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Short communication

Plant products as topical microbicide candidates: assessment of in vitro and in vivo activity against herpes simplex virus type 2

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Abstract

There is considerable interest in developing topical microbicides; products to be used intravaginally by women for protection against sexually transmitted diseases. Many compounds derived from plants have been shown to have antimicrobial properties. We examined 19 such compounds in vitro by plaque reduction assay to determine their activity against a common sexually transmitted pathogen, herpes simplex virus type 2. Compounds with an $ED_{50} \leq 7.0$ mg/ml were tested for efficacy in vivo. Four compounds, carrageenan lambda type IV, cineole, curcumin, and eugenol, provided significant protection ($P < 0.05$) in a mouse model of intravaginal HSV-2 challenge. Eugenol, which provided the greatest protection in mice was also evaluated using the guinea pig model of genital HSV-2 infection where it also demonstrated significant protection. Based on these results, several plant-derived compounds appear to warrant further evaluation as potential microbicides.

Keywords: Herpes simplex virus; Genital herpes; Topical microbicides; Vaginal microbicides; Natural products; Plant products; Sexually transmitted diseases; Nonoxynol-9; N-9; Carrageenan; Cineole; Curcumin; Eugenol

Sexually transmitted diseases (STDs) are a global public health concern (Alexander, 1996). The development of safe, inexpensive, and effective intravaginal microbicides is one strategy for reducing the spread of STDs (Rosenthal et al., 1998). Much of the current work has focused on nonoxynol-9 (N-9), a nonionic detergent found in many over-the-counter spermicides. While N-9 has been reported to have in vitro and in vivo antimicrobial activity, a recent clinical trial demonstrated that it was ineffective in preventing acquisition of HIV, gonorrhea, or chlamydial infection (Roddy et al., 1998a). Other clinical studies found that repetitive use of N-9 containing

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products can cause injury to the cervicovaginal epithelium (Kreiss et al., 1992; Zekeng et al., 1993), possibly placing the user at increased risk of acquiring some STDs. The ineffectiveness of N-9 combined with serious safety concerns makes the identification of novel, safe, effective, broad-spectrum microbicides of considerable interest. The safety and effectiveness of potent antimicrobial drugs derived from natural sources (Becker, 1980) led us to hypothesize that some naturally occurring products might make effective topical microbicides. This concept is supported by recent experimental studies that show that plant- and animal-derived compounds have activity in vitro and in vivo against selected sexually transmitted pathogens (Harmsen et al., 1995; Qu et al., 1997; Zeitlin et al., 1997; Lampe et al., 1998). To further explore natural products as potential microbicides we selected plant-derived compounds reported in the literature to have antimicrobial properties and evaluated these agents for their in vitro and in vivo activity against HSV-2.

Plant-derived chemicals (Table 1) were purchased from Sigma (St Louis, MO). Stocks of HSV-2 strain MS (American Type Culture Collection, Rockville, MD) were prepared in HeLa cells (American Type Culture Collection, Rockville, MD) maintained in Basal Medium Eagle (BME) supplemented with 10% fetal bovine serum (FBS). Stocks of HSV-2 strain 186 (obtained from Dr R. Thompson, University of Cincinnati, OH) were prepared in primary rabbit kidney cells maintained in BME with 10% FBS as previously described (Stanberry et al., 1987). All virus stocks were stored frozen (-80°C). The ED_{50} (dose required to inhibit virus replication by 50%) was determined by plaque assay where equal volumes (0.5 ml) of HSV-2 strain MS (approximately 10^6 pfu/ml) and the specific chemical or vehicle were mixed and incubated for 1 h at 37°C. Serial 1:10 dilutions of the mixtures were prepared and 0.2-ml samples were inoculated onto confluent HeLa cells grown in 24-well plates. Plates were incubated at 37°C in 5% CO_2 for 1 h and gently shaken every 15–20 min. After 1 h the medium was removed and replaced with BME with 10% FBS. Plates were incubated for 72 h at 37°C in 5% CO_2, then fixed and stained with ACCUSTAIN (Sigma, St. Louis, MO). Plaques were counted and the ED_{50} (the concentration of drug that reduced the plaque number by 50% relative to the appropriate controls) was calculated. All assays were run in quadruplicate. Under these assay conditions cellular cytotoxicity was not a problem in enumerating the plaques.

