly asymptomatic but can also cause painful blisters or
89 ulcers in the genital or anal area (Roitzman et al., 2007). In addition, HSV-2 infection can increase
90 the risk of HIV acquisition by approximately three-fold and genital herpes can occur in 60-90% of
91 HIV-infected people (Freeman et al., 2006; Feng et al., 2013). HSV infections are lifelong for
92 ability of virus to establish latency in the neurons of the sensory ganglia, therefore antiviral drugs,
93 such as acyclovir, famciclovir, and valacyclovir, can reduce the severity and frequency of
94 symptoms, but cannot cure the infection (Cunningham et al., 2006). Another important sexually
95 transmitted virus is HPV, especially types 16 and 18, that cause 528000 cases of cervical cancer and
96 266000 deaths each year. To date, no anti-HPV drugs are available to cure HPV lesions and current
97 treatments are ablative. If for HIV and HSV-2 infections no vaccines are currently available, in
98 recent years two safe HPV vaccines have been introduced in routine immunization programmes in
99 65 countries (<http://www.who.int/en/>). Populations of developing countries with the highest rates of
100 STIs often do not have access to adequate health services. Therefore, despite the presence of
101 antiviral drugs for HIV and HSV-2 and HPV vaccines, their very high costs limit the administration

102 in people living in low-socioeconomic settings. In this contest, medicinal plants, as *Vachellia*
103 *nilotica*, have been extensively used to treat infectious diseases in India and Africa. This study was
104 undertaken to explore whether the ethnomedical use of *V. nilotica* to treat STI could be
105 substantiated by an antiviral activity against HSV-2, HPV-16 and HIV-1. Here, we report on the
106 cytotoxicity, the antiviral spectrum of activity of three *V.nilotica* bark extracts and the probable
107 mechanisms of antiviral action of *V. nilotica* methanol bark extract.

108

109 **2. Materials and methods**

110 *2.1. Plant material*

111 The bark of *Vachellianilotica* L. were collected from the medicinal plant garden of Birla Institute of
112 Technology, Mesra, Ranchi and authenticated by Botanical Survey of India, Central National
113 Herbarium, Botanical Garden, Howrah (Letter No. CNH/Tech.II/2015/18/275 dated 23-04-2015). A
114 specimen has been deposited at the herbarium (voucher no, SM-5).

115

116 *2.2. Preparation of extracts*

117 The fresh bark of *Vachellia nilotica* L. was completely dried at 40–45°C and pulverized in a knife
118 grinder to fine powder (250 µm- particle diameters). The powdered bark (250 g) was then
119 successively extracted by soxhlation using chloroform and methanol. Water extract of bark was
120 prepared by decoction. Extracts were filtered and evaporated to dryness using a rotator evaporator
121 under controlled temperature and reduced pressure. The obtained extracts were lyophilized and
122 stored in desiccators (Silva et al., 2010).

123

124 *2.3. Phytochemical investigations of V. nilotica methanolic extract*

125 The plant extract was submitted to preliminary phytochemical screening according to the methods
126 previously described (Manosroi et al., 2010). Briefly, Dragendorff's reagent, Ammonia solution,
127 Killer kiliani test, Shinoda test, Molisch test, FeCl₃, Frothing test, and Salkowski test were
128 performed to detect alkaloids, anthraquinone glycosides, cardiac glycosides, flavonoids,
129 carbohydrate, tannins, saponins and steroids/terpenoids, respectively.

130

131 *2.4. Cells*

132 African green monkey kidney cells (Vero) (ATCC CCL-81) were cultured in Eagle's minimal
133 essential medium (MEM) (Gibco/BRL, Gaithersburg, MD) supplemented with heat inactivated 10%
134 fetal calf serum (FCS) (Gibco/BRL). The human cervical carcinoma cell lines HeLa (ATCC CCL-

135 2) were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL,
136 Gaithersburg, MD) supplemented with heat-inactivated 10% FCS. The 293TT cell line, derived
137 from human embryonic kidney cells transformed with the simian virus 40 (SV40) large T antigen,
138 was cultured in DMEM supplemented with heat-inactivated 10% FCS and nonessential amino
139 acids. 293TT cells allow high levels of protein to be expressed from vectors containing the SV40
140 origin due to over-replication of the expression plasmid (Buck et al., 2005). C8166 CD4+T
141 lymphoblastoid cell line was maintained in RPMI 1640 (GIBCO, Grand Island, NY) supplemented
142 with 10% FCS at an optimal cell density of 0.5 to 1.5x10⁶ cells/ml. All media were supplemented
143 with 1% antibiotic-antimycotic solution (Zell Shield, Minerva Biolabs GmbH, Berlin, Germany) and
144 cells were grown at 37°C in an atmosphere of 5% of CO₂.

