Prevalence and prevention of sexually transmitted viral infections in women from the Bolivian Amazonas

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To my beloved family

"The major problems in the world are the result of the difference between how nature works and the way people think"

"Los mayores problemas del mundo son el resultado de la diferencia entre el funcionamiento de la naturaleza y el pensamiento humano"

Gregory Bateson

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ABSTRACT

This thesis investigates the prevalence of sexually transmitted viral infections in women living in the Amazonas region of Bolivia and explores whether Bolivian medical plants can affect the immune system and prevent or treat infections with Herpes simplex virus type 2 (HSV-2) and inflammation. My PhD project was financed by the Swedish International Development Cooperation Agency (SIDA), and I did my work alternately in Bolivia and in Sweden. In La Paz, Bolivia, I work at the Universidad Mayor de San Andrés. The university there has a large collaboration project together with international and local organizations who work in a cooperative program that aims to improve the health of women, especially in poor rural areas of the Amazonas that are inhabited mainly by indigenous tribes. Part of this thesis work was carried out in Bolivia as fieldwork during the dry seasons. This involved the collection and preparation of human biological samples and medical plants that were later used in clinical laboratory assessments and experimental studies at the University of Gothenburg, Sweden.

In the cross-sectional study of 394 indigenous participants in **Paper I**, 64% were found to be positive for at least one viral sexually transmitted infection. The seroprevalence of HSV-2 was 53% and that of hepatitis B virus (HBV) was 10.3%. Of the women with antibodies to HBV, 16% also had HBV antigen in their blood, indicating ongoing infection. The frequency of high-risk human papillomavirus (HPV) infection was 27%, with the most prevalent high-risk HPV types being HPV 56, 39 and 31, followed by HPV 16 and 18. None of the participants were seropositive for HIV. For **Papers II** and **III**, plants used in traditional Tacana medicine as anti-infectious and anti-inflammatory remedies were collected with the help of a local guide. Hydro-ethanolic extracts of *Equisetum giganteum*, *Croton lechleri*, *Uncaria tomentosa*, *Copaifera reticulata*, *Tipuana cf tipu*, *Mangifera indica* and *Erythroxylum coca* efficiently blocked HSV-2 infection of cell cultures without any significant cytopathic effects. In

Paper II, we show that *E. giganteum*, *C. lechleri*, *U. tomentosa*, and *C. reticulata* can prevent HSV-2 infection in a mouse model genital herpes, and we also demonstrate that extracts of these plant efficiently block viral attachment and entry but not viral replication post-entry. In **Paper III**, we show that extracts of *T. tipu* and *M. indica* not only block viral infectivity, but are also efficient antiviral agents when administered after viral entry in Vero cells. *T. tipu* also promotes anti-viral immunity by inducing the production of type III interferons, and it primes for both inflammatory (IL-1β) and chemotactic (CXCL10) chemokines in human peripheral blood mononuclear cells. In **Paper III,** we show that several of these plants have anti-inflammatory properties, as they block LPS-induced inflammasome activation and subsequent release of IL-1β.

These studies reveal that infections with HPV, HBV and, in particular, HSV-2 are common in women in the Bolivian Amazonas, and that the pattern of high-risk HPV types differs from that covered by the HPV vaccine Gardasil. Several medicinal plant extracts are identified as promising anti-HSV-2 microbicides, and some of these plants can also modify anti-viral and inflammatory responses.

Keywords: Sexually transmitted infections, indigenous women, Bolivian Amazonas, HSV-2, HBV, HPV, medical plants, microbicides, cytokines

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SAMMANFATTNING PÅ SVENSKA

I mitt avhandlingsarbete har jag dels studerat hur vanligt det är med sexuellt överförda virusinfektioner bland kvinnor i Amazonas i Bolivia och dessutom undersökt om medicinalväxter från Amazonas kan påverka immunförsvaret och användas för att förhindra och/eller behandla genitalherpes och inflammation. Mitt doktorandprojekt finansierades av den svenska biståndsorganisationen SIDA, och jag utförde mitt arbete omväxlande i Bolivia och i Sverige. I La Paz i Bolivia arbetar jag vid Universidad Mayor de San Andrés. Universitetet har ett stort samarbetsprojekt med internationella och lokala organisationer med en uttalad målsättning att förbättra kvinnors hälsa. Man har ett särskilt fokus på fattiga kvinnor på landsbygden i Amazonas där befolkningen domineras av den inhemska ursprungsbefolkningen. En betydande del av mitt avhandlingsarbete var fältarbete i Bolivia. Vi rekryterade friska kvinnor ute i byar och mindre städer, föreläste för dem om sexuellt överförbara virusinfektioner, intervjuade dem om deras levnadsvanor och tog sen både blodprover och cervixprover. Dessutom var vi på expeditioner i djungeln tillsammans med en lokal guide och samlade in medicinalväxter som den ursprungliga Tacana-stammen använder för att förebygga och behandla olika sjukdomar. Alla biologiska prover och växtextrakt skickades till Göteborgs universitet där jag sedan gjorde kliniska laboratorieanalyser och experimentella studier.

I en tvärsnittsstudie på 394 friska kvinnor (**arbete I**) kunde jag visa att 64% av kvinnorna hade minst en sexuellt överförd virusinfektion. 53% av kvinnorna hade antikroppar mot herpes simplex virus typ 2 (HSV-2) och 10% av kvinnorna hade antikroppar mot hepatit B-virus (HBV). 16% av de kvinnor som hade antikroppar mot HBV hade också HBV-antigen i blodet vilket kan betyda att de hade en kronisk infektion. 27% av kvinnorna hade en pågående infektion med ett hög-risk humant papillomvirus (HPV). De vanligaste hög-risk HPV-typerna de var infekterade med var HPV 56, 39 och 31 medan HPV 16 och 18 (som är de vanligaste hög-risk HPV i t.ex Sverige) var mindre vanliga. Ingen av kvinnorna HIV-positiv.

I **arbete II** och **III** använde vi vatten/etanol-extrakt av de olika växter vi samlat in för att studera deras antivirala och antiinflammatoriska egenskaper. Vi identifierade sex växter som kunde blockera en HSV-2-infektion *in vitro*: *Equisetum giganteum, Croton lechleri, Uncaria tomentosa, Copaifera reticulata, Tipuana cf tipu, Mangifera indica* och *Erythroxylum coca*.

I **arbete II** visade vi att *E. giganteum, C. lechleri, U. tomentosa och C. reticulata* kunde förhindra HSV-2 infektion *in vivo* i en musmodell för genitalherpes. De

fyra växtextrakten blockerade virusets förmåga att infektera celler, men hade dessvärre ingen effekt som behandling av en etablerad infektion. I **arbete III** testade vi därför ytterligare växter och identifierade två extrakt, *T. tipu* och *M. indica,* som inte bara blockerade virusinfektion utan även kunde användas för att behandla redan infekterade celler. Av dessa var extraktet från *T. tipu* extra intressant eftersom det aktiverade ett anti-viralt immunsvar i primära humana vita blodkroppar. Efter exponering utsöndrade de vita blodkropparna den anti-virala cytokinen interferon lambda och de började även transkribera inflammatoriska (IL-1β) och kemotaktiska (CXCL10) cytokiner. I **arbete III** visade vi också att flera av växtextrakten hade antiinflammatoriska egenskaper eftersom de kunde blockera LPS-inducerad inflammasom-aktivering och efterföljande frisättning av IL-1β.

Sammantaget visar jag i mitt avhandlingsarbete att HPV, HBV och i synnerhet HSV-2 har en hög prevalens hos kvinnor som lever i Amazonas i Bolivia och att HPV-infektion framför allt orsakades av andra HPV-typer än de som ingår i HPV-vaccinet Gardasil. Jag identifierade även flera medicinalväxter som skulle kunna användas som anti-HSV-2-mikrobicider, och visade att flera av dessa växtextrakt kunde påverka det anti-virala och det inflammatoriska immunsvaret.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Marianela Patzi-Churqui**, Katty Terrazas-Aranda, Jan-Åke Liljeqvist, Magnus Lindh, Kristina Eriksson. Prevalence of viral sexually transmitted infections and HPV high-risk genotypes in women in rural communities in the Department of La Paz, Bolivia. *Manuscript in-press. BMC Infectious Diseases, 2020.*

II. **Marianela Patzi-Churqui**, Liza Lind, Karolina Thörn, Alexandra Svensson, Otto Savolainen, Katty Terrazas-Aranda, Kristina Eriksson. Extracts of *Equisetum giganteum L* and *Copaifera reticulata Ducke* show strong antiviral activity against the sexually transmitted pathogen herpes simplex virus type 2. *J Ethnopharmacology. 2017;*210:192-197.

III. **Marianela Patzi-Churqui**, Alexandra Svensson, Karolina Thörn, Otto Savolainen, Roger Carvajal-Saravia, Katty Terrazas-Aranda, Kristina Eriksson. Antiviral and immunomodulatory potentials of medical plants *Tipuana cf tipu (Benth) Kuntze*, *Erythroxylum coca Lam* and *Mangifera indica L*. *Manuscript.*

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ABBREVIATIONS

STI	Sexually transmitted infection
HSV-2	Herpes simplex virus type 2
HPV	Human papillomavirus
HR-HPV	High risk - Human papillomavirus
LR-HPV	Low risk - Human papillomavirus
HIV	Human immunodeficiency virus
AIDS	Acquired immune deficiency syndrome
HBV	Hepatitis B virus
HCV	Hepatitis C virus
Anti-HBc	Anti-hepatitis B viral core (antibody)
HBsAg	Hepatitis B surface antigen
UNAIDS	Joint United Nations Programme on HIV/AIDS
ICO	Institut Català d'Oncologia
WHO	World Health Organization
PAHO	Pan American Health Organization
IARC	International Agency for Research on Cancer
DBS	Dried Blood Spots
DCCS	Dried Cervicovaginal Cell Spots
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
ORF	Open reading frame
MASL	Meters above sea level
ELISA	Enzyme-linked immunosorbent assay
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
OR	Odds ratio
CI	Confidence interval

PBS	Phosphate-buffered saline
Ig	Immunoglobulin
Ab	Antibodies
pDC	Plasmacytoid dendritic cell
NK	Natural killer (cell)
IFN	Interferon
IL	Interleukin
CXCL10	Chemotactic cytokine IFN-γ-inducible protein 10
NLRP3	Nod-like receptor protein 3
TLR	Toll-like receptor
CD	Cluster of differentiation
TORCH	Toxoplasmosis, Rubella, Cytomegalovirus and Herpes simplex
CIN	Cervical intraepithelial neoplasia
FAO	Food and Agriculture Organization of the United Nations
UMSA	Universidad Mayor de San Andrés
SELADIS	Instituto de Servicios de Laboratorio de Diagnóstico e Investigación en Salud
CIPTA	Consejo Indígena del Pueblo Tacana
CIMTA	Consejo Indígena de Mujeres Tacana
SIDA	Swedish International Development Cooperation Agency
TCO	Tierras comunitarias de origen
SUMI	Seguro universal materno infantile (Universal maternal and children insurance)

1 INTRODUCTION

1.1. Bolivia

Bolivia has a geographical territory that comprises different zones: plains or lowlands (59%); Andean or highlands (28%); and sub-Andean or middle lowlands (13%). The country has nine departments, a population of around 11 million inhabitants (67% urban and 33% rural) (Table 1), 36 recognized ethnic or indigenous nations, and 40%–70% of the population self-identify as indigenous [1, 2]. Owing to its geographic diversity, with altitudes ranging from 90–6,542 meters above sea level, the country has a variety of ecologic regions, extending from tropical rainforests to valleys to mountainous areas.

Categorized as a low-to-middle-income country with continuing economy growth, Bolivia has a history of rural areas being excluded from investments and of social exclusion being suffered by indigenous people and women. Due to these social problems and economic difficulties in 2009, the Constituent Assembly included and recognized the indigenous autonomic regions, and this is reflected in the new name for the country: Plurinational State of Bolivia [2].

1.1.1. Indigenous populations

The population of Bolivia is multiethnic, including Amerindians, Mestizos, Europeans, and Afro-Bolivians. However, the principal division of the indigenous people is by territory, with the Andeans settled in the Andes and Altiplano, and people settled in the lowlands, mainly in tropical areas and the Chaco (hot semiarid lowlands). Spanish is the official language and Aymara, Qhechua and Guarani are the most commonly spoken native languages among the 36 native indigenous or ethnic groups.

The indigenous populations have traditionally lived in harmony both with other people and with nature and the environment. As a consequence, native populations now have the opportunity to hold the territory through a collective agreement. This is called Native community lands of origin (*Tierras comunitarias de origen*; TCO or TIOC in Spanish). Until 2011, 190 TIOCs were assigned to indigenous groups [3].

In the Amazonas, one of the indigenous groups located in part of the rainforest is the **Tacanas**, a tribe dispersed in small communities around the northwestern zone of Bolivia in the departments of La Paz and Beni. This group has its own language, and like the vast majority of languages spoken by other indigenous groups, it is being lost. In order to continue living in harmony both

organizations of men and women (*Consejo Indígena Tacana* and *Consejo Indígena de Mujeres Tacana*) work together with the government and UMSA on a strategy for the improvement and development of the region [4].

1.1.2. Women in rural areas

According to the Food and Agriculture Organization of the United Nations (FAO) despite the progress made in reducing poverty in recent decades, 736 million people live in extreme poverty, representing 10% of the global population, with 820 million still suffering from hunger. Furthermore, 80% of the poorest populations live in rural areas of developing countries [5]. Between 2007 and 2014, extreme poverty in rural areas in Bolivia fell from 63.9% to 36.1% [6].