As shown in Table 1, all compounds demonstrated some activity in vitro against HSV-2. Hypericin, a naphthodianthone derived from St John's Wort, was the most potent compound tested with an in vitro ED_{50} of 0.0039 mg/ml. The other product with a very low ED_{50} (0.082 mg/ml) was cinnamon oil, a mixture of cinnamaldehyde, cinnamyl acetate and eugenol. Seven terpenes were tested and had activity that ranged from moderate (citral, 0.51 mg/ml) to poor (menthol, 7.2 mg/ml). The three terpenoids tested also had activity in vitro ranging from moderate (origanum oil, 0.52 mg/ml) to poor (borneol, 16.0 mg/ml). The two phenolic compounds, caffeic acid and safrole had ED_{50} values of 0.54 and 3.1 mg/ml, respectively. The two related sulfated polysaccharides, carrageenan lambda type IV and iota type V had similar ED_{50} values at 2.4 and 1.4 mg/ml. As with the terpenes and terpenoids, the phenolic vanillyloids had activity ranging from moderate (curcumin, 0.32 mg/ml) to poor (ferulic acid, 8.0 mg/ml).

To further explore their potential as microbicides, compounds with ED_{50} values ≤ 7 mg/ml were evaluated for protective efficacy in vivo in a mouse model of genital HSV-2 infection. Female Swiss Webster mice weighing 18–21 g (Harlan, Indianapolis, IN) were treated with 0.1 ml of a suspension containing 3 mg medroxyprogesterone acetate (Pharmacia and Upjohn, Kalamazoo, MI) 7 days and 1 day prior to HSV-2 inoculation. Mice were anesthetized with sodium pentobarbitol and the vagina first swabbed with a moistened calcium alginate swab, then a dry swab and then 15 μl of the test compound was instilled vaginally followed 20 s later by inoculation of 15 μl of virus suspension containing 10^4 pfu of HSV-2 strain 186. Oils were tested undiluted (100%) and other compounds were tested at 100 mg/ml or at concentrations determined by the limits of solubility of the test compound. Because of limited

Table 1
Chemicals tested against HSV-2 in vitro and in vivo in mice[a]

Chemical	Molecular weight	Common source	Type	Active against	Reference	ED_{50} (mg/ml)	Concentration tested in vivo	No. without disease[b]		*P*-value
								Control	Chemical	
Borneol	154.24	Rosemary	Terpenoid	Gram-positive bacteria, HIV, fungi	Aruoma et al., 1996; Hammerschmidt et al., 1993	16.0[e]	ND	–	–	–
Caffeic acid	180.15	Green coffee	Phenolic	HIV, HSV	Dimitrova et al., 1993; Kreis et al., 1990	0.54[d]	18 mg/ml[g]	3/16	8/16	NS
Carrageenan lambda type IV	NA	Seaweed	Sulfated polysaccharide	HIV, HSV, CMV	Baba et al., 1988	2.4	10 mg/ml	0/15	8/15	<0.005
Carrageenan iota type V	NA	Seaweed	Sulfated polysaccharide	HIV, HSV, CMV	Baba et al., 1988	1.4	10 mg/ml	0/15	2/15	NS
Cineole	154.3	Eucalyptus	Terpene	Gram-positive bacteria, gram-negative bacteria	Pattnaik et al., 1997	6.9[e]	100%	1/15	7/16	<0.05
Cinnamon oil	NA	Cinnamon	Aldehyde	Gram-positive bacteria, fungi	Hili et al., 1997; Montes-Belmont and Carvajal, 1998	0.082[e]	100%	4/15	4/15	NS
Citral	152.2	Lemon grass	Terpene	Gram-positive bacteria, gram-negative bacteria, fungi	Pattnaik et al., 1997	0.51[e]	100%	3/15	6/16	NS
Curcumin	368.37	Tumeric	Phenolic vanillyloid	HIV	Mazumder et al., 1995	0.32[e]	100 mg/ml[h]	5/15	12/16	<0.05
Eugenol	164.2	Clove	Phenolic vanillyloid	Gram-positive bacteria, gram-negative bacteria, fungi	Himejima and Kubo, 1992; Hao et al.,, 1998	1.5[e]	100%	2/15	14/16	<0.001
Ferulic acid	194.2	Wheat	Phenolic vanillyloid	Fungi	Dowd et al., 1997	8.0[e]	ND	–	–	–
Geraniol	154.3	Rose	Terpene	Gram-positive bacteria, gram-negative bacteria, fungi	Pattnaik et al., 1997	7.0[e]	100%	1/15	5/16	NS
Hypericin	504.5	St. John's Wort	Naphtho-di-anthrone	HIV, HSV	Lavie et al., 1995; Cohen et al., 1996	0.0039[c]	1 mg/ml[i]	3/15	6/15	NS

Table 1 (Continued)