145 2.5. *Viruses*

146 HSV-2 strain (ATCC VR-540) and a HSV-2 strain with phenotypic resistance to acyclovir were
147 used. The resistant strain was generated by serial passage in the presence of increasing
148 concentrations of acyclovir as previously described by Field et al. (1980). The resistant virus was
149 then plaque purified, and the antiviral susceptibility was tested as described in Donalisio et al.
150 (2016). Viral strains were propagated, collected and titrated by plaque assay on Vero cells. HIV-
151 1IIIb strain stock was prepared in C8166 cells as previously described (Bon et al., 2013). The viral
152 stocks were titrated with an HIV-1 gag p24 antigen ELISA kit (Biomerieux, Marcy L'Etoile,
153 France).

154 2.6. *HPV PsV production*

155 Plasmids and 293TT cells used for pseudovirus (PsV) production were kindly provided by John
156 Schiller (National Cancer Institute, Bethesda, MD) or bought at Addgene (Cambridge, MA).
157 Detailed protocols and plasmid maps for this study can be seen at
158 <http://home.ccr.cancer.gov/lco/pseudovirusproduction.htm>. HPV-16 PsVs were produced
159 according to previously described methods (Buck et al., 2005). Briefly, 293TT cells were
160 transfected with plasmids expressing the papillomavirus major and minor capsid proteins (L1 and

161 L2, respectively), together with a reporter plasmid expressing the green fluorescent protein (GFP),
162 named pfwB. Capsids were allowed to mature overnight in cell lysate; the clarified supernatant was
163 then loaded on top of a density gradient of 27 to 33 to 39% Optiprep (Sigma-Aldrich, St. Louis,
164 MO) at room temperature for 3 h. The material was centrifuged at 28,000 rpm for 18h at room
165 temperature in an SW41.1 rotor (Beckman Coulter, Inc., Fullerton, CA) and then collected by
166 bottom puncture of the tubes. Fractions were inspected for purity as described in Cagno et al. (2015)
167 and the L1 protein content of PsV stocks was determined by comparison with bovine serum
168 albumin standards in Coomassie-stained SDS-polyacrylamide gels.

169 *2.7. HSV inhibition assay*

170 The effect of extracts on HSV-2 infections was evaluated by plaque reduction assay as described in
171 Donalisio et al. (2014). Briefly, increasing concentrations of extracts were added to cells before,
172 during and after infection. HSV-2 or acyclovir resistant HSV-2 were used at MOI of 0.0003
173 pfu/cell. After 24h of incubation at 37°C, cells were fixed and stained with 0.1% crystalviolet in
174 20% ethanol and viral plaques counted. The effective concentrations producing 50% and 90%
175 reduction in plaque formation (EC50 and EC90) were determined using Prism software by
176 comparing drug-treated with untreated wells. The selectivity index (SI) was calculated by dividing
177 the CC50 by the EC50 value. As control, Vero cells were treated with Acyclovir (Sigma-Aldrich).

178 *2.8. HPV GFP-based assay*

179 HeLa cells were seeded in 96-well plates at a density of 8,000 cells/well in 100 µl of DMEM
180 supplemented with 10% FBS. The next day, serial dilutions of extracts were added to preplated
181 cells for 2 hours at 37°C, after the incubation time the extracts were removed and mixtures of
182 extracts and dilutions of PsV stocks at a multiplicity of infection (MOI) of 0.05 (determined by
183 calculation of the fraction of cells positive for reporter protein expression in untreated cells) were
184 added. The percent of infection was calculated as described elsewhere (Savoia et al., 2010). As
185 control, cells were infected and treated with heparin (Laboratori Derivati Organici S.p.A., Milan,
186 Italy).