In Bolivia, "poverty has a rural face and a woman's face"

According to the National Institute of Statistics of Bolivia in the census of 2012, women living in rural areas made up close to 16% of the total population (Table 1). While the majority of indigenous people are living in rural areas, in urban and rural areas. the proportions of indigenous women are approximately 45% and 55% of the total women, respectively [7].

Table 1. Distribution of men and women in urban and rural areas of Bolivia.

	Urban (%)	**Rural (%)**	**Total (%)**
Women	34.53	15.57	50.10
Men	32.95	16.94	49.90
Total	67.49	32.51	100.00

Different groups (rural or indigenous) of inhabitants still encounter disparities with regard to health and educational services because, for example, these services are not adapted to their culture. In this context, rural women and particularly indigenous women are at high risk of being excluded or ignored. Results from a survey have shown that all women feel discriminated against by different factors (Fig. 1), and that indigenous women are 2–3-times more affected than non-indigenous women [8].

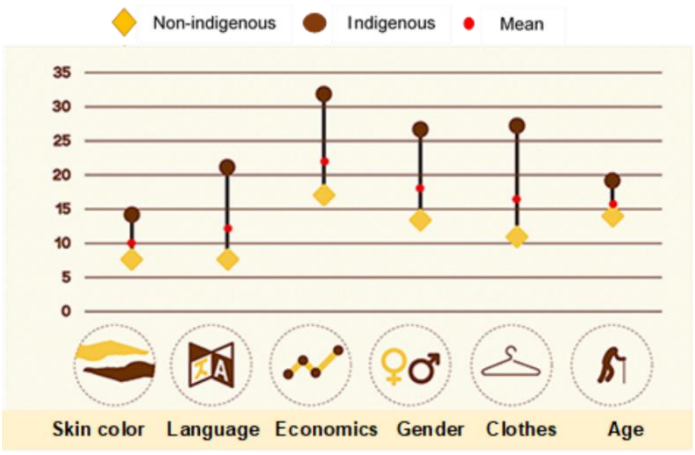

Figure 1. *Factors related to discrimination. Right to reprint obtained from World Bank: Challenges and Constraints to Gender Equality and Women's Empowerment, 2015.*

Even in urban areas, women are less likely than males to finish high-school, and indigenous rural women are 5-times less likely to finish or complete school, as compared to men from urban areas, according to data from the census in 2012 [2, 8]. Furthermore, when it comes to seeking healthcare, 6 out of 10 women in rural areas give birth with the help of a doctor or nurse, while the remainder prefer to use (the more widely available) midwives.

1.1.3. Healthcare in Bolivia

Public healthcare strategies

Healthcare system comprise multiple public and private organizations and the implication is that people who have jobs have access to healthcare services. Close to 66% of the overall population of Bolivia does not have healthcare insurance, with just 20% of the urban and 10% of the rural population having any health insurance service [7, 9].

The improvements in health seen in the Bolivian population in recent years can be attributed to increased investment in equipment, hospitals, and vaccination programs, as well as improved health policies. An example of such improvements in public health care is the introduction in 2008 of the Unified Family, Community and Intercultural Health System (*Salud familiar comunitaria intercultural*; SAFCI), which integrates traditional medicine into conventional medical practice.

In particular, the short-term Compulsory Social Security policy benefits the health of children under the age of 5 years, pregnant women, older adults, and people with disabilities. In 2003, universal maternal and children insurance (SUMI) was introduced for pregnant women, and this covers the costs of follow-up appointments and the hospital stay when giving birth. In 2009, the Juana Azurduy bonus (18$) was given every 2 months to mothers who had children under the age of 2 years. By law, women who have a job in the public or private sector are entitled to maternal leave of 45 days before and after giving birth. While a universal healthcare was intended to be implemented in Bolivia in May 2019, the political instability halted the process.

According to the population census conducted in 2012, life expectancy for Bolivians is 69 years (65 and 70 years for men and women, respectively). The prevalences of vaccine-preventable diseases have decreased since the introduction of these vaccines, and starting in 2006, the number of vaccines offered by the national program has increased up to 13, including vitamin A supplementation. In 2011, vaccines were made more available for children and populations at risk, such as patients with chronic diseases (according to the schedule presented in Table 2). The latest vaccination program to introduced was against HPV in March 2017 [10].

Table 2. General immunization schedule in Bolivia.

Vaccine	**Schedule**	**Planned coverage**	**Actual coverage 2018 (%)**	**Comment**
Bacille Calmette-Guérin vaccine (BCG)	At birth	Entire population	90	-
Diphtheria, Tetanus, Pertussis, Haemophilus influenza, and Hepatitis B vaccine (Pentavalent)	2, 4, 6, 18 months; 4 years	Entire population	89	HBV vaccination introduced from 2000
*Measles, mumps and rubella **(MMR)** vaccine*	12–23 months	Entire population	95	
Polio vaccine	2, 4, 6, 18 months; 4 years	Entire population	89	
Pneumococcal conjugate vaccine	2, 4, 6 months	Entire population	83	
Rotavirus vaccine	2, 4 months;	Entire	87	
Tetanus and diphtheria toxoid for older children/ adults vaccube	1st contact;+1,+6 months; +1, +1 year	Entire population	83	Children older than 7 years and pregnant women
Yellow fever vaccine	12 months;	Entire population		
*Human **p**apillomavirus vaccine*	≥10 years, after 6 months of the 1st	Girls aged 10-12 years	61	From March 2017
Influenza vaccine	6-11, 12-23 months			At-risk groups
Hepatitis B adult-dose vaccine	1st contact, +1, +6 months			Healthcare workers and at-risk groups

Infectious diseases

Due to the general lack of sanitation and widespread malnutrition in Bolivia, acute respiratory and diarrheal infections are the most prevalent diseases causing death in children under the age of 5 years. The reported incidence of Tuberculosis in 2014 was 70.8/100,000, showing a continue decrease of the disease[6]. Water- and food-borne infections are also common from intestinal parasites. In children, the most common parasitic infections are caused by *Blatocystis hominis*, *Entamoeba coli*, *Endo-limax nana* and *Giardia lamblia.* Vector-borne infections, such as malaria, Chagas disease, Leishmaniasis, Chikungunya, Zika and Dengue, remain endemic and are most prevalent in the tropical and sub-tropical regions of the country [11-13]. Dengue virus infections increase in number of outbreaks and disease severity every year, and sporadic outbreaks of deadly Hantavirus and Chapare virus have occurred in 2019 [14]. Sexually transmitted infections (STIs) other than HIV and syphilis are poorly monitored, and not much has been reported on their prevalences. Education regarding STIs has attracted wide attention due to the HIV epidemic, which has affected populations at risk, such as men who have sex with men, sex workers, homeless persons, and children from infected mothers. The program for HIV/AIDS in Bolivia that screens all pregnant women reported in 2016 a higher prevalence in major cities, such as Santa Cruz, Cochabamba and La Paz (see HIV section) [15]. The incidence and mortality rates of cervical cancer in Bolivia are among the highest in South America, and the reference centers reported in 2007 and 2008 prevalences of close to 10% for Chlamydia, 3% for syphilis, 5% for trichomoniasis, and 0.5% for gonorrhea [16].

Maternal and child mortality rates

Although new policies were introduced that decreased significantly (by >50%) the mortality rates in the period 1990–2013, Bolivia has the third highest maternal mortality rate in Latin America, after Haiti and Guyana. In 2012, Bolivia had a maternal mortality rate of 160/100,000, with the principal cause of death being birth-related complications [17]. The infant mortality rate in Bolivia has declined to around 44/1,000, and the major causes of death are infections such as diarrhea and pneumonia [6, 16, 18] .

1.2. Sexually transmitted infections

More than 30 microbial agents, including bacteria, parasites, fungi and viruses can be transmitted through sexual contact, and eight of these agents are the main causes of STIs. Syphilis (caused by *Treponema pallidum*), gonorrhea (*Neisseria gonorrhoeae*), chlamydia (*Chlamydia trachomatis*), and trichomoniasis (*Trichomonas vaginalis*) are all curable, whereas viral STIs, involving herpes simplex virus (HSV) and human immunodeficiency virus (HIV), are currently incurable, with treatment available to reduce viral replication. Although Hepatitis B (HBV) and human papillomavirus (HPV) infections can be cleared by the host, the persistence or chronicity of these infections lead to liver cancer and cervical cancer, respectively, which are difficult to treat. Viral STIs can be transmitted through sexual contact during vaginal, oral or anal sex, as well as from mother to child at birth. In particular, HBV and HIV are also transmitted through contact with contaminated blood, syringes, and other sharp objects. The usual symptoms of infection are vaginal discharge, urethral discharge, genital ulcers, genital warts and abdominal pain. However, in the infected population, the symptoms or signs may not be obvious initially, which means that the virus can be spread unknowingly. Due to the absence of symptoms, the diagnosis of STIs is quite difficult and complications related to the disease appear when the infection has established chronicity or latency, particularly in cases of viral STIs [19]. The only barrier method to prevent STIs is the use of condoms.

In high-income countries, counseling, sex education, screening tests, treatment, vaccination and prevention programs are more available than in low- and middle-income countries, and these are the main reasons for the differences in the prevalence of STIs. For example, syphilis is more prevalent in low-income countries, while chlamydia is more prevalent in high-income countries [20, 21]. In the countries of South America and the Caribbean, the prevalences of STIs (with the exception of HIV) are underestimated and the clinical management remains challenging [22].

The transmission of STIs from mother to child can result in adverse outcomes from low-birth-weight, neonatal conjunctivitis to sepsis and death. Untreated infections with HSV or *T. pallidum* can increase 3-fold the risk of HIV acquisition. Similarly, infections with *N. gonorrhoeae* and *C. trachomatis* that are not treated can lead to pelvic inflammatory disease and infertility in women [23, 24]. Furthermore, meningitis, cirrhosis, liver cancer and cervical cancer, as well as AIDS are major complications of untreated viral STIs.

1.2.1. HSV-2

Herpes simplex virus type 2 (HSV-2), which is a double-stranded DNA virus of the Herpesviridae family, is composed of a capsid, tegument and envelope. The diameter of the virus is around 120 nm and the viral genome has more than 74 open reading frames (ORFs). The difference between the two types HSV lies in their amino acid identity, which is about 50%. After HSV-2 binds to the cell membrane, its envelope fuses with the cytoplasmic membrane and its capsid is transported to the nuclear membrane, where it injects its DNA through the nuclear pores. Transcription of the viral proteins is driven by the host RNA polymerase, which facilitates DNA replication. Finally, the DNA is packed into the capsid, which then exits the endoplasmic reticulum and the new virions are released by endocytosis [25]. HSV-2 is associated with genital ulcers, with the virus usually being transmitted between individuals by mucosal contact. After infection, HSV-2 can be present in ***latent*** form for life or it can enter a **lytic** stage in which virions released from the ganglia travel through the sensory nerves to the genital mucosal or epithelial cells and replicate, producing **genital ulcers**. This process is called ***reactivation*** and is frequently present as **asymptomatic shedding**. If the viral infection is uncontrolled, it can lead to **meningitis** (Figure 1). As in other STIs, women are at higher risk of acquiring HSV-2 than men, seropositivity increases with age, and a deficient immune system can increase the number / frequency of recurrences **[26]**.

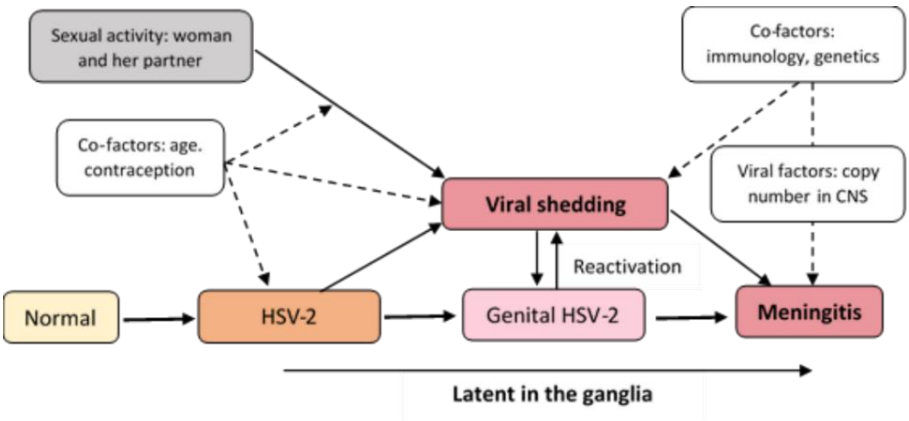

Figure 2. *Pathways from HSV-2 infection to meningitis: associated risk and cofactors involved in the reactivation, recurrence, and latency of the infection.*

Burden of disease

It is estimated that worldwide more than 500 million people suffer from genital infections with HSV-2. In 2012, the general seroprevalence of HSV-2 in people aged 15–49 years was estimated to be 14.4% in the Americas, and 4% in Europe, while the highest prevalence was in African populations at 31% [27]. The HSV-2 seroprevalence differences between South America and Caribbean countries and it varies owing to behavioral and social conditions. It increases with age [27] and it is usually higher in rural than in urban settings. For rural populations in Colombia and Haiti, for example, the prevalences are higher than in the corresponding urban populations [28]. In rural areas of Haiti and Costa Rica, the prevalences of HSV-2 infection have been reported as 42% and 38%, respectively [29, 30]. Until now, no studies of HSV-2 prevalence have been carried out in rural or urban areas of Bolivia.