Chemical	Molecular weight	Common source	Type	Active against	Reference	ED_{50} (mg/ml)	Concentration tested in vivo	No. without disease[b]		*P*-value
								Control	Chemical	
(+)-Limonene	136.2	Lemon	Terpene	Gram-positive bacteria, gram-negative bacteria, fungi	Pattnaik et al., 1996	3.61[e]	100%	3/15	2/16	NS
Linalool	154.3	Basil	Terpene	Gram-positive bacteria, gram-negative bacteria	Pattnaik et al., 1997	1.38[e]	100%	1/15	5/16	NS
Menthol	156.3	Peppermint	Terpene	Gram-positive bacteria, gram-negative bacteria	Pattnaik et al., 1997	7.2[e]	ND	–	–	–
Origanum oil	NA	Wild marjoram	Terpenoid	Gram-positive bacteria, fungi	Locoste et al., 1996; Montes-Belmont and Carvajal, 1998	0.52[e]	100%	1/15	0/16	NS
Safrole	162.2	Sassafras	Phenolic	Gram-positive bacteria, fungi	Himejima and Kubo, 1992	3.1[e]	100%	1/15	0/16	NS
Tea tree oil	NA	Tea tree	Terpenoid	Gram-positive bacteria, gram-negative bacteria	Carson et al., 1995; Hammer et al., 1996	2.7[e]	100%	2/15	3/16	NS
Thymol	150.2	Thyme	Terpene	Gram-positive bacteria, fungi	Locoste et al., 1996; Montes-Belmont and Carvajal, 1998	0.8[f]	100 mg/ml	1/15	0/16	NS
KY containing Nonoxynol 9	–	–	–			0.018	2.2%[j]	1/10	5/10	NS

[a] NA, not available (multiple components). ND, not done. *P*-value as determined by Fisher's exact test. Non-significant differences ($P > 0.05$) are indicated by NS.

[b] Disease was defined as including hair loss and erythema around the perineum, chronic urinary incontinence, hind-limb paralysis and/or death. ED_{50} vehicle was Eagles basal medium (BME) with 10% fetal bovine serum (FBS) unless noted:

[c] BME with $\leq 5\%$ dH_2O + NaOH, pH 11;

[d] BME with $\leq 50\%$ 0.1 N NaOH, pH 8.5;

[e] BME with $\leq 10\%$ DMSO;

[f] BME with $\leq 50\%$ peanut oil. In vivo vehicle was BME, control animals treated with PBS unless noted:

[g] 0.1 N NaOH, pH 7, control animals treated with 0.1 N NaOH, pH 8.5;

[h] 80% PEG 1000, 10% PEG 6000, 10% dH_2O, control animals treated with vehicle alone;

[i] dH_2O, pH 11, control animals treated with PBS, pH 11;

[j] control animals treated with KY without N-9.

availability and high cost, hypericin was tested at 1 mg/ml. Mice were assessed to day 21-post inoculation (PI) for signs of disease including hair loss and erythema around the perineum, chronic urinary incontinence, hind-limb paralysis and/or death. None of the compounds caused apparent vaginal irritation or distress following intravaginal administration of a single dose. As shown in Table 1, four of the 16 compounds tested in vivo provided significant protection ($P < 0.05$) against disease caused by intravaginal HSV-2 challenge. The four compounds were carrageenan lambda type IV, cineole, curcumin, and eugenol. Our findings with carrageenan lambda are similar to those of Zacharopoulos and Phillips (1997) who recently reported that this plant-derived compound had topical microbicide activity in a similar mouse model of genital HSV infection. The data in Table 1 indicate that activity in vitro did not necessarily predict efficacy in vivo. For example, cinnamon oil, which had one of the lowest ED_{50} values (0.082 mg/ml), did not protect in vivo, while cineole with a relatively high ED_{50} value (6.9 mg/ml), provided significant protection in vivo ($P < 0.05$).

