Biological Activity Assessment of a Novel Contraceptive Antimicrobial Agent

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ABSTRACT: Microbicides are a new category of compounds being developed as a prophylactic approach for the prevention of transmission of sexually transmitted diseases (STDs), including the human immunodeficiency virus (HIV). These are primarily being developed as women-controlled methods, with the target of designing new compounds or formulations that can be used without the knowledge of a male partner. Microbicide screening can be initially based on their hyaluronidase-inhibiting (HI) activity, as this enzyme plays a major role in the sperm and microbe penetration into the substrate. Derivatives of hesperidin, a citrus flavonoid glycoside, have been reported in the literature for their HI effects. Hesperidin was thereby sulphonated under strictly controlled conditions and the active fraction isolated and characterized, based on its HI activity. This derivative was screened for antimicrobial and enzyme-inhibitory activities, specifically for the reproductive tract. Sulphonated hesperidin (SH) was found to completely inhibit the sperm enzymes hyaluronidase, giving an indication toward its contraceptive effects. It was also been found to inhibit various sexually transmitted pathogens, including *Chlamydia trachomatis, Neisseria gonorrhoea,* HIV, and Herpes Simplex virus type 2 (HSV-2). Its safety assessment was based on its noninterference in sperm motility and its penetration through the cervical mucus, and no effect on the growth of *lactobacilli,* the normal vaginal flora. It was also found to be nontoxic to the HIV substrate cells (MT2 cells). The study concludes that sulphonated hesperidin can be developed as a potential microbicide for a dual prophylaxis of contraception and transmission of STDs and AIDS.

Key words: Hesperidin, microbicides, hyaluronidase, STDs, HIV.

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As per the recent UNAID statistics (http://www.unaids.org/wad2004/index.html), about 37.8 million adults (globally) are living with human immunodeficiency virus (HIV) and close to half of them are women. HIV, the etiologic agent for acquired immunodeficiency syndrome (AIDS) has become the major threat to the security and development of many parts of the world. Young women are especially vulnerable to get this and similar infections. As of now, there is no conclusive treatment to eliminate this virus from the body once the infection has taken place. Timely use of anti-HIV drugs can prevent opportunistic infections and can keep one healthy for a few years. Prevention is considered to be the best option, about 28 times more effective than treatment (Marseille, 2002). Attempts to develop vaccines against HIV have not been very successful so far due to the ever-changing variants.

Microbicides, a new category of prophylactics, are being developed simultaneously as an alternative approach for prevention of transmission of HIV and other sexually transmitted diseases (STDs), and are getting wide attention as indicated by the recently held meeting Microbicides-2004 in London (March, 2004). These are topical pharmaceutical products, applied to the genital area, with the capacity to prevent transmission of STDs, particularly the HIV and with or without sperm-inhibitory activity, thereby serving as contraceptives as well (Reich, 2001). Topical microbicides can provide excellent potential for a female-controlled, preventive option, which would not require negotiation, consent or even knowledge of the partner. Both women and men would benefit, as these can be bidirectional (Zerodie and Holschneider, 2001). An ideal microbicide will effectively inhibit STD pathogen transmission while causing limited disruption to the structural integrity and function of the healthy cervico-vaginal epithelium without inhibiting the vaginal lactobacillus, the most prevalent component of the reproductive tract's dynamic ecosystem. These beneficial bacteria help protect the vagina from pathogenic microbes (Klebanoff and Coombs, 1991).

HIV/AIDS is slowly, but gradually, turning into a female-oriented pandemic. In almost all the regions of the world, the proportion of women living with HIV has increased over the years. East Asia experienced the sharpest increase, of 56% since 2002, followed by eastern Europe and central Asia at 48% (http://www.thebody.com/unaids/

women_challenge.html). In sub-Saharan Africa, about 76% of the young people living with HIV are females. The sociocultural factors, higher anatomical susceptibility, and lack of a voice in sex-related decisions are some of the factors that make women more prone to HIV infection.

Microbicide, as a new prophylactic tool, can play a significant role by empowering women to protect themselves, especially in cases of a noncooperative male partner. Although there are about 60 microbicides in different stages of preclinical and clinical development, a safe and efficacious preparation is not yet available to those needing them urgently. As a result, research need to be continued to identify new agents and formulations, and present research is an effort in that direction.

Hyaluronidase-inhibiting activity has been used as a powerful tool in the initial screening of microbicide compounds. The enzyme hyaluronidase has been shown to affect the dispersal of follicle cells surrounding the ova in rabbits. It was found that testicular hyaluronidase is required to disperse follicular cells surrounding the ova, thus permitting sperm penetration (Pincus et al, 1948).