Diagnosis

Although genital ulcers are visible, it is difficult to identify the etiology based solely on a clinical inspection. Therefore, suspected infections with HSV-2 must be confirmed by direct or indirect detection of the virus. Samples that contain virus, such as swabs from the genital area, blood, tissues, and cerebrospinal fluid, are used for analyses of viral materials and antiviral responses. Detection of **viral DNA** can be performed by polymerase chain reaction (PCR). Virus isolation can be achieved by cell tissue culturing. Viral antibody detection is also possible by immunofluorescence tests, although the sensitivity and specificity of such tests are quite limited. Enzyme-linked immunosorbent assays (ELISAs) are used to determine the levels of immunoglobulins (Ig) **IgG** or **IgM** according to the course of the infection. Western blotting is the Gold standard method for the detection of type-specific antibodies against viral glycoprotein (**gG2**). These serologic tests use serum or plasma to determine whether the tested person has had contact with the virus and is an asymptomatic carrier [31, 32]. Given that the seroprevalences in different settings differ, some studies have shown that the available commercial tests give inaccurate results due to the fact that the reactivities against the antigen preparations affect the sensitivities and specificities of these methods [32-34].

Prevention

Since the virus is shed intermittently in the genital tract irrespective of disease status, the infection can be transmitted also by persons who have no symptoms of disease (Fatahzadeh and Schwartz, 2007). Genital herpes is a major risk

factor for subsequent HIV infection (Freeman et al., 2006; Strick et al., 2006). This underlines the need to develop efficacious HSV-2 preventive regimens. Currently, there is no cure or vaccine for HSV-2, with the only treatments being drugs such as **acyclovir** and **penciclovir**, which inhibit the viral DNA polymerase. These drugs can be used as a prophylactic treatment in infected individuals to prevent transmission of the virus to uninfected partners [35, 36].

Current situation in Bolivia HSV infection prevention

When pregnant women present with or are referred with any risk factor associated with congenital infections, apart from HIV or syphilis, the TORCH (Toxoplasmosis, Rubella, Cytomegalovirus and Herpes simplex) screen test is required, the cost of which is not always covered by the general insurance for pregnant women. The HSV tests used in Bolivia generally detect both HSV-1 and HSV-2 antibodies, and since HSV-1 is endemic in this region, HSV-2 infection is not monitored at all. There is a scarcity of studies or reports regarding the prevalence of STIs other than HIV in Bolivia, and the only recent study conducted on female prisoners from Cochabamba (a semitropical region of Bolivia) gave a prevalence of HSV-2 of 62% [37].

1.2.2. HPV

Human papillomaviruses are non-enveloped, icosahedral viruses of approximately 50–60 nm in diameter. These viruses belong to five different genera (alpha, beta, gamma, mu and nu) in the *Papillomaviridae* family [38]. The double-stranded DNA genome encodes six early genes: *E1* and *E2*, which are involved in viral replication and amplification; *E4*, which is responsible for viral release; *E5*, an oncogene involved in immune evasion; and *E6* and *E7*, which are oncogenes that inhibit tumor suppressors p53 and retinoblastoma Rb in the cell host. Two late genes, *L1* and *L2*, are responsible for the assembly of the capsid [39].

To date, 225 HPV types have been identified and assigned unique numbers by the International HPV Reference Center [40]. However, the most relevant HPV types in relation to cancer development according to the International Agency for Research on Cancer **IARC** (Table 3) are classified as Class 1 carcinogens, and HPV68 is designated as a Class 2A carcinogen [38].

Table 3. Classification of HPV genotypes according to the IARC, together with the available vaccines and their targets.

Classification	**HPV types**	**Vaccines available**
Class 1, carcinogenic with high risk	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59	2v: 16, 18 4v: 16, 18, 6*, 11* 9v: 16, 18, 31, 33, 45, 52, 58, 6*, 11*
Class 2A, probably carcinogenic	68	
Class 2B possibly carcinogenic	26, 53, 66, 67, 70, 73, 82	

**Low-risk HPV types.*

The 13 HPV types in class 1 and class 2A are considered to be high-risk HPV (**HR-HPV**) types due to their detection in more than 5% of all human cancers worldwide, and HPV16 and HPV 18 are responsible for ~85% of cases of **cervical cancer** [41]. HPV6 and HPV11, which belong to the alpha virus genus, as well as the other HR-HPV viruses are low-risk HPV (**LR-HPV**) types that are responsible for the development of **condylomas** (genital warts) [38].

HPV is highly transmissible in men and women, being mainly spread through sexual or skin contact, and the infection is generally asymptomatic. Ninety percent of HPV infections are cleared within 1–2 years, and the persistence of an infection of the HR-HPV type(s) leads to pre-cancerous lesions (CIN1, CIN2, CIN3) and cancer in situ progressing between 10–20 years after the initial infection [42, 43] (Fig. 3). Risk factors associated with the acquisition of the infection, as well as co-factors associated with the development of cancer are: sexual activity, early sexual debut, multiparity, lack of condom use, contraception use, smoking, immune suppression, and the carriage of certain genetic polymorphisms in humans [42].

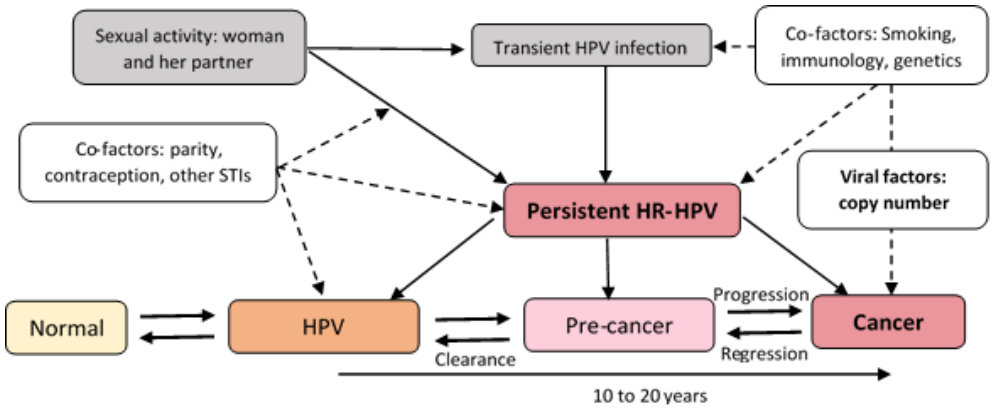

Figure 3. *The pathways from HPV to cancer, showing the associated risk factors and co-factors involved in the persistence of HPV infection and the development of cancer.*

Burden of disease

HPV infection is the most common STI, and almost 300 million people are infected with HPV viruses as single or multiple (more than 1 HPV type) infections. Cohort studies have estimated that most people have had an infection with HPV at some point in their life, with a high prevalence seen in persons at the age of 25 years and a second peak in prevalence at ~45 years of age in certain countries [44]. The last report from the Catalan Institute of Oncology (ICO) and IARC's Globocan in June 2019 reported close to 600,000 new cases of cervical cancer, ~300,000 deaths, and a mortality rate of 6.9 per 10,0000 worldwide. The global prevalence of HPV infection is around 10%, although it varies between regions, being much higher in developing regions. In the African continent, the prevalence of HPV infection is ~23% and in Latin and Central America the prevalences are 14% and 20%, respectively [44, 45].

Cervical cancer is the second-most-common cancer in developing regions, while it is the eleventh-most-common cancer in more-developed regions [46]. Although there has been a decrease in the incidence of cervical cancer in Latin America following the implementation or improvement of prevention programs, as reported for the period 2000–2012, cervical cancer remains one of the major causes of death, particularly in Bolivia [47, 48]. The distribution of different HR-HPV types also differs between regions; the most common HPV types worldwide in women without cervical lesions are HPV16, 42, 58, 31, 18, 56, 81, 35, 33, and 45 [49], and the five most-common HPV types in women with invasive cervical cancer are HPV16, 18, 45, 31 and 33 [44].

Diagnosis

Using cytology and histology, the most frequently available test is the Papanicolao (**PAP**) smear test, which detects pre-cancerous lesions, which are then categorized according to severity as Cervical Intraepithelial Neoplasia (CIN), i.e., CIN1, CIN2, and CIN3), or as invasive carcinoma [43].

The molecular detection of viral DNA or RNA in exfoliated cells or biopsied tissues is used to assess an ongoing infection. Furthermore, it allows the detection of different viral genotypes in single or multiple infections. The specific or general primers used in the tests target HPV DNA for the *L1* and *E6/E7* genes, and the *MY09/11* consensus gene, respectively [50, 51].

Comparing tests for cervical screening, cytology testing was found to have a sensitivity of 76% in CIN3 cases and the level of sensitivity was increased when using repeated screening tests, whereas primary screening of HPV has ~95% sensitivity in a single sample test [52]. The screening for cervical cancer recommended in the European guidelines entails HPV primary testing every 3rd year between 23 and 49 years of age, and every 7th year between 50 and 64 years of age [53]. When the primary screening gives a positive result, it is followed by cytological triage, HPV detection once more and/or genotyping. **HPV typing** is used for follow-up treatments and to monitor treatment efficacy and HPV type-specific **persistence**.

The measurement of antibodies (Ab) is mainly done in vaccine research. HPV serology assays allow for the detection of specific HPV vaccine antibodies, their titers, and neutralization capacities.

Prevention

The primary prevention measures involve prophylactic vaccines, which are based on virus-like particles (VPLs). The major capsid protein L1 assembles VLPs that induce neutralizing antibodies against HPV [39]. The currently available vaccines are: bivalent (Cervarix) against HPV16 and 18; quadrivalent (Gardasil 4) against HPV6, 11, 16 and 18; and nonavalent (Gardasil 9) against HPV6, 11, 16, 18, 31, 33, 45, 52 and 58 (Table 3). The vaccines confer some cross-protection against HPV types 31, 33 and 45, which are genetically related to HPV types 16 and 18 [54].

Secondary prevention involves the screening for precursors of cervical cancer, mainly using the PAP smear test and screening for HPV as mentioned above.

In efforts to eliminate the vaccine-targeted HPVs, organized prevention programs have included giving the vaccine to teenage girls at school and cervical screening for women. The achieved decrease in the prevalence of HPV is attributed to the high levels of coverage of both these programs [54, 55].

Current situation in Bolivia regarding HPV infections and cervical cancer

Although new policies have been introduced to improve the general health programs, Bolivia still has the highest rates in South America of cervical cancer, with a yearly incidence of 34.8/100,000 and a mortality rate of 18.2/100,000 according to the Information Centre of HPV and Cancer, Catalan Institute of Oncology [56]. Two previous studies have reported a prevalence of between 8% and 20% in urban and rural regions, and have identified HPV16, 31, 51 and 58 as the most common HPV types [57, 58]

A cytology-based screening program for women in the age range of 25–64 years was introduced in Bolivia in 2006. In this program, the PAP smear test is performed every 3rd year after two consecutive annual negative tests. This program reaches mostly urban areas, and has had relatively poor coverage of 20% [59], which recently has improved to 33% [56]. Unfortunately, 50% to 80% of the screened women do not attend the follow-up appointments [60]. The vaccination with Gardasil 4 of schoolgirls in the age range of 10–12 years started officially in 2017; the general levels of coverage, without distinction being made between rural and urban areas, have been reported as 88% in 2017 and 61% in 2018 [61].

1.2.3. HBV

Hepatitis B virus (HBV) is an enveloped, partially double-stranded relaxed circular DNA (rcDNA) virus from the Hepadnaviridae family, the virions of which are 30–42 nm in diameter. The overlapping open reading frames (ORFs) in HBV are: *P* (polymerase), *S* (surface), *C* (core) and *X* (HBx protein). These ORFs encode: P, the polymerase reverse transcriptase; S, the lipid envelope containing Hepatitis B antigen (HBAg); C, the core or pre-core protein; and X, the HBx protein, which is more related to viral infectivity [62].

With a tropism for hepatocellular cells, HBV virions can bind to the cell surface through the HBAg glycoprotein and enter into the target cell. Thereafter, the rcDNA is converted into cccDNA, which assembles into a mini-chromosome, which is the template for the viral mRNAs that will be exported from the nucleus. Finally, mature virions or incomplete **viral antigen particles** are assembled in the endoplasmic reticulum and are released into the

bloodstream [63]. These antigen particles, which contain or lack the genome, are present in the serum of infected individuals. HBV is transmitted vertically from mother to child at birth and horizontally through sexual contact, as well as through contact with blood, semen or infected sharp materials.

The clinical presentation of HBV infection, depends on the infectious dosage, host age, and host immune system. The **acute infection** phase, which is asymptomatic in 2/3 cases, can last 1–4 months and viremia becomes detectable. When infection occurs viral replication is controlled by specific neutralizing antibodies and cell-mediated responses (seroconversion). When there is no clearance of the infection and it persist for more than 6 months, it is classified as a **chronic infection**. The phases according to the new terminology are: HBeAg-positive chronic infection, HBeAg-positive chronic hepatitis B, HBeAg-negative chronic infection, and HBeAg-negative chronic hepatitis B, referred previously as immune tolerant, immune reactive/clearance, inactive carrier, and reactivation phases respectively. A **latent** or **occult infection** is defined as an infection with viral DNA replication but without the presence of HBV surface antigen (**HBsAg**), and this type of infection can persist for years. If these infections are not compensated/cleared or treated, they can progress to cirrhosis, which culminates in hepatocellular carcinoma, [64]. The different biomarkers that are present according to the course of infection, except for HBV "e" antigen (HBeAg), are described in Table 4.

Phylogenetic analyses of HBV strains isolated from various regions of the world have identified nine different genotypes (A–I), and some studies have reported the presence of genotype J. These genotypes have different associations with the progression of HBV infection or HCC [65].