187 *2.9. HIV inhibition assay*

188 Viral stocks were assessed at concentration of 1000 ng p24/ml. HIV-1_{IIIb} laboratory strains (5 ng/ml
189 of HIV-1 gag p24) was pre-incubated for one hour at 37°C with increasing concentrations of
190 extracts (0, 0.025, 0,25, 2,5, 25 µg/ml) and then added to C8166 cells (0.5x10⁶ cells/ml) for 2 hours
191 at 37°C. After three washes in PBS, the cells were seeded at 5x10⁵ cells/ml into fresh medium plus
192 the drug concentration used in the pre-incubation. The HIV-1 gag p24 amount was determined
193 seven days post-infection in the culture supernatants with the HIV-1 p24 antigen ELISA kit
194 (Biomerieux). Mock-infected C8166 cells in medium with or without DMSO were used as the
195 negative controls. As further control, we have treated the HIV-1_{IIIb}-infected C8166 with serial
196 dilutions of Tenofovir (NIBSC, London, UK).

197 *2.10. Cell viability assay*

198 HeLa and Vero cells were seeded in 96-well plates and, the next day, treated with serially diluted
199 extracts. After 24h (Vero cells) or 72 h (HeLa cells) of incubation cell viability was determined
200 using the Cell Titer 96 Proliferation Assay Kit (Promega, Madison, WI, USA), according to the
201 manufacturer's instructions. Absorbances were measured using a Microplate Reader (Model 680,
202 BIORAD) at 490 nm. The percent of viability was calculated in comparison with the untreated
203 control, where medium and extract solvent was added. The viability of the C8166 cells in presence
204 of scalar concentrations of extractor Tenofovir was analysed by the Trypan Blue exclusion
205 technique at day 7 post-infection.

206 *2.11. Mechanism of action assays against HSV-2 infection*

207 Vero cells were seeded in 96-well plates or 24-well plates in MEM supplemented with 10% FBS,
208 for virus inactivation assay and all other assays, respectively. The following day:

- 209 - In virus inactivation assay, extract (33µg/ml) was added to aliquots of 10⁵ PFU HSV-2 and
210 incubated at 37°C for 2 h. After incubation, samples were titrated on Vero cells at high
211 dilutions, at which the extract was not active.

- 212 - In time of addition assay, serial dilutions of extract were added on cells 2h before infection or
213 during infection or post-infection. After the incubation time described before, viral plaques were
214 counted.
- 215 - In attachment assay serial dilutions of extract or heparin were mixed with HSV-2 and added to
216 cooled cells and incubated for 2h at 4°C to ensure viral attachment but not entry. After two
217 gentle washes, cells were overlaid with 1.2% methylcellulose medium, shifted to 37°C for 24h
218 and successively plaques were counted.
- 219 - In entry assay, HSV-2 at MOI of 0.01 PFU/cell was adsorbed for 2h at 4°C on pre-chilled
220 confluent cells. Cells were then washed with cold MEM three times to remove unbound virus,
221 treated with different concentrations of extract, and incubated for 3h at 37°C. Un-penetrated
222 viruses were inactivated with acidic glycine for 2 min at room temperature. Cells were then
223 washed with warm medium three times and treated.

224 *2.12. HPV inactivation assay*

225 HeLa cells were seeded in 96-well plates in 100 µl of DMEM supplemented with 10% FBS. The
226 following day a mixture of 11 µg/ml of extract and HPV-16 PsV or HPV-16 PsV (10⁵ focus
227 forming units) alone was incubated for 2 hours at 37°C and subsequently titrated on cells. 72 hours
228 later the infection was measured.

229 *2.13. Data analysis*

230 All results are presented as the mean values from three independent experiments. The EC50 and
231 EC90 values for inhibition curves were calculated by regression analysis using the software Graph-
232 Pad Prism version 5.0 (GraphPad Software, SanDiego, California, U.S.A.) by fitting a variable
233 slope-sigmoidal dose-response curve. For virus inactivation assays the viral infectivity in presence
234 and absence of extract was compared using a one-way analysis of variance (ANOVA) followed by

235 Bonferroni test, if P values showed significant differences in virus titers. Significance was set at the
236 95% level.