Hyaluronidase inhibitors (HI) have also shown activity against mammalian spermatozoa (Anderson et al, 2002). In vitro and in vivo studies have proved HI prevents the follicle cell-dispersing activity of sperm (Martin and Beiler, 1952). Inhibition of sperm/enzymes will lead to the disruption of the fertilization process and thereby prevent conception. Flavonoids that inhibit sperm testicular hyaluronidase also inhibit microbial hyaluronidase, lysozyme, and tyrosinase (Rodney et al, 1950). Hesperidin, a flavonoid glycoside, possesses a wide range of pharmacological properties and is available as an inexpensive byproduct of citrus cultivation (Garg et al, 2001). Derivatives of hesperidin, particularly sulphonated and phosphorylated derivatives, have been studied by various scientists as hyaluronidase inhibitors (Beiler and Martin, 1948; Preston et al, 1953; Joyce et al, 1979; Joyce and Zaneveld, 1985; Joyce et al, 1986). Though this pharmacological activity has been reported, no effort has been made toward the validation of a synthetic procedure, isolation and characterization of the active fraction, and a detailed microbiological study as an effective microbicide, of the same. In an attempt to develop a potential microbicide, sulphonation of hesperidin was attempted under carefully controlled conditions. The active moiety (on the basis of HI activity) was separated from the mixture and characterized by the use of modern analytical techniques. The compound (sulphonated hesperidin [SH]) was then evaluated for various biological activities, including sperm enzyme (hyaluronidase) inhibition, inhibitory effect against different STD-causing microbes, lactobacillus inhibition, and sperm-function tests to assess the safety of the compound.

Materials and Methods

Materials

Hesperidin (97%) was obtained from Sigma-Aldrich Inc (St Louis, Mo). Sulphuric acid (98%), used for sulphonation, was obtained from SD Fine Chemicals Ltd (Mumbai, India). All other reagents were of analytical grade. The materials and methods for the biological assays have been discussed in detail in the relevant references (Reissig, 1955; Anderson et al, 2000).

Synthesis of Sulphonated Hesperidin

Sulphonation of hesperidin was carried out under strictly controlled conditions. Reactions were carried out at different temperatures (0°C to 60°C), over a range of reaction times (1–8 hours), using different sulphonating agents (concentrated sulphuric acid, chlorosulphonic acid) of varying concentrations (90%–100%), and in an appropriate solvent (chloroform, nitrobenzene, dimethylformamide, ethyl acetate), on a shaking water bath. The final reaction conditions were selected after evaluating each derivative for its HI activity and those resulting in the best activity, yield, and physical properties were selected. The sulphonic acid formed was precipitated as a sodium salt to get SH.

The final reaction conditions that gave a mixture of significant HI activity (99%–100%) and yield (68%) consisted of concentrated sulphuric acid (98%) as a sulphonating agent and ethyl acetate in equal proportions. The reaction was carried out for 5 hours at 40°C and finally quenched in absolute alcohol. The compound was precipitated as a sodium salt.

Separation and Characterization of the Active Component

The precipitated product consisted of a mixture of 3 components as ascertained by thin-layer chromatography (TLC). The chromatographic system consisted of precoated RP C-18 silica plates (Macherey-Nagel, Germany) as the stationary phase and methanol:n-butanol:water:ammonia (4:3:2:1) as the mobile phase. The analysis was carried out at 20°C. The mixture was separated into individual components using preparative TLC. The analytical TLC system was extrapolated to preparative scale using preparative RP C-18 silica plates, thickness 1 mm (Macherey-Nagel), using the same mobile phase and conditions. The active constituent was determined based on the HI activity of the 3 components and the most active fraction (R_f .89) was designated as SH.

SH was characterized based on various modern analytical techniques, including ultraviolet spectroscopy, infrared spectroscopy, nuclear magnetic resonance spectroscopy (^{1}H and ^{13}C), Matrix Assisted Laser Desorption-Time of Flight mass spectroscopy, and elemental analysis. Using the spectral details and analyses obtained, a molecular formula and a structure was assigned to the active moiety.

Biological Activity Studies of SH

Enzyme (Hyaluronidase)/Inhibition Studies—Hyaluronidase inhibition assay was based on the colorimetric determination of *N*-acetylglucosamine (Reissig, 1955) and involved quantification of the hydrolysis of its natural substrate, the hyaluronic acid, by
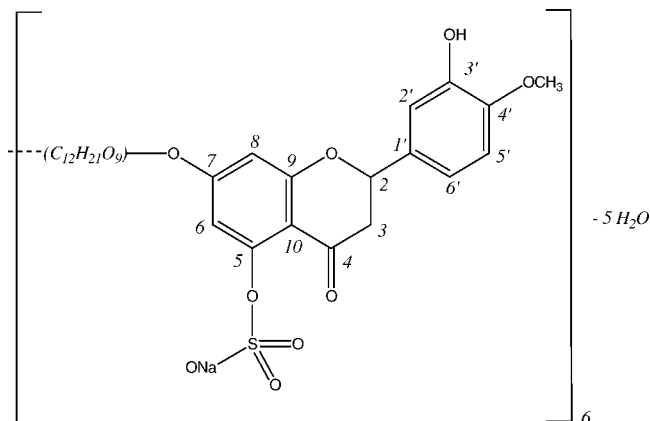
Figure 1. Chemical structure of sulphonated hesperidin (SH).

bovine testicular hyaluronidase. The enzyme was incubated with the test agent for 5 minutes at 37°C and reaction was started by adding the hyaluronic acid. A dose-response study involving the inhibition of enzyme by different concentrations of the test agent and a reversibility of inhibition study to assess the reversibility of the enzyme activity were also conducted.