Burden of disease

The following prevalences of HBV infection have been estimated by the WHO: 6.2% in the western Pacific; 6.1% in African regions; 3.3% in the eastern Mediterranean; 2% in South–East Asia; 1.6% in European regions; and 0.7% (the lowest) in the Americas (South and North) [66]. However, in some countries of Central and South America, the prevalence of HBV can be intermediate (from 2%) to high (>8%) [67, 68]. Approximately 250 million people have chronic HBV infection, and in 2015 approximately 0.8 million HBV-related deaths were reported [62, 66].

Diagnosis: biomarkers

HBV virus can be detected by measuring serological markers (antibodies or antigens) and detecting the presence of viral material (**DNA**) (Table 4). These markers can be used to diagnose and distinguish the stage of infections, as well as to determine if the individual had resolved the infection or if they had received immunization against HBV [69]. Overall, the presence of HBV surface antigen (**HBsAg)** represents chronicity due to the replication of infectious particles. Hepatitis B surface antibody (**anti-HBs)** is the only HBV marker detected in people who have immunity through vaccination, and it is present together with Hepatitis B core (**anti-HBc)** antibodies, particularly of the **IgG** subtype, in people who have past or resolved previous HBV infection. Some HBsAg-negative people are positive for anti-HBc IgG but not for anti-HBs, particularly in cases of occult infection, which can reactivate.

Table 4. Biomarkers of HBV infection.

	Anti-HBc (IgG)	Anti-HBc (IgM)	HBsAg	Anti-HBs	HBV DNA
Acute infection	+/-	+	+	-	+
Chronic infection	+	+/-	+	-	+
Immunity from vaccination	-	-	-	+	-
Latent or occult infection	+	-	-	+/-	+
Past or resolved infection	+	-	-	+/-	-

Prevention

HBV vaccines that are based on the A2 genotype show cross-protection against other genotypes. They are present as single-antigen vaccines to groups at risk, or as combined vaccines given to children after 6 weeks and up to 6 years of age [70]. Combined vaccines include the pentavalent vaccine, which is given to children and protects against HBV and four other infectious diseases, and the hepatitis A and B vaccines for adults.

Distribution of the vaccine is according to the prevalence of infection in the specific region. In low-income regions with high prevalence, universal vaccination is available for all children, while in high-income regions, screening for HBV on mothers who are suspected of being infected. Prophylactic treatment is given if they are found to be HBV-positive, and selective vaccination plus administration of hepatitis B immunoglobulin is administered to the children born from the HBV-positive mothers [70].

Analogs of nucleotides or nucleosides, such as Tenofovir or Entecavir, inhibit the reverse transcription of the viral RNA to DNA, which suppresses in the serum the numbers of HBV [68]. This antiviral treatment is currently applied to patients with chronic inflammation, to reduce complications such as cirrhosis and hepatocellular carcinoma. However, it has been shown that the long-term use of certain drugs can cause renal damage and drug resistance [71].

Current situation in Bolivia regarding HBV infections

Few studies of HBV prevalence were carried out in Bolivia before the introduction of the vaccine program in Year 2000. Between 1992 and 1996, the prevalences of HBV in certain indigenous populations, including urban Cochabamba, have been high (approximately 9%), in similarity to other countries in the same region [68, 72]. In South America, the main HBV genotype detected is HVB/F, but there are also HBV/B and HBV/C genotypes that might have been introduced by Asian immigrants who settled in nearby regions [73]. A recent study has shown that 0.5% of the female prisoners in prisons in Cochabamba have chronic Hepatitis B infection (i.e., are HBsAg-positive) [37].

1.2.4. HIV

Human immunodeficiency virus is a retrovirus that is a member of the *Lentivirus* genus in the *Retroviridae* family. HIV virions are ~100 nm in diameter, composed of two copies of non-covalently linked single-stranded RNA that is enclosed by a conical capsid composed of the viral protein p24. The RNA genome is complex with many ORFs, due to the many accessory proteins. Viral structural proteins are encoded by long ORFs, whereas smaller ORFs encode regulators of the viral life cycle. Viral replication occurs mainly in CD4+ cells and macrophages, and after fusion and entry of the virus into the cell, the viral RNA is transcribed into DNA. The viral DNA migrates to the nucleus where it is integrated into the host DNA, followed by transcription of the new HIV RNA [25].

There are two strains or types of HIV: HIV-1 is the most prevalent worldwide, while HIV-2 is mainly found in West Africa. HIV-1 is the cause of the AIDS pandemic. The virus infects CD4+ T cells, and after Day 10, the virus is detectable in the blood and its titer increases exponentially until Day 30. Although the adaptive immune response shows relatively good control of the infection, the ineffective antibodies allow the virus to escape, resulting in the destruction of the CD4+ T cells and leading to immunodeficiency and chronic inflammation [74]. In order to reduce the number of neonatal infections that

are acquired by the child from the mother, screening tests for HIV and syphilis are applied to every pregnant woman in Bolivia.

Burden of disease

According to the Joint United Nations program on HIV/AIDS (UNAIDS), ~38 million people are living with HIV, which may cause acquired immune deficiency syndrome (AIDS). Overall, 1.7 million new cases have been reported and 770,000 people have died from AIDS [75]. The majority of these cases are found in Sub-Saharan African countries, at a prevalence of 4%. In Latin America, the prevalence is 0.2%–0.7%, and the treatment coverage has increased to 63% [76].

Diagnosis

Serology testing can detect antibodies to HIV-1 and HIV-2 by ELISA, a simple/rapid test, and Western blotting. These methods have high sensitivity and specificity. Viral infection can be detected by PCR amplification of the viral RNA or the DNA of HIV or the p24 antigen. Dried blood spots (DBS) from infants can also be used for testing for HIV infections [77]. Measurements of CD4+ cells and viremia (number of RNA copies) are required to evaluate the degree of immunodeficiency and the extent of destruction of the immune system, respectively [78].

Prevention

As a barrier method, the male condom remains the cornerstone of HIV prevention, with an efficacy of up to 95% when used correctly.

Currently, there is no vaccine or cure available for HIV infections. However, there exist antiretroviral therapies (ART), pre-exposure prophylaxis, and topical microbicides. Approximately 25 antiretroviral drugs have been approved and used in different combinations, showing high-level viral suppression and effective treatment when administered after the person has tested positive for HIV [79].

Microbicides, which are topical products that can be applied to the genital tract in order to prevent viral infections, have been broadly investigated against HSV-2 and HIV. Currently, there are more than 50 microbicide candidates. The most extensively studied of these is Tenofovir, a nucleotide analog that inhibits viral reverse transcription, which has been shown to confer 39% and 51% protection against HIV and HSV-2, respectively [80]

Current situation in Bolivia *regarding HIV prevention*

The HIV prevalence among Bolivian adults in the age range of 15–49 years is 0.3%. In 2018, UNAIDS reported that about 22,000 people were living with HIV in Bolivia, with a general prevalence of 0.3%, and that only 44% of those infected were receiving treatment. HIV-1 subtype B is the predominant subtype in Bolivia [81]. A more-recent report indicates an increase in HIV incidence [82]. In general, the knowledge and awareness levels regarding disease transmission and treatment are low in urban areas [83], and even lower in rural communities. The program for HIV/AIDS in Bolivia has shown a higher prevalence in major cities, such as Santa Cruz, Cochambamba, and La Paz, with total prevalences in pregnant women of 1.3%, 1%, and 0.3%, respectively [15], and with an increase in the number of new cases [82].

With the goal of ending the AIDS epidemic, the "90-90-90: treatment for all" program intends by Year 2020 that 90% of the people living with HIV will be diagnosed, on treatment, and virally suppressed. In Bolivia, however, the latest report from UNAIDS in 2018 [84] showed that among the people living with HIV, an unknown percentage were unaware of their status, 44% were accessing treatment, and 33% were virally suppressed.

1.3. Novel antivirals

Antiviral therapy is not sufficiently efficient for certain viral infections, mostly due to emerging resistance. Therefore, the need to find new effective drugs has increased. Until now, there has not been much success with developing a vaccine for HIV or HSV, and HSV remains particularly challenging due to its latent lifelong infection of the host.

Most people are unaware of the extent to which plant-based medicines belong to the contemporary pharmacy. In order to adapt to environmental hazards, plants produce a vast array of products that have antimicrobial activities [85, 86]. Many plants also have immunomodulatory activities that complement treatments of infection diseases [87, 88].

Antivirals based on medical plants contain numerous compounds, which have diverse actions on viral infections. Many compounds, such as polyphenols, flavonoids, coumarins, phenolic acids, anthocyanins, polysaccharides, terpenoids and alkaloids [89, 90], have demonstrated antiviral activities and have been used as prophylactic therapies [91]. Specific compounds, such as curcumin [92], propolis [90], Ficus species [93], and *Matricaria recutita*, have anti-HSV-2 activities [94].

A microbicide based on the marine red alga *Griffithsia* has shown strong activity against HIV, HSV, and other viruses, with no significant harmful effects on the mucosal microbiome of the genital tract [95].

1.4. Traditional Medicine

Traditional medicine, known as ethno-medicine or folk medicine, encompasses the knowledge, skills, and practices from mostly indigenous people around the world who use it to prevent, diagnose and treat physical and mental illnesses [96]. The ancient knowledge has come from indigenous communities – natives who pass the knowledge from generation to generation verbally or in some form of writing. Traditional medicine is practiced at different levels all over the world and the WHO supports the integration of traditional and modern medicine as a means to provide affordable treatments [97]. As many pharmaceutical drugs are derived from **medicinal plants** [98], it is important to maintain the fund of ancient knowledge of herbal medicines.

History

The first traces of plants used for medical purposes were found in the grave of Shanidar Neanderthal, dating back to 60,000 B.C. [99]. Papyrus documents dating from 1,500 B.C. to 1,800 B.C. reveal that in ancient Egypt the treatment of diseases was based on herbal mixtures, magic practices and prayers. Hippocrates in 470 B.C. stated that disease is the result of natural causes, thereby separating medicine from religion [100]. For thousands of years, plants have been used extensively in different regions of the world, with traditional Chinese medicine, Ayurveda and Unani medicine from India, and traditional aboriginal medicine from Australia being the most-studied. In South America, the ancient medicine is from the Andes and the Amazonas, with the Inca civilization (c. 1400–1533 A.D.) using natural remedies. As this civilization did not have written languages, this knowledge did not spread outside the Inca Empire until the Spanish arrived in the Americas. A priest, Bernabe Cobo, who on a mission landed in America in 1596 wrote several descriptions of "some kinds of medicine" and how herbs were used on broken bones, and Cristobal Molina in 1565 observed in the Inca traditions the broad usage of plants, particularly Coca leaves [101, 102]. It is conceivable that most of the knowledge on medicinal plants has been lost, as almost 90% of the native population were killed by smallpox, measles, and other diseases introduced to the Americas by the European conquerors.

1.4.1. Herbal preparations

The herbalist is the person who had or has the knowledge about the uses of plants, often called healers and midwives (*curanderos* and *parteras* in Spanish). Herbal medicine include the use of herbs, which are crude plant materials from flowers, leaves, stem wood, bark, roots, and fruits, and herbal preparations, which include herbs and some other treatments or products, such as oils, alcohol or honey. Herbal preparations are produced by different methods of extraction, which allow separation of the chemical constituents from the insoluble parts of the plant [103]. The most commonly used methods are maceration, percolation, infusion, and decoction. Maceration involves the use of the plant material (generally, a powder) and solvents, which are mixed together for a certain period, after which the liquid obtained is separated by filtration or centrifugation [104]. The most broadly used method is maceration, which can extract hydrophobic and non-hydrophobic chemical compounds by using solvents or mixtures of solvents [105]. Using ethanol or methanol, it is possible to extract the majority of polyphenols, terpenoids, tannins, and lignins, which have antimicrobial, antioxidant, and anti-inflammatory activities [106].

Ethnobotanical records and pharmacopeias from all over the world have reported on the broad use of alcohol in medicinal preparations, and that is the case with tinctures and liqueurs that contain herbs or a mixture of herbs and spices [107, 108]. These preparations were subsequently used for enjoyment rather than as medicines. Examples of this are gin and tonic, which contains quinine, an antimalarial agent derived from the bark of Cinchona, a plant used by the Incas [109, 110], and other distilled drinks, such as aquavit and brandy [111].

1.4.2. Medicine from the Tacanas

The Amazonas basin constitutes 66% of the Bolivian territory and it is home to a wide variety of plants used as traditional medicines. One of the indigenous groups located in the Amazonian rainforest is the **Tacanas**, which have tried to preserve the knowledge of their traditional medicine by sharing part of the ethnobotany within the generations and, more recently, with academic organizations. More than 450 plant species have been identified as used by the Tacanas, and the majority of these are used to treat inflammatory and infectious diseases [112, 113]. More than 50% of these plants are used for skin problems and gynecological disorders, although this is most likely an underestimation as

the information was only collected from males [114]. Three recent studies and interviews with the Tacanas have allowed updating of the pharmacopeia and the identification of 46 additional species of plants, which are used to treat endemic parasitic diseases in the region [115-117].

1.4.3. Plants used in this thesis

We selected seven different plants based on their reported use in the treatment of infectious diseases related to the female genital tract and inflammatory disorders: *Equisetum giganteum L, Copaifera reticulata Ducke, Croton lechleri Müll. Arg., Uncaria tomentosa (Willd. ex Schult.) DC, Tipuana cf tipu (Benth) Kuntze, Erythroxylum coca Lam,* and *Mangifera indica L* (Figs. 4 and 5).