237

238 **Results and discussion**

239 *V.nilotica* chloroform, methanol and water extracts were prepared obtaining w/w extraction yields
 240 of 6.27, 9.37 and 10.25% w/w, respectively, as previously described. In a first set of experiments,
 241 we tested the antiviral activity of three *V.nilotica* bark extracts against HSV-2, HPV-16 and HIV-1.
 242 Since extracts were resuspended in DMSO to a final concentration of 25 mg/ml before use, control
 243 samples with equal volumes of DMSO were included in the assay in order to rule out any cytotoxic
 244 effect ascribable to the solvent.

245 **Table 1. Antiviral activity of *V.nilotica* bark extracts against HSV-2, HPV-16, HIV-1 and**
 246 **acyclovir resistant HSV-2 strain**

Virus	Extract	EC50*(µg/ml) – 95% C.I. #	EC90[§](µg/ml)– 95% C.I.	CC50[†](µg/ ml)	SI[‡]
HSV-2	Chloroform	12.3 (7.72-19.6)	35.9 (11.4 -62.7)	189	15.4
	Methanol	4.71 (3.11-7.12)	8.07 (2.66-24.4)	144	30.6
	Water	10.2 (7.95-12.9)	20.1 (8.77-46.0)	190	18.6
	Acyclovir	0.64 (0.48-0.86)	3.17 (1.69-5.95)	>300	>468
HPV-16	Chloroform	3.89 (1.69-8.88)	23.5 (4.15-43.4)	211	54.2
	Methanol	1.80 (1.42-2.27)	5.48 (3.63-8.28)	58.7	32.6
	Water	5.51 (4.58-6.63)	15.1(10.1-22.1)	115	20.9
	Heparin	2.20 (1.83-3.01)	4.3 (3.05-5.01)	>300	>136
HIV-1_{mb}	Chloroform	-	-	74	-
	Methanol	-	-	74	-
	Water	-	-	74	-
	Tenofovir	0.21 (0.12 -0.37)	1.09(0.36- 3.29)	>14.3	>68.3
HSV-2 ACVr[¥]	Methanol	6.71 (3.85-11.7)	10.5 (5.99-18.5)	144	21.5
	Acyclovir	62.0 (57.7-65.2)	> 500	> 300	> 5.00

247
 248 EC50* half maximal effective concentration
 249 C.I. # confidence interval

250 EC90[§]90% effective concentration
251 CC50[†] half maximal cytotoxic concentration
252 SI[‡] selectivity index
253 [¥] HSV-2 acyclovir resistant strain
254