Evaluation of dose-response for hyaluronidase inhibition. Different concentrations of the test agent were prepared, ranging from 0 to that concentration that would inhibit hyaluronidase by approximately 80%–95% (0.0125–0.2 mg/mL). The HI study was carried out as per the previously described method (Reissig, 1955) with each test concentration. The absorbance was converted to percentage inhibition, taking the absorbance of minimum concentration (0.0125 mg/mL) as 100%. Thereby, the values for inhibition concentrations (ICs) IC_{20}, IC_{50}, IC_{80}, and IC_{100} were determined. A dose-response graph was plotted between concentration of the test agent and percentage inhibition of hyaluronidase.

Determination of reversibility of hyaluronidase inhibition. Reversibility of inhibition was approximated by the previously published method (Ackermann and Potter, 1949), in which the level

of inhibited enzyme activity was determined in the presence of different amounts of the enzyme. Reversibility was determined by plotting the activity in the presence of a fixed concentration of inhibitor as a function of amount of enzyme assayed (ie, activity on *y*-axis, amount of enzyme on *x*-axis). If line of regression passed through the origin, inhibition was considered to be reversible. If the line passed below the origin, inhibition was considered to be irreversible.

Antimicrobial Testing—Antimicrobial assays were divided into viral and bacterial assays. HSV and HIV were selected as representative viral candidates and chlamydia and gonorrhea as bacterial candidates for sexually transmitted pathogens. The microbes were exposed to SH and the mixture was inoculated into the target cells. SH was removed by centrifugation or dilution before the microbe was allowed to replicate within the target cells (Anderson et al, 2000). Therefore, inhibition in SH-treated samples was due to inhibition of either viral/bacterial binding or entry into the target cells or both.

Viral inhibition. For studying HSV inhibition, infectivity of CaSki cells by HSV-2 was measured (Anderson et al, 2000) after treating the virus with different concentrations of SH ranging from 0.1 to 100 µg/mL. HIV-1 (lymphotropic and monocytotropic strains) infectivity in the presence of SH was evaluated with a viral binding inhibition assay. Serial dilutions of SH, ranging from 3.2 to 100 µg/mL, were added to MT-2 cells and, after the specified period of incubation, were studied for synctia formation.

Bacterial inhibition. Infection of HeLa cells by *C trachomatis* in the presence and absence of SH was evaluated as described by Cooper et al (1990). Serial dilutions of elementary bodies were added to different concentrations of SH (1–1000 µg/mL). After inoculation into the HeLa cell monolayers, chlamydia-induced inclusions were measured by immunofluorescence after reacting the cultures with a Kallsted chlamydia culture confirmation fluorescein-conjugated antibody (monoclonal).

Inhibition of *N gonorrhoeae* growth on agar by SH was measured as described earlier by Anderson et al (1998). SH (1–1000 µg/mL) was incorporated directly into gonococcal agar, and this

Table 1. *Biological activities of sulphonated hesperidin (SH)*

| Class	Property	Potency	Comments
Enzyme inhibition	Hyaluronidase inhibition	Very high	Complete inhibition of sheep testicular hyaluronidase at 200 µg/mL and 50% at 50 µg/mL
Antimicrobial	Anti-HIV	High	Complete inhibition at 100 µg/mL
	Anti-HSV-2	High	100% inhibition at 100 µg/mL
	Antigonococcal	Moderate	33% inhibition at 100 µg/mL and 48% at 1000 µg/mL
	Antichlamydial	Moderate	85% inhibition at 1000 µg/mL
Safety	Lactobacilli inhibition	None	No effect on the growth, 108% growth of the test as compared with the control at 5 mg/mL
	Sperm function:		
	• Sperm mobility	None	No effect on sperm motility at a concentration of 25 mg/mL; sperm motility was greater than 95%
	• Cervical mucous penetration	None	Inhibition of migration at a concentration of 1 mg/mL is less than 5% and even, at 5 mg/mL, inhibition is only about 7%
	Host cell toxicity (HIV)	None	50% of MT-2 cells were inhibited at a concentration greater than 3000 µg/mL
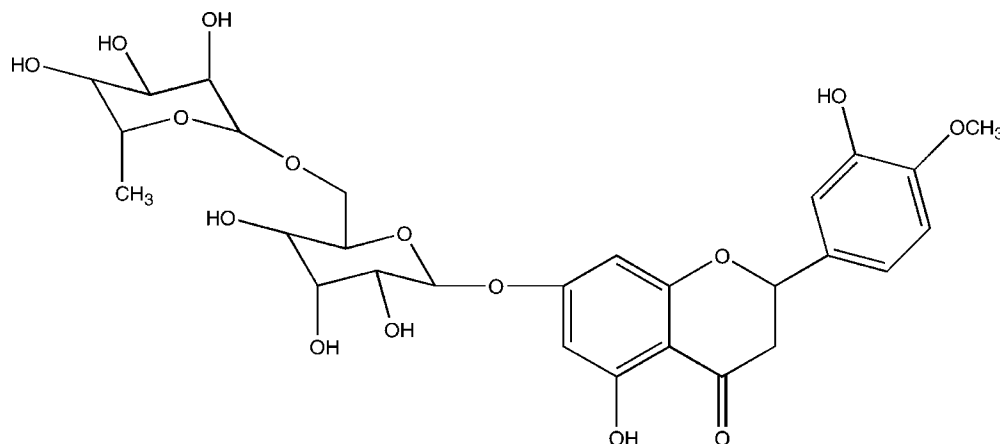
Figure 2. Structure of hesperidin (hesperetin 7-rhamnoglucoside).