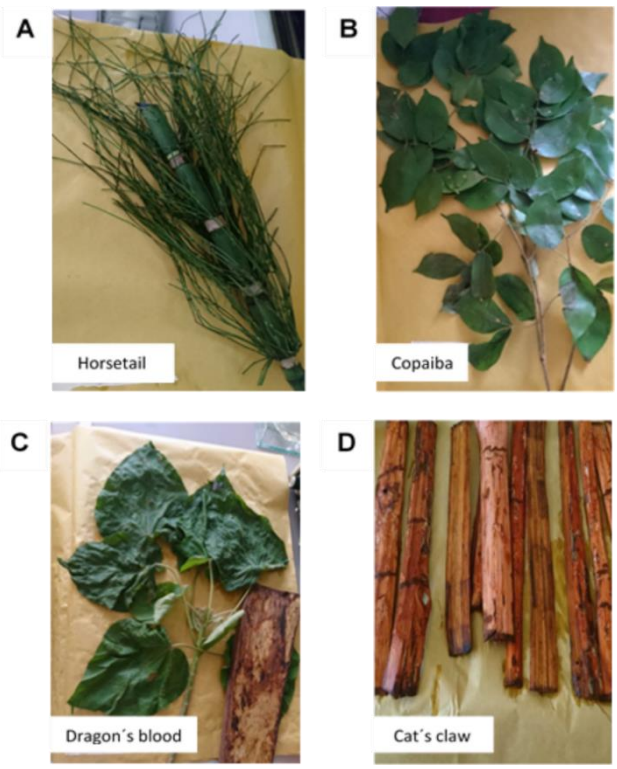

Figure 4. *Medicinal plants used in this thesis: Equisetum giganteum or horsetail* ***(A)****; Copaifera reticulata Ducke or copaiba* ***(B)****; Croton lechleri Müll. Arg. or dragon's blood* ***(C)****; and Uncaria tomentosa (Willd. ex Schult.) DC. or cat's claw* ***(D)****.*

Equisetum giganteum L or giant horsetail (*cola de caballo* in Spanish) is a common native plant of Central and South America that is widely used in

traditional medicine (Fig. 4 A) [118]. Pharmacologic studies have shown that extracts of *Equisetum giganteum L* have both antibacterial [119] and anti-inflammatory properties [120].

Copaifera reticulata Ducke is a flowering plant of the legume family. The oils from this family of plants (often referred to as Copaiba oils) have well-documented antibacterial properties [121]. The plant is widely used by Tacana women to treat genital infections [112].

Croton lechleri Müll. Arg. (dragon's blood) and ***Uncaria tomentosa (Willd. ex Schult.) DC.*** (cat's claw) are used in alternative medicine throughout South America (Fig. 4 C and D). Both plants and in particular the stem bark have high contents of terpenoids, which are believed to mediate some of their antimicrobial and anti-inflammatory activities [122]. The sap from the flowering plant *Croton lechleri Müll. Arg.*, which has the aspect of blood, is used by indigenous tribes as a vaginal antiseptic [123]. In addition, the woody wine of *Unicaria tomentosa (Willd. ex Schult.) DC.* is widely used in folk medicine, including by the Tacanas, for many purposes, including the treatment of viral infections [124].

Tipuana cf tipu (Benth) Kuntze, which is also known as Tipa, Rosewood or Pride of Bolivia, is a common tree of the *Leguminosae* family *(Papilionoideae)* that grows in Bolivia and several other countries of South America (Fig. 5 A). The stem bark of this plant is used by people in the Amazonas to treat cancer of the uterus [117], as well as to treat gastritis and wounds [125].

Figure 5. *Medicinal plants used in this thesis: Tipuana cf tipu (Benth) Kuntze or tipa* ***(A)****; Erythroxylum coca Lam or coca* ***(B)****; and Mangifera indica L or mango* ***(C)****.*

Erythroxylum coca Lam, better known as coca, is a widely used medicinal plant in both the Bolivian highlands and the Amazonian lowlands (Fig. 5 B). The most common way to utilize this plant is to chew the leaves, so as to relieve fatigue or tiredness, although in the traditional medicine it is also ingested for gastrointestinal problems and acute mountain sickness, and is applied topically to treat skin inflammation and wounds [114, 126, 127]. Studies have shown that it has antimicrobial activities against both Gram-positive and Gram-negative bacteria [128]. Most of the research performed on this plant has highlighted the main anesthetic activity of the alkaloid cocaine, which was studied by Albert Nieman 1860 following a suggestion of Sigmund Freud [129], indicating it as a cure for all ailments, and even leading for a time to its incorporation into "Coca-Cola" [126].

Mangifera indica L or mango is a well-known plant and food that has been ascribed many medicinal properties and that is used in traditional medicine in many countries around the world, in particular in Asia and South America (Fig. 5 C). Extensive research has been carried out on the medical uses of the fruit and leaves, and they collectively show that the plant has anti-inflammatory, antioxidant and antimicrobial properties [130]. The active substance of *Mangifera indica L* has been identified as the natural phenolic compound mangiferin [131]. Mangiferin extracted from mango leaves has direct antiviral effects on many viruses, including the sexually transmitted HSV-2 [132].

1.5. Antiviral immunity

The human immune system response viruses involves mainly cytotoxic cells, interferons (IFNs), and antibodies. It comprises initially the **recognition of the virus (viral DNA or RNA)** through pathogen recognition receptors, including Toll-like receptors (TLRs), retinoic acid inducible gene 1 (RIG)-like receptors, and NOD-like receptors (NLRs). The DNA from HSV-2 can be sensed by TLR9, and TLR2 can sense glycoproteins. Other receptors involved are IFI16, RIG-I, cGAS, and absent in melanoma 2 (AIM2). The virus acts via the TLRs and RIG-1 to induce the transcription of factor nuclear factor-kappa B (NF-κB) and interferon regulatory factors 3 and 7 (IRF3, IRF7) [133].

After viral recognition, the antiviral **innate immune response** is manifested via phagocytes, **cytotoxic cells**, and the production of mediators, principally interferons (**IFNs**), pro-inflammatory cytokines, and **chemokines**. Through the release of cytokines, cytotoxic T cells, natural killer (NK) cells DCs, and pDCs are recruited to the site of infection. Interferons have the ability to inhibit

viral replication in cells. Type I IFNs (**IFN-α** and **IFN-β**) induce intracellular signaling and NK cell cytotoxicity, while **IFN-λ** or IL-28/29, a Type III IFN, has a similar albeit much more potent effect than the type I IFNs on mucosal surfaces [134, 135].

Viruses can also be neutralized by the **adaptive immune response**. This involves CD4+ helper T cells, which recruit and activate phagocytes and CD8+ cytotoxic lymphocytes, and IFN-γ (a type II IFN), which potentiates pro-inflammatory signaling by priming macrophages for antiviral action, i.e., an antigen-specific response from specialized B-lymphocytes. In this case, **antibodies** produced by the B cells will neutralize viruses in the blood and mucosal surfaces before they can infect the cells, and this mechanism of protection is called *memory* because it will be the response to future infections [136].

1.5.1. Inflammation

Inflammation is generally a response to infection or an endogenous product that is classically defined by signs such as rubor (redness), calor (warmth), dolor (pain), and tumor (swelling), and functio laesa (loss of function). This acute response acts to eliminate the cause of injury, clear the affected cells, and repair the damaged tissue. If this process is not controlled the result is chronic inflammation [137].

Components of pathogens are recognized by cells in the skin or in the bloodstream, including epithelial cells, fibroblasts, macrophages, dendritic cells, monocytes, and lymphocytes. These cells sense pathogen-associated molecular patterns or damage-associated molecular patterns through pattern recognition receptors (PRRs). The activation of PRRs induces signaling pathways that regulate **pro-inflammatory cytokines**, mainly via NF-κB or via the inflammasome [138]. This activation can be specifically directed by exposure to viral (**DNA)** or bacterial (lipopolysaccharide, **LPS**) components, DNA damage, oxidative stress (**mitochondrial ROS**) or other pro-inflammatory cytokines [139]. The pro-inflammatory cytokines produced are IL-1 and TNF family members, whereas anti-inflammatory cytokines, including IL-10 and TGF-β, are also induced.

1.5.2. The NLRP3 inflammasome

A major component of the inflammatory cascade is the inflammasome, which is a multimeric complex that controls activation of the pro-inflammatory cytokines **IL-1β** and **IL-18**. Secretion of these two cytokines occurs in response to **pyroptosis**, which is a type of programmed, pro-inflammatory cell death. The inflammasome is activated through one of several NLRs (NLRP1, NLRP3, NLRC4, AIM2), which assemble the inflammasome together with an adaptor apoptosis-associated speck-like (ASC) protein and a zymogen or pro-form caspase 1. The NLRP3 inflammasome is the most intensively investigated complex and it can be activated by micro-particles, ATP, cholesterol, reactive oxygen species (ROS), and microbial toxins. Assembly of the NLRP3 inflammasome can be driven via **caspase 1**-dependent or independent signaling, with subsequent cleavage of IL-1β and IL-18 into their active forms [140, 141].

Furthermore, after activation of caspase 1 (**priming**), the cleavage of Gasdermin D, an executioner protein, permeabilizes the plasma membrane, leading to a process known as **pyroptosis.** Gasdermins also form pores at the **mitochondrial membrane** level and release many substances, including cytochrome c, which could increase the levels of ROS and play a role in apoptosis [142]. IL-1β and IL-18 are pleiotropic cytokines that play roles during viral infections. Consequently, the viruses have developed ways to avoid inflammasome activation. For example, HSV-1 has a decoy caspase 1 and can probably block NLRP3/IFI16 inflammasomes [143]. Moreover, AIM-2 inflammasome oligomerization is inhibited by the HSV-2 tegument protein VP22 [144].

It has previously been shown that several plant extracts, as well as purified plant fractions have anti-inflammatory properties and can reduce the secretion of IL-1β by human cells [145]. More specific studies have shown that dietary plant flavonoids, such as quercetin, can inhibit NLRP3 and AIM-2 inflammasome activation [146].

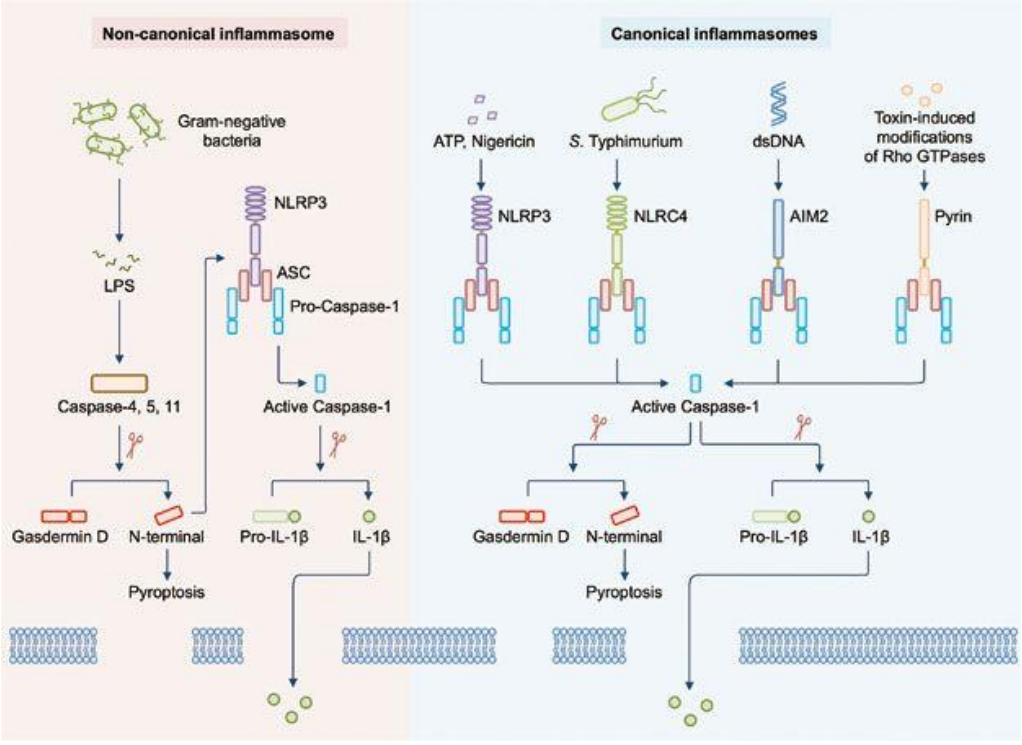

Figure 6. *NLRP3 inflammasome and pyroptosis driven via a caspase-1-dependent or –independent process, maturation of the pro-inflammatory cytokines IL-1β and IL-18, and the role of Gasdermin D in pyroptosis. Rights to reprint from natureresearch group.*

2. AIM

The overall aims of this thesis were to investigate the prevalence of sexually transmitted viral infections among women from rural communities of the Amazonas in La Paz, Bolivia and to explore the biological effect of medical plants used as natural anti-viral and anti-inflammatory remedies.

The specific aims of the papers were to investigate:

- The prevalence of viral STIs in women from the Bolivian Amazonas
- If extracts from medical plants can act as anti-virals against herpes simplex virus type 2 infection.
- If plant extracts have anti-viral and immunomodulatory properties.

3. MATERIAL AND METHODS

All the methods are described in detail in the appended papers. Therefore, this section provides a brief overview of the methods used. I performed all of these methods with four exceptions: 1) viral detection by PCR and serology by Western blotting were performed in collaboration with Magnus Lindh and Jan-Åke Liljequist at the Department of Clinical Virology at Sahlgrenska University Hospital; 2) Endotoxin assay by the Department of Clinical microbiology at Sahlgrenska University Hospital; 3) the chemical compositions of the plant extracts were analyzed by mass spectrometry in collaboration with Otto Savolainen of the Department of Biology and Biological Engineering, Chalmers University of Technology; 4) the collection of all the plant samples and the preparation of some plant extracts were performed by the person responsible of the project in La Paz, Bolivia, Katty Terrazas-Aranda and personnel/researchers at Unidad de Virología, Inmunidad e Infección, Insituto SELADIS, Universidad Mayor de San Andrés (UMSA).