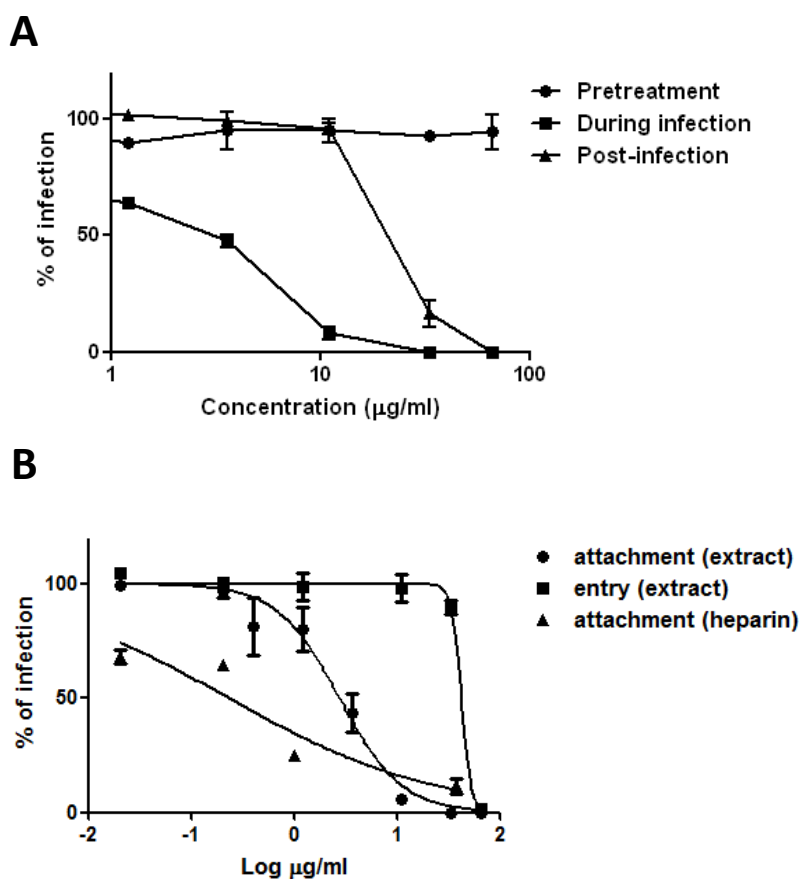
255

256 Results shown in Table 1 reveal that the three extracts were active against HSV-2 and HPV-16 PsV
257 although at different extents. Chloroform, methanolic and water extracts inhibited HSV-2 infection
258 with selectivity indexes of 15.4, 30.6 and 18.6, respectively, whereas they exerted anti-HPV-16
259 activity with selectivity indexes of 54.2, 32.6 and 20.9, respectively. These data indicated that the
260 antiviral activity was not due to cytotoxicity. Acyclovir and heparin were tested in parallel as
261 reference inhibitors for HSV-2 and HPV-16 respectively and exerted the expected antiviral activity.
262 By contrast, when *V. nilotica* bark extracts were tested on C8166 T CD4+ lymphoblastoid cells
263 against HIV-1_{IIIb} strain infection, no suppression of HIV infection was observed. In the same assay,
264 the reference drug tenofovir strongly inhibited HIV-1 infection in a dose-response manner (Table
265 1). The lack of anti-HIV-1 activity of extracts is in accord with the study by Hussein et al., that
266 reported no HIV-1 inhibitory activity of methanol and water bark extracts from Sudanese *Acacia*
267 *nilotica*. However, it must be noted that, despite the lack of HIV-1 inhibitory activity, the extracts of
268 the bark and the pods have been reported to exert an anti-HIV-1 protease activity (Hussein et al.,
269 1999).

270 Although aqueous extract is mainly used in traditional medicine to treat STIs, our results
271 demonstrated that methanolic extract is the most active one with the lowest EC50 of 4.71 and 1.80
272 µg/ml against HSV-2 and HPV-16 infections, respectively. Therefore, the *V. nilotica* bark
273 methanolic extract was chosen for further studies aimed at investigating its major mechanism(s) of
274 action.

275 Of note, the methanolic extract proved active also against an acyclovir-resistant HSV-2 strain
276 (HSV-2 ACV-r) that was previously generated in our laboratory (Donalisio et al., 2016). This

277 finding might indicate that the active components of the extract act through a different mechanism
 278 of action to that of acyclovir, an inhibitor of viral DNA polymerase.
 279 To explore whether the extract directly inactivates HSV-2 virus particles, 10^5 PFU of HSV-2 and
 280 33 $\mu\text{g/ml}$ of extract (a dose inhibiting 90% of infectivity in previous assays, i.e. EC_{90}) were
 281 incubated at 37°C for 2 h and, then, the samples were titrated on cells at high dilutions, at which the
 282 extract was no longer active. A 66% of inhibition of viral titers was observed indicating that the
 283 anti-HSV-2 activity of the extract depends, at least in part on the inactivation extracellular virus
 284 particles (data not shown).



285

286

287 **Fig. 1. Mode of anti-HSV-2 activity of methanol bark extract.** A) Time-of-addition assays. Cells
 288 were treated with methanol bark extract for 2 h before infection (pre-treatment), for 2 h during
 289 infection or by adding the extract immediately after infection (post-infection). The number of
 290 plaques in the treated samples is expressed as a percentage of control (DMSO treated). B) HSV-2
 291 entry and attachment assays. The methanol bark extract of *V. nilotica* was added to the cell culture
 292 during virus-cell binding (attachment assay) or virus-cell penetration (entry assay). Heparin was
 293 used as a known inhibitor of attachment. The number of viral plaques in the treated samples is

294 expressed as a percentage of control (DMSO treated). Each point represents mean and SEM for
295 triplicates.