was inoculated with dilutions of *N gonorrhoeae* and the colonies enumerated after overnight incubation at 37°C in 5% CO_2.

Safety Assessment—Sperm-function tests, growth of normal vaginal flora (*Lactobacillus gasseri*) in the presence of SH were chosen for safety assessment. In addition, cytotoxicity to target cells (MT-2 cells in HIV-inhibition testing) also helped in ascertaining the safety index of the compound.

Sperm-function tests. Sperm-function tests included inhibition of cervical mucus penetration by human spermatozoa and sperm immobilization assays (Anderson et al, 2000, 2002). Cervical mucus penetration was determined by measuring the distance through which the most progressive spermatozoa migrated through bovine cervical mucus in the presence of the test agent. A sperm immobilization assay was conducted to assess the spermicidal activity of the compound. Sperm motility in the presence of the test agent confirms the noncytotoxic nature of the compound if the sperm are not killed and are still mobile. In this assay, different concentrations of the test agent were mixed with freshly ejaculated semen and the percentage of motile spermatozoa was determined by brightfield microscopy (400×) just before and 30 seconds after the mixing. Data was presented as the percentage of motile spermatozoa. If inhibition was less than

90% when the test agent was present at 5 mg/mL, the test agent was considered inactive and, thereby, nontoxic to the sperm.

Lactobacillus inhibition. Growth of *Lactobacillus gasseri* was estimated turbidiometrically, as described by Anderson et al (1998). SH was added to an active culture of the microbe under anaerobic conditions. Suspensions with and without SH were measured for absorbency at 550 nm, beginning at 120 minutes of incubation at 37°C and at 20-minute intervals for a total of 260 minutes. Doubling times of growth were calculated from plots of ln(absorbency) vs time.

Toxicity to host cells. Host cells (MT2 cells) were examined at the end of the experiment for signs of test agent-induced damage (confluence and general condition). Modulation of virus-induced cytopathic effects were measured by determining percent reduction in optical density with an XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide) dye-reduction assay. Virus-induced cytopathic effects and mechanical artifacts were verified by microscopic observations. Undamaged host cells confirmed the nontoxicity of the compound to the host cells and thereby also to the vaginal membrane cells.
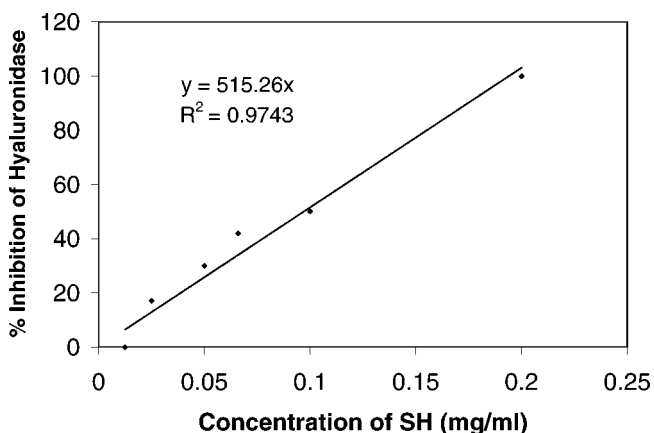
Results

Sulphonation of Hesperidin

It was observed that sulphonation of hesperidin led to a compound with completely changed physical and chemical properties. Sulphonation occurred at only 1 position in each parent unit and 6 sulphonated units polymerized

Figure 3. Dose response for sulphonated hesperidin (SH) on the inhibition of hyaluronidase.