3.1. Site of the study

The studies described in this thesis were conducted in the rural provinces of Abel Iturralde (the lowlands of the Amazonas at altitudes of 150–600 meters above sea level), and the Caranavi and Nor Yungas regions (the adjacent central valleys at less than 1,000 meters above sea level) in the Department of La Paz in Bolivia. For **Paper I**, participants were recruited in the following places (Fig. 7 A):

- Villages: Tumupasa, San Silvestre and Santa Rosa de Maravilla (mostly indigenous population with <1,200 inhabitants);
- Small town: San Buenaventura (population of approximately 8,000); and
- Large town: Caranavi (population of approximately 50,000).

For **Papers II** and **III**, plants were collected from the territory assigned to the Tacana ethnic group, which is located in the north of La Paz and is part of the Department of Beni (outline in yellow in the map). Specifically, the yellow spots on the map indicate the surrounding areas in the Madidi National Park and the Suapi community where plants were found and collected (Fig. 7 B).

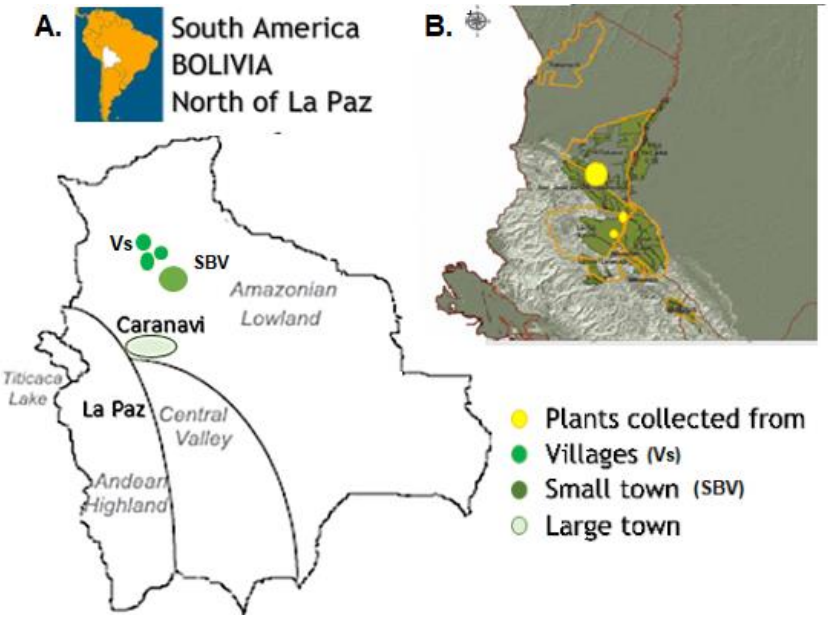

Figure 7. *Location map of Bolivia and the department of La Paz in Bolivia, participant women from rural places in green (A) and places where plants were collected (B)*

3.2. Female volunteers

Through six field trips, mostly during the dry season (July to October), to villages in the Amazonas during 2015 and 2017, and two trips to Caranavi in March 2018 and May 2019, personnel and researchers (teams of 3–5 people) from UMSA visited the study sites. The field trips lasted 4–7 days and involved meetings with local authorities to spread information explaining our objectives and the benefits of our study. Thereafter, female volunteers were recruited via direct invitations to small mother groups, unions, local authorities, and indigenous leaders or via general invitations transmitted by radio and television.

The interviews and collection of samples were performed in the nearest health center or hospital. After the participants gave their acceptance, they signed an informed consent form and we obtained demographic and lifestyle data through a questionnaire and a personal interview. The questions posed to the participants were about their age, social identification (indigenous group), current occupation (housewife, employee, merchant, artisan, student, professional), place of living, place of origin, number of children, current

family planning method (use of hormonal contraception, intrauterine device, calendar, tubal ligation, condoms), family disease history, possible health problems, if they had undergone or not undergone any cervical cancer screening test, and if they knew the results from the test (follow-up appointment). The total number of participants was 394, and they received extended oral and written information regarding STIs. We collected 389 blood samples and 376 cervicovaginal cell samples. Women under the age of 18 participated with the consent of their guardian, who signed the form after receiving the information. The ethical committee at Universidad Mayor de San Andrés in La Paz, Bolivia approved the project study "Infecciones virales tropicales" with an ethical permission CEI-UMSA 0215. The biological material shipment to and analysis in Sweden was in agreement between the main coordination at University of Gothenburg and Universidad Mayor de San Andrés.

Sampling was performed using filter papers that preserved the **biological material (proteins and DNA)**. This method of sampling is commonly used in field studies, due to the lack of sanitary services in remote areas [147-149]. For blood collection, capillary blood was collected from the ring finger by finger pricking. After cleaning the ring finger with 70% ethanol, we collected whole blood (dots) onto filter papers, which were kept at room temperature until we returned to La Paz. For the collection of genital cell samples, exfoliated cervicovaginal cells were collected by swabbing the lateral walls with a sterile cotton swab, after which the material was transferred into cell essential media (D-MEM medium), and kept at 4ºC until arrival to La Paz no more than 1 week later. Cells were centrifuged for 10 minutes, suspended in 150 µL of medium and transferred onto filter papers to dry as cervicovaginal cell spots.

Once in La Paz, the dot blood spots **(DBS)** and dried cervicovaginal cells spots **(DCCS)** were stored at -20ºC until they were transported to Sweden for further analysis. In Sweden, the biological sample samples and volumes as described in table 5. Briefly, a diameter of the filter papers were punched or cut, soaked and eluted in buffer at 4ºC for one or 24 hours, after which the filter paper was removed, the specimen was eluted by centrifugation, transferred into a new tube and stored at -20 ºC for further analysis. Serology and the isolation of DNA plus PCR genotyping were carried out on the materials eluted from the DBS and DCCS, respectively. DNA isolation was performed according to the manufacturer's instructions and the concentrations were quantified by Nanodrop.

Table 5. Characteristics for the elutions of the biological material

Material	*Diameter*	*Buffer*	*Vol. of Buffer*	*Time of elution*	*Vol. obtained*	*Purified material*
DBS	5 mm	ELISA diluyent	300 µL	24 hours	250 µL	--------
DCCS	2.5 cm	PBS	600 µL	1 hour	400 µL	150 µL DNA

3.3. Collection of plants

Plants were selected from studies of traditional Tacana medicine and based on recent reports/interviews carried out with Tacana healers and midwives, who described the species and parts of the plants used for specific pathologies [112, 117]. We selected plants that were used to treat infectious diseases related to the female genital tract, and to treat inflammatory disorders (Table 6). The plants were collected with the help of one or two local Tacana guides and researchers/personnel involved in the Biological Integral Program-Botanical Garden who were familiar with the species. The Bolivian National Herbarium of UMSA in La Paz confirmed and certified the scientific identification of the collected plants using the International Plant Name Index Database. None of these species was classified as endangered species according to the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), and the samples collected were minimum amounts just for scientific purposes.

Table 6. Selected plants studied in this thesis

Medical plant English/Spanish	Scientific name	Family	Part of plant used	Traditional use
Horsetail/Cola de caballo	*Equisetum giganteum L*	Equisetaceae	Roots and bark	Diuretic, pain in the kidney, appendicitis, prostate problems, genital infections
Dragon's blood/ Sangre de grado o drago	*Croton lechleri Müll.Arg*	Euphorbiaceae	Bark	Diarrhea, genital infections, vaginal antiseptic (sap), wound healing
Cat's claw/ Uña de gato	*Uncaria tomentosa (Willd. ex Schult.) DC.*	Rubiaceae	Bark	Antiemetic, rheumatism, regulate problems in the uterus from women, infections, wound healing
Copaiba/Copa ibo	*Copaifera reticulata Ducke*	Leguminosae Caesalpinoideae	Leaves	Genital infections, skin healing, cervical cancer (oil)
Rose wood or pride of Bolivia/Tipa o palo rosa	*Tipuana cf tipu (Benth) Kuntze*	Leguminosae Papilionoideae	Bark	General problems in women, especially cancer in the uterus, gastritis, wound healing
Mango	*Mangifera indica L*	Anacardiaceae	Leaves	Diabetes, common flu, cancer, pain, wound healing, dysentery
Coca	*Erythroxylum coca Lam*	*Erythroxylaceae*	Leaves	Any place with pain, stomach ache, antiemetic, diarrhea, acute mountain sickness, wound healing

Preparation of plant extracts

We prepared the plant extracts according to the guidelines from the WHO [104]. After collection, the plant materials (branches, leaves and stem bark) were transported to the laboratory in La Paz with a delay of no more than 48 hours. Every species was stored in two batches, one for the extraction and one for the herbarium. The selected parts of the plants (leaves or roots or stem bark) were first washed extensively in clean tap-water, three times (5 minutes each) to get rid of dust, sand and debris, leached in sterile water, and then with 70% ethanol (30–60 seconds). Using a knife and a brush, the external part of the stem bark was removed, and washed as described above. All the plants then were dried outdoors in the shade until they were completely dry; this required 10–14 days for the leaves and more that 30 days for the stem bark. Thereafter, the dried plant material was cut into smaller pieces and ground to a powder using an automatic canonical grinder. The extraction process consisted of **maceration** of the powdered plant material in 50% ethanol and 70% ethanol for *Equisetum giganteum* at a weight:volume ratio of 1:10 for 72 hours at room temperature. The liquid material was separated from the insoluble plant material by centrifugation at 1,500 rpm for 5 minutes and filtration with Whatman filter paper No. 1, after which the ethanol was evaporated by incubation at 65°C in a rotary evaporator. The remaining final product was frozen at -80ºC and lyophilized. The lyophilized plant extracts were stored at -20ºC until their transport to Sweden. In the laboratory in Sweden, we dissolved the extracts in dimethylsulfoxide (DMSO) to generate a stock solution, which was stored at -20ºC. All the dilutions used in our experiments contained less than 1% DMSO, which at high doses can be cytotoxic.

A minimum of 10 mg of the powdered material from the plant extracts was sent for analysis by tandem mass spectrometry (GC-MS/MS) [150, 151] and high-resolution mass spectrometry coupled to high-pressure liquid chromatography (HPLC-HRMS)) [152]. These methods allowed us to separate, identify and quantify the compounds, so as to obtain an estimation of the chemical constituents of the extracts, using GC-MS/MS for volatile compounds of low molecular weight, while HPLC-HRMS was used for polar compounds and large molecules.

Cytotoxicity analyses of the plant extracts

By measuring the release of lactate dehydrogenase (LDH) from cells (either PBMCs or VERO cells) that were treated with different doses of the plant extracts, we could determine whether the plant extracts could damage the plasma membrane of the cells.

In addition to LDH measurements, we also investigated whether the plant extracts could cause mitochondrial damage. This was achieved using flow cytometry to detect the superoxide levels in live cells. This assay is based on a fluorescence reaction, whereby cells, after treatment with plant extracts, are incubated with a derivate of ethidium (MitoSOX), a probe that can be oxidized by superoxide and produce fluorescence, which is then visualized in a flow cytometer.

3.4. Antiviral and immunomodulatory assays

Plaque assays to assess the antiviral properties of the plant extracts

We performed a HSV-2 plaque reduction assay to quantify the numbers of infectious viruses. The procedure is well-established in VERO cell monolayers that are cultured for around 72 hours at 37°C in a humidified atmosphere that contains 5% CO_2 until they reach 70%–80% confluence. The monolayers are then infected with HSV-2 for 1 hour at 37°C. Then, 2% methylcellulose is added to block the spreading of the virus, and after 3 days of incubation, the plates are washed and stained with crystal violet to visualize the plaques. We used this assay to assess several different aspects of the antiviral activities expressed by the plant extracts:

- *The ability of plant extracts to block HSV-2 infection*. Serial dilutions of the plant extracts were added to VERO cells concomitantly with addition of the virus.
- *Virucidal effects of the plant extracts*. HSV-2 was pre-incubated with plant extracts for 30–60 minutes and then added to the cells.
- *Direct effects of the plant extracts on target cells*. VERO-cells were pre-treated with plant extract for 30-60 minutes prior to HSV-2 exposure
- *Abilities of the plant extracts to block the different stages (binding, entry, post-entry) of HSV-2 infection*. We used a synchronized assay (described below; Figure 9) in which plant extracts were added at different time-points during the viral infection.

For the synchronized assay, the cells were cooled to 4°C and inoculated with HSV-2 at 4°C for 4 hours. At this temperature, the virus can **bind** but not enter into the cells. Unbound virus was then removed by washing, and the cultures were shifted to 37°C for 30 minutes to allow **viral penetration**. Thereafter, non-penetrating viruses was inactivated by treating the cells with citrate buffer (pH 3.0) for 30 seconds followed by washing, after which fresh medium was added and the plates were processed as described above. Plant extracts were

added either together with the virus (to assess the effect on binding), directly after removal of unbound viruses (to assess the effect on viral entry) or after the citrate buffer treatment (to assess the effect on **viral post-entry** replication).