296

297 To investigate if bark extract is also able to target cell-surface-virus interactions or intracellular
298 steps of the HSV-2 replicative cycle, we performed a set of time-of-addition assays in which the
299 sample was added to the cells only before (pre-treatment), during, or after infection, and viral
300 plaques were counted after an incubation time of 24 h. Figure 1A shows that pre-treatment with the
301 bark extract did not affect cell susceptibility to HSV-2 infection thus excluding the possibility that
302 components of the extract form stable interactions with one or more cellular components,
303 preventing their interaction with viral glycoproteins. By contrast, a strong antiviral activity was
304 observed when the extract was added during infection with EC₅₀ values of 1.94µg/ml. A modest
305 dose-dependent effect occurred when the extract was added post-infection (EC₅₀ of 22.5µg/ml).
306 These data bring out several considerations: 1) bark extract does not target cell-surface; 2) the
307 extract inhibits probably also early steps of the virus replicative cycle since a significant reduction
308 in the number of viral plaques was also observed when the extract was added to methylcellulose
309 medium during infection. To confirm this hypothesis, an attachment and entry assays were
310 performed (Ghosh et al., 2016). As described in Figure 1B, bark extract inhibited HSV-2 attachment
311 to Vero cells generating a dose-response curve with EC₅₀ of 2.74 µg/ml. A dose response curve
312 was also obtained treating cells with heparin, a known inhibitor of attachment (EC₅₀: 0.23 µg/ml).
313 By contrast, performing the entry assay, a significant reduction of infectivity was only observed at
314 highest tested concentration (66 µg/ml). Then, we carried out studies to elucidate whether the
315 mechanism of action was similar against HSV-2 and HPV-16 PsV infections.

316 Interestingly, 11 µg/ml of *V. nilotica* extract was able to completely abrogate the infectivity of
317 HPV-16 pseudovirions in the virus inactivation assay indicating that bark extract exerts a virucidal
318 activity as main mechanism of action against HPV-16 (data not shown).

319 Summing described results, we demonstrated the ability of *V. nilotica* methanol bark extract to exert
320 a specific virucidal action against HPV-16, whereas more than one mechanism of action against
321 HSV-2 infection, including a partial inactivation of the infectivity of virus particles and an
322 inhibition of viral attachment to cells. These multiple antiviral actions can be attributed to several
323 bioactive metabolites of the plant. A preliminary phytochemical screening detected saponins and
324 flavonoids as main constituents and tannins in traces.

325 Further work remains to be done to investigate which component is responsible for biological
326 activity and elucidate their mechanisms of action.

327

328 Overall, our findings support the traditional use of *V. Nilotica* extracts for the treatment of sexually
329 transmitted diseases due to HSV-2 and HPV-16 infections, but not due to HIV-1 infection.

330 Interestingly, previous studies proved an antiviral activity of extracts of *Acacia nilotica* also against
331 Hepatitis C virus, that can also be transmitted sexually, even if this mode of transmission is much
332 less common (Hussein et al., 2000; Rehman et al., 2011; <http://www.who.int/en/>). Further studies
333 are required to investigate the therapeutic potential of *V. nilotica* extracts for the treatment of HSV-
334 2 and HPV infections.

335

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338 **Conflict of interest**

339 There is no conflict of interest associated with the authors of this paper.

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342

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450

451 **Author contributions** ^[1]_[SEP]

452 M.D. was responsible for all activities involving HSV-2 under the ^[1]_[SEP]
453 supervision of D.L.

454 V.C. was responsible for all activities involving HPV under the ^[1]_[SEP]
455 supervision of D.L.

456 A.C. and M.R. produced the viral stocks and were responsible of the cell viability assays.

457 D.G. and G.M. were responsible for all activities involving HIV.

458 M.G. collected and identified the plant and produced the plant extracts.

459 D.L. and M.G. conceived the study. M.D., V.C., and D.L. developed the interpretation of the
460 experiments. M.G., V.C., M.G. and D.L. wrote the paper.

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