Table 2. *Anti-HIV evaluation of sulphonated hesperidin (SH)**

Compound	CTS IC_{50} (μg/mL)	VBI-IIIB IC_{50} (μg/mL)	VBI-BaL IC_{50} (μg/mL)
Control	>100	<1	7.2
SH	>3000	31.2	6.2

* IC_{50} indicates 50% inhibitory concentration; CTS, cytotoxicity assay; VBI-IIIB, viral entry inhibition assay (lymphocytotropic strain); and VBI-BaL, viral entry inhibition assay (monocytotropic strain).
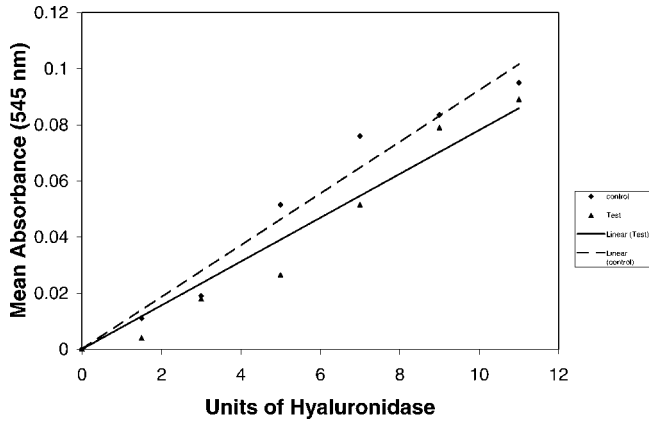
Figure 4. Reversibility of hyaluronidase-inhibiting (HI) activity of sulphonated hesperidin (SH).

Figure 5. Effect of sulphonated hesperidin (SH) on HSV-2 infectivity.

(accompanied with dehydration reactions), to form a polymeric, high molecular weight, sulphonated compound (SH), which had a molecular weight of 4182 Dalton and a molecular formula $C_{168}H_{188}O_{98}S_6Na_6$ (Figure 1), as compared to hesperidin (Figure 2), which has a molecular weight of 610 Dalton and a molecular formula ($C_{28}H_{34}O_{15}$). There is also evidence in the literature that high molecular weight, polymeric, polyanionic compounds are good inhibitors of hyaluronidase, thereby, supporting the activities of SH (Pantitschko and Kaiser, 1951; Spensky and Rogers, 1954).

Biological Activities of SH

The results of all the biological tests conducted on SH have been compiled in Table 1. SH was found to completely inhibit sheep testicular hyaluronidase at a concentration of approximately 200 μg/mL. This was nearly 50-fold higher as compared with unsulphonated hesperidin (2.9% at 200 μg/mL). Inhibition was dose dependant (Figure 3), with an IC_{20} (concentration that inhibits enzyme activity by 20%) of 0.0388 mg/mL, IC_{50} of 0.0970 mg/mL, IC_{80} of 0.1552 mg/mL, and complete inhibition (IC_{100}) achieved at 0.194 mg/mL. The effect was also reversible in nature. The curve fit to the data obtained when SH was present in the assay passed through the origin (y-intercept = −0.0072: 90% confidence interval = −0.0159

to 0.00148). The y-intercept for control assays (no SH) was −0.0011 and the 90% confidence interval was −0.0123 to 0.00905. These data are consistent with reversible inhibition as seen in Figure 4. Inhibition of hyaluronidase indicates its likely potential as a vaginal contraceptive.

SH was found to be very effective against the enveloped viruses, including HIV-1 and HSV-2. It inhibited both strains of HIV (lymphotropic and monocytotropic) at very low concentrations. The IC_{50} for HIV-1$_{IIIB}$ (lymphocytotropic strain) infection was found to be 31.2 μg/mL and for HIV-1$_{BaL}$ (monocytotropic strain) was 6.2 μg/mL (Table 2). SH was found to be even more active against HSV-2. The anti–HSV-2 activities of SH have been described in Figure 5. IC_{50} was determined to be approximately 2 μg/mL and IC_{100} 93 μg/mL.

SH was found to inhibit the infection of HeLa cells by *C trachomatis* in a dose-dependent manner, with an IC_{50} of approximately 310 μg/mL and IC_{87} (considered as complete inhibition) of 1000 μg/mL, or 1 mg/mL. Higher concentrations were required to inhibit chlamydia infec-

Table 3. *Inhibition of cervical mucus penetration by sulphonated hexperidin (SH)*