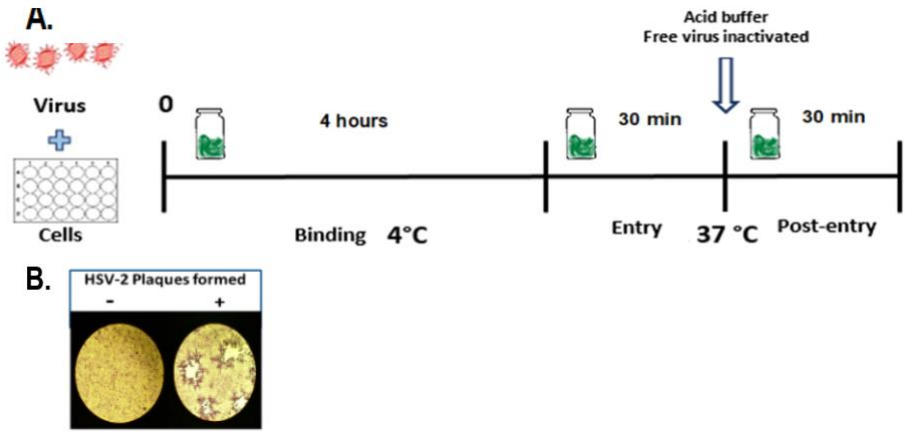

Figure 8. *Synchronized plaque assay: Plant extracts were added at the indicated time points for binding, entry and pots-entry (A). Plaques formed on cell monolayers (B).*

Murine model of HSV-2 infection to assess the antiviral properties of plant extracts in vivo

In **Paper II**, we used female **C57BL/6** mice, which after pretreatment with medroxyprogesterone acetate (Depo-Provera) are susceptible to intravaginal infection with HSV-2. The HSV-2 mouse model has been extensively used for research and shows many features that resemble those of the natural infection. However, the infection does not spontaneously re-occur in mice. Instead, a low-dose viral inoculum gives a subclinical infection that leads to latency in the central nervous system (CNS), whereas at higher viral doses the mice develop meningoencephalitis and die [153, 154]. In our studies, mice were infected intravaginally with 5×10^3 PFU of HSV-2 strain 333, which led to a non-lethal clinical infection with readily detectable levels of virus in both the genital tract and the CNS (Fig. 9).

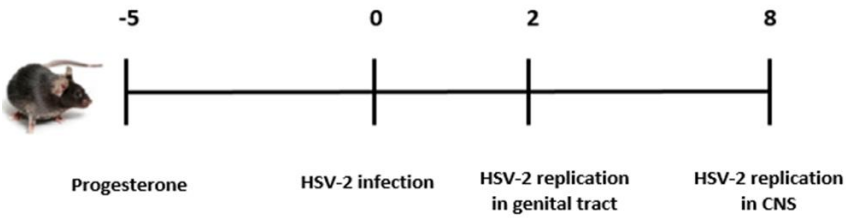

Figure 9. *Mouse model for HSV-2infection: Mice were pretreated with Depo-Provera 5 days before infection, and at 2 and 8 days post-infection, vaginal washes and spinal cords were taken, respectively, to evaluate the numbers of viral DNA copies.*

Mice were scored for the severity of disease on a scale of 0 to 5: healthy (0), genital erythema (1), moderate genital inflammation (2), genital lesion and/or generally bad condition (3), hind-limb paralysis (4), and death or sacrifice (5). Samples (vaginal washes and homogenized spinal cords) were assessed by PCR for HSV-2.

Direct effects of plant extracts on innate immunity

In order to study the antiviral and anti-inflammatory properties of plant extracts, PBMCs isolated from the buffy coats of blood donors were either stimulated with plant extracts using endotoxin (lipopolysaccharide, LPS) as a positive control or were pretreated with plant extracts for 30 minutes prior to exposure to LPS. After incubation for 3–24 hours, the concentrations of cytokines released into the supernatants and the cytokine mRNA expression levels in the cells were measured. For the antiviral tests, we focused our efforts on assaying the type I and type III IFNs, as well as the chemokine CXCL10, which is induced by these cytokines. For the anti-inflammatory tests, we focused on cytokines released following activation of the inflammasome, i.e., IL-1β and IL-18. In the blood, the major producers of IL-1β and IL-18 in response to PAMPS or DAMPS are monocytes [155], whereas plasmacytoid dendritic cells are the main producers of type I and type III IFNs [135]. Both of these cell types are present in the PBMC fraction.

3.5. Detection methods

Serologic methods

Enzyme-linked immunosorbent assays (ELISAs) are widely used for the diagnosis of viral infections because they can detect and quantify the presence of antibodies and antigens. The serologic test used here, which normally uses

serum as the sample, is based on an immune principle whereby specific enzyme-conjugated antibodies bind to the target protein and a detection system (chromogen/substrate) indicates the presence of the target in a quantifiable manner. Western blotting involves a similar immune-based detection system that utilizes specific antibodies, although the target antigens are first separated by electrophoresis and transferred to a membrane, upon which a band of a specific size can be revealed after binding to a labeled antibody.

To detect a previous infection, in **Paper I**, IgG antibodies were assessed against **HSV-2 glycoprotein G2, HBV core protein**, and **HIV** using commercial ELISA kits. We also assessed the presence of **HBV antigens** in the serum samples, to identify active/chronic infections (Table 2). Equivocal samples for HSV-2 were confirmed with the Gold standard method of Western blotting, where we specifically detected antibodies against glycoprotein G-2 (gG-2) [156]. Due to the presence of red blood cells in the DBS samples, the cutoff values provided by the manufacturers of the ELISA kits were multiplied by a factor of 1.5, to eliminate false-positive samples [34, 157]. In **Paper III**, we used ELISA to measure in the supernatants the levels of IFN-α, IFN-β, IFN-λ, IL-1β, IL-18 and CXCL10 using commercial ELISA kits. The samples (supernatants from cell cultures) were diluted with the sample buffer solution from the commercial kits or with a solution of 0.5% bovine serum albumin in PBS.

Endotoxin assay

For **Papers II and III**, the endotoxin concentration assay was performed with Limulus amebocyte lysate (LAL). This test is based on the clotting reaction between an aqueous extract of blood cells or amebocytes from the horseshoe crab (Limulus Polyphemus) and PAMPs, in this case *Escherichia coli* O55:B5. A standard curve and dilution of the samples (plant extracts) at the concentrations used were run in duplicate. The curve and samples were read at 405 nm after 30 minutes of kinetic reaction.

Real-Time PCR detection

Quantitative PCR (qPCR) is widely used in clinical diagnostics and research. This method has the ability to amplify the DNA material in a sample together with primers, DNA polymerase, nucleotides and ions, and through cycles that consist of DNA denaturation, primer annealing, and extension. To monitor DNA amplification, we used a fluorescent detection system called 'probe-based detection' in which fluorescence is due to the addition of a fluorophore and a quencher present on the primer sequences. This system detects the cycle

in which the signal is significant or stronger due to the amount of amplified DNA that reaches a threshold or is above the background levels. This cycle (Ct value) is used to calculate the relative levels (RQ) of expression of the initial material from samples compared to an endogenous or reference gene, which is present in all the samples [158]. The **RQ** values of the samples were calculated and normalized to a standard that consisted of a pool of cDNA from 10 adults, with the value set to 1. In this thesis, DNA and RNA were extracted using silica-based spin columns, and the eluted material was quantified. For the latter analyses, mRNA was first converted to cDNA using a two-step RT-PCR with random hexamer primers.

In this thesis, we used this method in: **Paper I** to detect an ongoing, high-risk HPV infection, using a Real-Time PCR assay that identifies 12 high-risk (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59) and two low-risk (6 and 11) HPV types, by targeting segments of the E6/E7 region [51]; **Paper II**, to detect HSV-2 DNA by amplifying a nucleotide segment of the gB region [159]; and in **Paper III** to quantify the relative mRNA expression levels of IL-1β, IL-18, CXCL10 and NLRP3 using *GAPDH* as the reference gene.

3.6. Statistical methods

The descriptive data in **Paper I** are presented as percentages, medians, prevalence estimates and 95% confidence intervals (CIs). Statistical comparisons were calculated using the χ^2 test. Associations between the dependent variable (viral STI) and independent variables were tested by binary logistic regression. This analysis was used to estimate crude and adjusted odds ratios and 95% CIs. Odds ratios were used to measure the association between the exposure and the outcome, while confidence intervals were used to estimate the precision of calculating the upper and lower limits in which the true population might lie [160]. Variables or categories that showed associations with the outcome at $p<0.1$ were included in an adjusted model to control for possible bias due to a cofounding variable. In **Papers II** and **III**, different treatments were compared using unpaired tests when we had used cell lines, and paired tests when we had used PBMCs. If the data were normally distributed we used the Student´s *t*-test and one-way ANOVA with Dunett´s post-test correction. If the data were not normally distributed we used the Mann-Whitney *U*-test and Kruskal-Wallis test. Most of the statistical analyses were carried out using the PRISM 7.0® GraphPad software, although for logistic regressions we used the SPSS ver. 24.0 IBM software. A p-value <0.05 was considered statistically significant.

4. RESULTS AND DISCUSSION

4.1. Prevalence of viral STIs in rural La Paz, Bolivia

Demographic data and prevalence of viral STIs

Very little is currently known about the prevalence of viral STIs in Bolivia owing to a scarcity of studies and a lack of follow-up reports from the reference centers. We have investigated the prevalence of viral STIs in 394 healthy women from rural and semi-rural areas in the Department of La Paz. Through our questionnaire, we have collected demographic data and also collected blood and cervicovaginal samples. To assess how socioeconomic factors and the traditional lifestyle influence the prevalence of viral STIs, we divided our study population into three groups according to residence: in small villages, in a small town, or in a large town. The median age of our study population was 34 years, the median number of children in this cohort was 2, and women living in villages tended to have more children than women living in towns. The majority of the participants were housewives, and they were particularly common in villages. More than 50% of the women did not use any family planning method, and about 4% of the women reported the use of condoms. While 69% of the women reported having had a cytological examination, 62% of those examined did not know the test results (see Table 1 in **Paper I**).

The prevalence of viral STIs in this group of women was analyzed by serology to detect antiviral antibodies and viral antigens in the serum, and by PCR to detect viral DNA in the cervicovaginal samples. For HPV, we focused our detection on the serotypes that can cause disease, i.e., the high-risk types that are associated with cervical cancer and the most common types that are associated with genital warts. As a consequence, our data on the overall prevalence of HPV infections (the low-risk HPV types) are incomplete.

HSV-2 was by far the most prevalent viral STI. Overall, 53% of the women had HSV2-specific antibodies, implying that these women had a chronic HSV-2 infection (Table 3). The percentage of HSV-2-infected women was higher in rural areas, and the prevalence increased with age and with the number of children born to these women.

High-risk HPV (HR-HPV) was the second most common viral STI, with 27% of the women having an ongoing HR-HPV infection (Table 3). In contrast to the pattern seen for HSV-2 infections, there were no significant differences in age, occupation, number of children, contraceptive use or place of living between the HPV-positive and HPV-negative women.

Overall, 10% of the women (in total, N=40) had been exposed to HBV (Table 3). Based on the presence or absence of anti-HBV antigen and antibodies, we estimate that 5% of those infected had an acute infection, 15% had a chronic infection, and 80% had a previous infection. These women belonged to different age-groups and were located in both villages and towns. None of the women had antibodies to HIV (Table 7), and they were therefore considered to be HIV-negative.

Table 7. Prevalence of viral STIs in rural and semi-rural populations of La Paz, Bolivia.

	Populations						**Total**	
	Villages		**Small town**		**Large town**			
	N=77		N= 73		N= 239		**N=394**	
Pathogen	**No.**	**(%)**	**No.**	**(%)**	**No.**	**(%)**	**No.**	**(%)**
*HPV**	17	(25)	14	(21)	70	(29)	101	(27)
HSV-2	52	(67)	41	(56)	112	(47)	255	(53)
HBV	4	(6)	5	(7)	29	(12)	38	(10)
HIV	0	(0)	0	(0)	0	(0)	0	(0)

* HPV prevalence was calculated for a total population of **376** participants: 69 participants from villages, 68 participants from a small town, and 239 participants from a large town.

Coinfections

Most of the women had a maximum of one ongoing viral STI. Overall, 36% were negative for any of the investigated viral STIs, 48% were positive for one infection, 16% were positive for two infections, and 0.5% were positive for three infections (see Table 5 in **Paper I**). There was no association between HPV-positive cases and the seroprevalence of HSV-2 or HBV, although the majority of the coinfections were for HSV-2 and HPV (see Tables 2–4 in **Paper I**).

HPV genotypes

For the 12 cases of HR-HPV that we detected in our PCR analyses, HPV 31, 39 and 56 were the most prevalent HPV types, in contrast to HPV 16 and 18, which are the more commonly found HPV types around the world (Fig. 10 A). In our cohort, the prevalence of HPV 31, 39 and 56 was more than 50% higher than the global prevalence, whereas the prevalence of HPV 16 and 18 was less

than half of the global estimate of prevalence (Fig. 10 B). Multiple infections with more than one HR-HPV were present in about 25% of the HPV-positive women (Fig. 10 C).