The most effective compound in vivo was eugenol (4-hydroxy-3-methoxy-allylbenzene). Its antiviral activity may be due to the phenolic nature of the compound. Polyphenolic complexes have been shown to damage protein envelopes in newly synthesized HSV virions (Serkedjieva and Manolova, 1992). Eugenol also has capsaicin-like properties that might contribute to its in vivo anti-HSV activity. Like capsaicin, eugenol activates C-type sensory nerve fibers, has antinociceptive properties, and activates a Ca^{2+}-permeable ion channel in dorsal root ganglion neurons (Chen et al., 1995; Ohkubo and Kitamura, 1997; Ohkubo and Shibata, 1997). Capsaicin, while lacking in vitro anti-HSV activity, has been shown to protect animals from genital HSV disease (Stanberry, 1990), and from HSV infection of the central nervous system (Ljungdahl et al., 1986; Herbort et al., 1989). It has been hypothesized that capsaicin alters the pathogenesis of HSV infection by interfering with essential virus–neuron interactions (Stanberry, 1990). While capsaicin has beneficial effects in animal models of HSV infection it is noxious when applied to mucous membranes and causes an unpleasant burning sensation. This property will likely preclude its development as a topical microbicide. Because eugenol is a related vanillyloid that appears less noxious we further examined its anti-HSV activity using the guinea pig model of genital herpes. Infection in this model, unlike in the mouse, does not result in death, and more closely mimics the natural course of disease in humans including limited primary infection, establishment of latent infection in sensory ganglia and both spontaneous and induced recurrent infections (Stanberry, 1992). For these experiments, 0.1 ml of eugenol was instilled into the vaginal vault of female Hartley guinea pigs followed 20 s later by instillation of 0.1 ml of virus suspension containing 10^6 pfu of HSV-2 MS strain. Vaginal swabs were collected on day 1 PI, and stored at -70°C until assayed for HSV-2 by plaque titration. Guinea pigs were assessed daily to day 14 PI for symptomatic primary genital infection. The severity of skin disease was quantified using a well-established lesion score scale (Stanberry, 1992). The area under the lesion score-day curve was used to measure disease severity. Asymptomatic infections were defined as recovery of virus from the vaginal swab specimens in animals that developed no signs of genital HSV infection. Table 2 shows that treatment with eugenol immediately prior to inoculation resulted in significantly fewer animals developing symptomatic primary disease ($P < 0.05$) compared to PBS-treated controls. However, in treated animals that developed symptomatic primary infection, severity was comparable to that in controls. In eugenol-treated animals that became infected there was a significant reduction ($P < 0.005$) in virus titer as compared to PBS-treated controls but the frequency of subsequent recurrent disease was not significantly reduced. These data indicate that eugenol does have direct antiviral activity. However, the results do not rule out the possibility that eugenol may have some capsaicin-like effect on HSV pathogenesis. Further studies evaluating the efficacy of eugenol in clinically useful vehicles appear warranted.

No effective topical microbicides are currently available. There are contradictory clinical data

Table 2
Effect of eugenol treatment on HSV-2 genital infection of guinea pigs

Group[a]	N	Infected[b]	Virus titer[c]	Primary disease		Recurrent disease	
				N	Severity[d]	N	Frequency[e]
Eugenol	10	6	4.56 ± 0.4*	5**	4.2 ± 1.1	6	8.67 ± 1.98
PBS	10	10	6.56 ± 0.31	10	5.5 ± 0.7	10	10.2 ± 1.84

[a] Treatment was administered immediately prior to intravaginal HSV-2 inoculation.
[b] Animals that were asymptomatic were defined as infected if virus was isolated from day 1 swabs.
[c] Mean ± S.E: log 10 of pfu/ml from day 1 swabs calculated using only animals from which virus was isolated.
[d] Mean ± S.E: severity measured as the area under the lesion score-day curve.
[e] Mean ± S.E: frequency as number of days on which recurrences were observed.
* $P < 0.005$ vs. PBS.
** $P < 0.05$ vs. PBS.

regarding the efficacy of N-9 containing products in preventing STDs (Roddy et al., 1998b); this combined with safety concerns associated with current commercial preparations has led to efforts to develop new N-9 containing formulations (Maguire et al., 1998). While this strategy may increase the efficacy of N-9 containing products it is uncertain that such an approach will substantially alter N-9's undesirable safety profile. Aside from N-9, there are few other microbicide candidates in clinical testing (Rosenthal et al., 1998). Two examples are C31G (a mixture of non-ionic detergents produced by Biosyn) and PRO2000 (a sulfated polymer developed by Procept). The safety and efficacy of these newer products have not yet been established. Given the current state of microbicide development, there is need for continuing drug discovery. As plants represent a source of potentially inexpensive and readily available materials, we examined 19 plant-derived compounds for their activity against HSV-2 in vitro and in vivo. Several compounds showed activity in vitro but performed poorly in vivo. This failure of in vitro results to predict in vivo efficacy has previously been noted by Zeitlin et al. (1997). In our experiments, when tested unformulated, four compounds, carrageenan lambda type IV, cineole, curcumin, and eugenol, afforded mice significant protection against disease caused by intravaginal HSV-2 challenge. We believe that these compounds warrant further evaluation to determine their spectrum of activity, vaginal irritant properties, efficacy in vivo when formulated in clinically useful vehicles, and mechanism of action. Our results, and those of others (Pauwels and De Clercq, 1996; Qu et al., 1997; Zacharopoulos and Phillips, 1997; Zeitlin et al., 1997; Lampe et al., 1998) support further exploratory studies of natural products in the search for safe and effective topical microbicides.

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