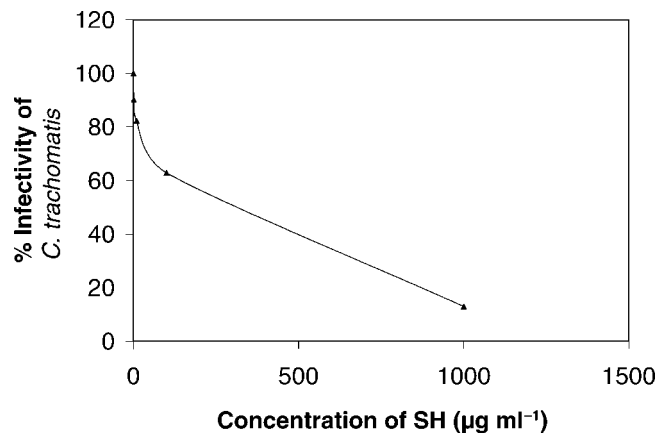| Compound	Concentration (mg/mL)	Sperm Migration; Mean ± SD (% of control)	Sample Size
0.9% NaCL (Control)	. . .	100 ± 0.0	9
SH	5.0	93.5 ± 2.6	9
	1.0	96.1 ± 1.4	9
	0.5	98.5 ± 1.4	9

Figure 6. Inhibition of *Chlamydia trachomatis* infectivity by sulphonated hesperidin (SH).
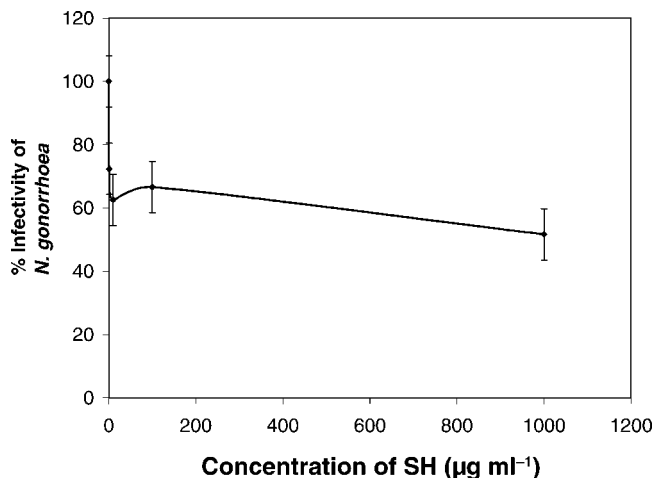
Figure 7. Inhibition of *Neisseria gonorrhoeae* by sulphonated hesperidin (SH).

Figure 8. Growth of lactobacillus in the presence of sulphonated hesperidin (SH) as compared with control.

tivity compared with that required for viral infectivity (Figure 6). SH also inhibited the growth of *N gonorrhoeae* on agar. Inhibition was dose dependent at concentrations ranging from 1 μg/mL to 1000 μg/mL. The calculated IC_{50} for gonococcal inhibition was approximately 1 mg/mL (Figure 7). Complete inhibition (IC_{100}) could not be achieved at the concentrations tested.

SH did not inhibit the migration of human spermatozoa through bovine cervical mucus (Table 3). Inhibition of migration at a concentration of 1 mg/mL was less than 5% and, even at 5 mg/mL, inhibition was only about 7%. Also, in contrast with its effect on the sperm enzyme hyaluronidase, SH minimally immobilizes the human spermatozoa. At a concentration of 5 mg/mL, SH (n = 9) inhibited the fraction of motile spermatozoa by only approximately 6%. This small effect is far less than sperm immobilization caused by the spermicide N-9 (IC_{50} = 88 μg/mL), as reported by Anderson et al (2000).

In contrast with the inhibitory effect of SH against sexually transmitted infection (STI)–causing organisms, it does not affect the growth of a commercial culture of the beneficial microbe *Lactobacillus gasseri* originating from a human vaginal isolate. The doubling time of *Lactobacillus* growth in the presence of 5 mg/mL SH was 142 minutes (90% confidence limits = 125.8–161.7 minutes). This was essentially the same (rather more) as the doubling time of 153 minutes (90% confidence limits = 136.6–173.5 minutes) seen for control cultures (Figure 8). The growth of *L gasseri* in the test sample was 108% that of the control. The concentration tested is several times higher than the concentrations of SH required for hyaluronidase inhibition or inhibition of viral or bacterial STI-causing microbes. Thereby, it is suggested that the compound is safe to the normal vaginal flora and would cause minimal interference with the vaginal environment.
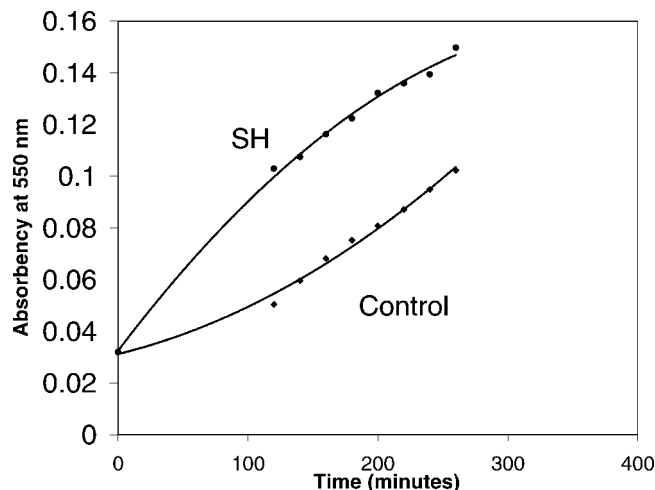
When tested for toxicity to the HIV host cells (MT-2)

used in the antiviral assays, no overt toxic effects were observed (IC_{50} > 3000 μg/mL). This nontoxicity to the host cells can be an indication that the compound would also be nontoxic to the epithelial cells of the vagina. SH was found to be even more active against HSV-2 as compared with HIV-1. The constants of inhibition included an IC_{50} of approximately 2 μg/mL and IC_{100} of 93 μg/mL (Table 2).

Discussion

Early work by other investigators have suggested various sulphated polymers with different molecular weights to have significant microbicidal and contraceptive properties. The objectives of the present study were to confirm similar findings for sulphonated hesperidin regarding its effects on the enzyme hyaluronidase and sperm functions and to define the extent of its antimicrobial properties.

Hyaluronidase is known to be present within and around the sperm acrosome. It eases the passage of spermatozoa through the cumulus oophorous and the zona pellucida, vestments that surround the unfertilized oocyte (Rogers and Bentwood, 1982; Yanagimachi, 1988). Hyaluronoidase inhibitors have long since been researched as antimicrobials and contraceptives (Pincus et al, 1948; Sieve, 1952; Joyce et al, 1979; Joyce and Zaneveld, 1985; Joyce et al, 1986). Also, different forms of hyaluronidase may help the penetration of target tissues by pathogenic microbes, including protozoal, bacterial, and viral pathogens. Thereby, hyaluronidase activities may support both mammalian fertilization and infection by certain STI-causing microbes.

It has been proven that SH inhibits hyaluronidase in a dose-dependent manner. The inhibition is also reversible

in nature. Hyaluronidase is a prerequisite to successful oocyte penetration by spermatozoa. Hyaluronidase inhibition thereby would prevent the sperm penetration and subsequently conception. However, the compound was found to be totally nontoxic to the spermatozoa. It inhibited the penetration of cervical mucus by the spermatozoa minimally. Inhibition of migration at a concentration of 1 mg/mL was less than 5% and, even at 5 mg/mL, inhibition was only about 7%. Also, it caused minimal immobilization of the sperm. At a concentration of 5 mg/mL, SH inhibited the fraction of motile spermatozoa by only approximately 6%. This small effect is far less than sperm immobilization caused by the spermicide N-9 (Anderson et al, 2000).

SH was found to effectively inhibit the enveloped viruses, including HSV-2 and HIV-1, with IC_{50} values in the submicrogram/milliliter range. Nonsulphonated hesperidin has been proven to be ineffective in inhibiting the HIV virus (Hu et al, 1994). The sulphonated derivative also inhibited other pathogens, including *C trachomatis* and *N gonnorhoeae,* in vitro. All these microbes are serious health hazards leading to difficult situations and cure.

A vaginally applied agent intended for contraception and prevention of STIs must have a high safety index, having minimal effects on the normal vaginal flora. The noninterference with the vaginal microflora, including *L gasseri,* is very significant in the case of vaginal preparations because the low pH and the hydrogen peroxide produced by the lactobacillus create a hostile environment for pathogenic microorganisms and help to maintain a healthy vaginal ecosystem (Mardh, 1991). In the case of SH, despite the inhibitory effects on the enzyme and the microbes, there was a general lack of cytotoxic effects on several cell types. It was found to be totally noncytotoxic to human sperm. The minimal effect on sperm mobility and cervical mucus penetration of sperm, by the test agent, is indicative of its nontoxicity to the sperm, even although it inhibits the sperm enzyme hyaluronidase. A nonspermicidal compound is very less likely to be toxic to the vaginal epithelial cells as well. This test was thereby important in assessing the safety of the test agent.

Also, it was found to be nontoxic to host cells of HIV (MT 2 cells).

Present studies show that SH has a high margin of safety. No evidence is seen toward the mediation of antimicrobial activities through cytotoxic mechanisms. Our results show that the host cells used in the viral and bacterial assays were not overtly damaged when SH was present in concentrations several times higher than those required to inhibit infectivity.

Because the molecular weight of SH was determined to be around 4000 Dalton, it is not likely to be absorbed when applied vaginally (Anderson et al, 2002). Poor systemic absorption reduces the expression of any yet-to-be-determined side effects. These observations, thereby, support an excellent safety profile for this compound.

We can thereby conclude that SH, with the assigned structure, is a potential candidate for preventing the spread of STDs and also conception. Its activities against the microbes, without affecting the normal, healthy cells (sperm, vaginal tissue) or the microbial flora of the vagina, confirm its high safety index. Its high solubility and stability are excellent properties for developing a good vaginal dosage form. Further studies on animals need to be done to get in vivo data and take the compound further toward development.

Acknowledgments

References

Ackermann WW, Potter VR. Enzyme inhibition in relation to chemotherapy. *Proc Soc Exp Biol Med.* 1949;72:1–9.

Anderson RA, Feathergill K, Diao X, et al. Evaluation of poly(styrene-4-sulfonate) as a preventive agent for conception and sexually transmitted diseases. *J Androl.* 2000;21:862–875.

Anderson RA Jr, Feathergill K, Kirkpatrick R, et al. Characterization of cysteamine as a potential contraceptive anti-HIV agent. *J Androl.* 1998;19:37–49.

Anderson RA, Jr, Feathergill KA, Diao XH, et al. Preclinical evaluation of sodium cellulose sulphate (ushercell) as a contraceptive antimicrobial agent. *J Androl.* 2002;23:426–438.

Beiler JM, Martin GJ. Inhibition of hyaluronidase action by derivatives of hesperidin. *J Biol Chem.* 1948;174:31–35.

Cooper MD, Rapp J, Jeffery-Wiseman C, Barnes RC, Stephens DS. *Chlamydia trachomatis* infection of human fallopian tube organ cultures. *J Gen Microbiol.* 1990;136:1109–1115.

Garg A, Garg S, Zaneveld LJD, Singla AK. Chemistry and pharmacology of the citrus bioflavonoid hesperidin. *Phytother Res.* 2001;15:655–669.

Hu CQ, Chen K, Shi Q, Kilkuskie RE, Cheng YC, Lee KH. Anti-AIDS agents. *J Nat Prod.* 1994;57:42–51.

Joyce C, Freund M, Peterson RN. Contraceptive effects of intravaginal application of acrosin and hyaluronidase inhibitors in rabbit. *Contraception.* 1979;19:95–106.

Joyce CL, Zaneveld LJD. Vaginal contraceptive activity of hyaluronidase and cyclooxygenase inhibitors in the rabbit. *Fertil Steril.* 1985;44:426–428.

Joyce CL, Mack SR, Anderson RA, Zaneveld LJD. Effect of hyaluronidase, beta-glucuronidase and beta-*N*-acetyl glucosaminidase inhibitors on sperm penetration of the mouse oocytes. *Biol Reprod.* 1986;35:336–346.

Klebanoff SJ, Coombs RW. Viricidal effect of *Lactobacillus acidophilus* on human immuno deficiency virus type 1: possible role in heterosexual transmission. *J Exp Med.* 1991;174:289–292.

Mardh PA. The vaginal eco-system. *Am J Obstet Gynecol.* 1991;165: 1163–1168.

Marseille E. Prevention 28 times more effective than treatment. *Lancet.* 2002;359:1851–1856.

Martin GJ, Beiler JM. Effect of phosphorylated hesperidin, a hyaluronidase inhibitor on fertility in the rat. *Science.* 1952;115:402.

Pantitschko M, Kaiser E. The activation and inhibition of hyaluronidase. *Biochem Z.* 1951;322:137–149.

Pincus G, Pirie NW, Chang MC. The effects of hyaluronidase inhibitors on fertilisation in the rabbit. *Arch Biochem.* 1948;19:388–396.

Preston RK, Avakian S, Beiler JM, Moss JN, Martin GJ. In-vivo and in-vitro inhibition of hyaluronidase by organic phosphates. *Exp Med Surg.* 1953;11:1–8.

Reich MR. The global drug gap: policy implications for microbicides. *AIDS.* 2001;15(suppl):14–17.

Reissig JL. A modified colourimetric method for the estimation of *N*-acetylamino sugars. *J Biol Chem.* 1955;217:959–966.

Rodney G, Swanson AL, Wheeler LM, Smith GN, Worrel CS. The effect of a series of flavonoids on hyaluronidase and some other related enzymes. *J Biol Chem.* 1950;183:739–747.

Rogers BJ, Bentwood BJ. Capacitation, acrosome reaction and fertilisation. In: Zaneveld LJD, Chatterton RT, eds. *Biochemistry of Mammalian Reproduction.* New York: John Wiley and Sons; 1982:203–230.

Sieve BJ. A new antifertility factor. *Science.* 1952;116:373–385.

Spensky PC, Rogers HG. Enzyme inhibition. *Nature.* 1954:3485.

UNAIDS. AIDS Epidemic Update. 2004. http://www.unaids.org/wad2004/index.html. Accessed November 29, 2004.

UNAIDS. The Complete HIV/AIDS Resource. 2004. http://www.thebody.com/unaids/women_challenge.html. Accessed on January 7, 2005.

Yanagimachi R. Mammalian fertilisation. In: Knobil E, Neill J, Ewing LL, Greenwald GS, Markert CL, Pfaff DW, eds. *The Physiology of Reproduction.* New York: Raven Press; 1988:135–185.

Zerodie D, Holschneider S. Expanding on preventive options: the role of the international community in providing an enabling environment for microbicides. *AIDS.* 2001;15(suppl):5–6.