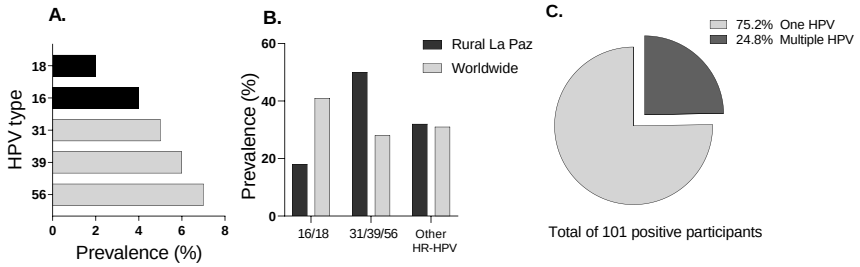

***Figure 10**. Prevalence of HPV genotypes in the cervical samples from 101 participants with detected HPV infection. Prevalences of the five most-prevalent HPV types in rural La Paz and worldwide (A). Distribution of HPV 16/18, HPV 31/39/56, and other HR-HPV types in rural La Paz compared with those found worldwide (data from Clifford at al. 2005). (B) Proportions of HPV-positive women in La Paz? who had a single or multiple HPV infections (C).*

Discussion: Prevalence of viral STIs

These results are in accordance with those from other studies showing that certain STIs are more common in poor rural populations [28, 161, 162]. It seems likely that the women living in these areas are not aware about how to prevent STIs as a result of limited access to healthcare and sex education. Overall, Bolivians have inadequate knowledge of preventive measures against viral STIs. Only 7% of rural Bolivian women have adequate knowledge as to how to prevent HIV/AIDS, as compared to 36% of those living in urban areas [83]. This may explain why frequency of the use of condoms was low in our study cohort, similar to the frequency reported for the general population of Bolivia [56]. The reported rate of cytological inspections related to Pap smear screening in the women was high (almost 70%), as compared with that in the general population (33%) [56, 59]. However, the importance of this difference in the rate of Pap smear testing is unclear, as few of the women received any follow-up information. Unfortunately, we did not ask more questions regarding other risk factors, e.g., number of partners or how long they have used contraception, as it is stigmatizing to talk about STIs in these populations and most women do not keep track of their consultations or the procedures that they underwent, e.g., Pap smear tests. On the other hand, the lifetime number of sexual partners could be a more relevant question to pose to the husbands of these women, as no differences in the prevalence of STIs were reported

between women with only one lifetime partner and women with more than three partners [163].

This study shows that the prevalences of viral STIs, with the exception of HIV, are high in rural populations located in the Amazonas. The seroprevalence of HSV-2 is higher in rural La Paz compared to the overall seroprevalence in South America and also compared to other rural communities in Haiti and Costa Rica, where the prevalence are 42% and 38%, respectively [30, 164]. A similar high prevalence (62%) was recently described in female prisoners in Cochabamba in the Bolivian lowlands, which supports our conclusion that HSV-2 infection is common in Bolivia [37]. Our data showing that HSV-2 is more prevalent in small villages, which are mainly inhabited by indigenous people, than in larger towns are in line with other studies from certain African populations, from Brazil [156, 165, 166], and from indigenous populations of Cape York, Australia [167]. I believe that better sanitary conditions, health services, and sex education are required for the control and management of genital STIs in these rural areas. Furthermore, male infidelity rates are high in indigenous populations in Bolivia [168], which is problematic because most of the women in our study had unprotected sex with their partner, and just 4% reported the use of condoms. We did not find any association between the use of hormonal contraception and HSV-2 infection, although it is important to bear in mind that prolonged hormonal contraception is considered to be a risk factor for the acquisition of genital herpes [169].

I was very pleased that we did not find any HIV cases in the population that we studied. This is in agreement with the report published by UNAIDS, which indicates a HIV prevalence of only 0.3% in the adult Bolivian population. This finding is also in agreement with the zero cases found in a study of approximately 1,500 people from urban and rural areas of Bolivia, which involved 885 healthy women [83]. The monitoring of HIV and AIDS in pregnant women in Bolivia has improved recently, and show that there is a higher prevalence of HIV in major cities such as Santa Cruz (1.3%), Cochabamba (1%), and La Paz (0.3%) [15], with an unfortunate increase in the number of new cases [82]. It is somewhat surprising that the HIV prevalence in Bolivia is so low given that there is a high number of HSV-2-positive cases and as a high frequency of unprotected sex, both of which are considered to be risk factors for HIV acquisition [170, 171].

The relatively high rate of HBV seroprevalence found in our study cohort is in agreement with data obtained from the Amazonas region of Colombia and other countries of South and Central America [67, 172, 173]. In contrast to earlier findings in other indigenous groups in the Amazonas and Cochabamba

in the 1990's in Bolivia, the prevalence of HBV core antigen seropositivity (indicating ongoing infection) has decreased from >30% [72] to close to 10%, a decrease that could potentially be attributed to the introduction of the HBV vaccine and/or improvements in sanitary services. However, vaccination for HBV started in Year 2000 and all the persons in this study, with the exception of a few women younger than 18 years, were considered to be unvaccinated. Moreover, the prevalence of HBV differs between regions and between groups.

In contrast to earlier studies that reported HPV prevalences of 8% and 18% in Bolivian rural and urban areas, respectively, we found a prevalence of 27% for ongoing HR-HPV infection, which indicates that HR-HPV infection is more prevalent in the Amazonas region than in other regions of Bolivia [57, 58]. Contrary to what we expected, we did not find a high frequency of the high-risk HPV types 16 and 18 or the same pattern of HPV types as that seen in urban regions of Bolivia [57] or other regions around the world [49, 174]. Although HPV types 16 and 18 are responsible for approximately 85% of cervical cancers worldwide, the persistence of other high-risk HPV types might contribute to the high rate of cervical cancer seen in Bolivia. Further studies will be needed to assess the pattern of HR-HPV types in rural women who develop cervical cancer. Luckily, the recent introduction of the HPV vaccine Gardasil 4 provides cross-protection against HPV 31, 33 and 45 [54]. However, it is not known if this vaccine protects against HPV 39 and 56.

This study did not find any association between HPV infection and other viral STIs, although the most common coinfection involved HPV and HSV-2. It is important to bear in mind that the presence of HSV-2 is considered a risk factor for the development of cervical cancer [175-177]. Thus, HSV-2 may play a role in the high incidence of cervical cancer in Bolivia. Since the majority of the participants were positive for at least one STI, this could also be a risk factor for acquiring a new STI.

4.2. Antiviral activity

We characterized the chemical compositions of these extracts and also screened them for cytotoxicity before we used the different plant extracts in any *in vitro* or *in vivo* model systems. None of the plant extracts used had strong cytopathic effects on Vero cells or PBMCs. All the extracts were also screened for LPS contamination using the Limulus assay, and LPS-contaminated extracts were discarded.

All the plant extracts were analyzed by GC-MS and revealed to contain more than 120 different molecules, which were categorized into five different families for the extracts of *Equisetum giganteum L, Croton lechleri Müll.Arg., Uncaria tomentosa (Willd. ex Schult.) DC., Copaifera reticulata Ducke, Tipuana cf tipu (Benth) Kuntze*, *Mangifera indica L.* and *Erythroxylum coca.* Sugars were the major constituents in all the plants, making up 50% of the dry weight. Phenolic compounds, including alcohols and organic acids, were the second-most abundant constituents. Extracts of *Copaifera reticulata Ducke* and *Tipuana cf tipu (Benth) Kuntze* also contained substantial amounts of amino acids. Additional HPLC-HRMS analyses performed on extracts of *Tipuana cf tipu (Benth) Kuntze*, *Mangifera indica L.* and *Erythroxylum coca* identified about 30 additional compounds, with flavonoids/polyphenols being more abundant in *Tipuana cf tipu (Benth) Kuntze* and *Mangifera indica L.*, while alkaloids dominated in the *Erythroxylum coca* extract (see Fig. 1 in **Paper II** and Fig. 1 in **Paper III**).

Effect of plant extracts in HSV-2 infection in vitro

An appropriate common cell-infection model was used to assess the antiviral activities of the plant extracts. In **Papers II** and **III**, we show that plant extracts of *Equisetum giganteum L, Croton lechleri Müll.Arg., Uncaria tomentosa (Willd. ex Schult.) DC., Copaifera reticulata Ducke, Tipuana cf tipu (Benth) Kuntze*, *Mangifera indica L.* and *Erythroxylum coca* have direct effects on the virus, thereby blocking subsequent infection (see Fig. 2 in **Paper II** and Fig. 3 A in **Paper III**).

Using a synchronized assay, we evaluated separately the entry, penetration and post-entry stages of HSV-2 infection. All the plant extracts (added at concentration of 100 μg/mL) could block viral attachment and entry into the cells, indicating that they might be used prophylactically to prevent viral infection. Even more interesting is the finding that *Tipuana cf tipu (Benth) Kuntze* and *Mangifera indica L.* were able to interfere with viral replication post-entry, indicating that these plant extracts might have value as post-infection treatments (Fig. 11).

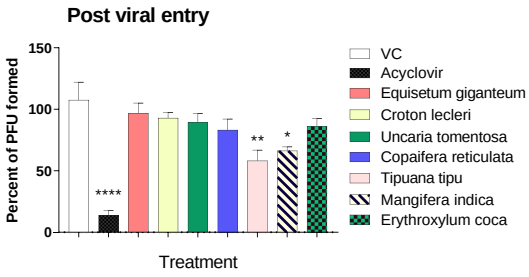

Figure 11. *Extracts of Tipuana cf tipu (Benth) Kuntze and Mangifera indica L. but not extracts of Equisetum giganteum L, Croton lechleri Müll.Arg., Uncaria tomentosa (Willd. ex Schult.) DC., or Copaifera reticulata Ducke block viral replication after viral entry into the cells. Virus control (VC) (Infected untreated VERO cells) and acyclovir treatments are used as controls. values are expressed as mean +/- SEM for n=3-6 and **** = p<0.0001, ** = p<0.01 and * = p<0.05 obtained using ANOVA with Dunnetts´s post-test.*

Effects of plant extracts on HSV-2 infection in vivo

In **Paper II**, we assessed the abilities of the plant extracts to block HSV-2 infectivity *in vivo* using a well-established murine model of genital HSV-2 infection. Female mice were infected with an intermediate dose of HSV-2 (5,000 PFU) and inoculated with HSV-2 or a mixture of the virus and individual plant extracts from *Equisetum giganteum L, Croton lechleri Müll.Arg., Uncaria tomentosa (Willd. ex Schult.) DC.* and *Copaifera reticulata Ducke*. All four plant extracts individually completely inhibited the viral infection. None of the animals that were treated with plant extracts developed any signs of disease (Fig. 12 A), and they had low or undetectable levels of viral DNA in both the vaginal washings and spinal cord (CNS) samples (Fig. 12 B and C). This can be compared to animals that were given HSV-2 alone; they showed high numbers of HSV-2 DNA copies in both the genital tract and in the CNS, and they also developed clinical signs of disease.

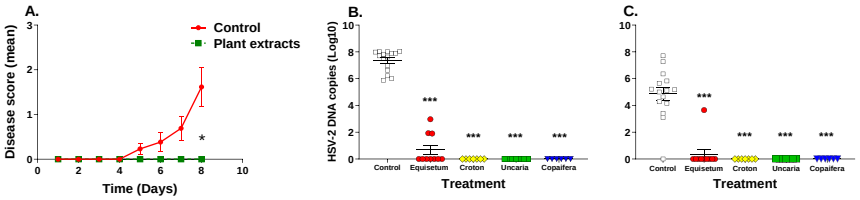

Figure 12. *Extracts of Equisetum giganteum L, Croton lechleri Müll.Arg., Uncaria tomentosa (Willd. ex Schult.) DC. or Copaifera reticulata Ducke reduce genital HSV-2 infection in mice. Assessed were: disease development (A); number of HSV-2 DNA copies in vaginal washes obtained on Day 2 post-infection (B); and number of HSV-2 DNA copies in spinal cord (CNS) samples on Day 8 of infection (C). Control group are untreated mice and the values are expressed as mean +/- SEM for n=10, and * = p<0.05 using log-rank (Mantel-Cox) test (A) and *** = p<0.001 using ANOVA with Dunnett´s post-test (B-C).*

Antiviral immune responses

Since *Tipuana cf tipu (Benth) Kuntze* and *Mangifera indica L.* were able to block viral replication post-entry, we were interested in the immunomodulatory capacities of these extracts. Therefore, we decided to investigate their abilities to induce an antiviral response. After stimulation of PBMC cells with the plant extracts for 24 hours, we found that the *Tipuana cf tipu (Benth) Kuntze* extract induced considerable albeit variable levels of IFN-λ (Fig. 13 A), while there was no IFN-α or IFN-β in the culture supernatants, indicating that this extract has a direct effect on the innate antiviral response. In support of this finding, we also detected increased transcription of the IFN-inducible CXCL10 gene in PBMCs exposed to this extract (Fig. 13 B).

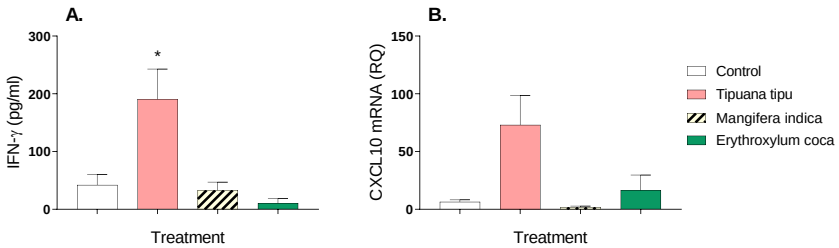

Figure 13. *Plant extracts from Tipuana cf tipu (Benth) Kuntze, but not extracts from Mangifera indica L. or Erythroxylum coca, induce in PBMCs IFN-λ release (A) and CXCL10 chemokine mRNA expression (B). mRNA levels were calculated versus GAPDH ratio. Data are presented as mean +/- SEM for n=3-6. * = p<0.05 using paired t-Test compared to control untreated cells.*

Discussion: Antiviral activity

Previous studies have reported on the use of medicinal plants as sources of novel treatments for diverse diseases based on the numerous and diverse compounds with antimicrobial activity that these plants contain [97]. In the research for this thesis, we evaluated seven plants that are commonly used in traditional Tacana medicine to treat infections of the female genital tract, particularly those caused by HSV-2.

We used hydro-alcoholic extracts that specifically enrich for polyphenols, flavonoids, phenolic compounds, terpenoids and alkaloids [89, 90]. The chemical compositions of the extracts differed not only for different parts of the plant, but also depending on the method of extraction used and environmental factors, such as the collection season and make-up of the soil [178]. However, our results using crude plant extract show that the compounds present in these mixtures/extracts do not have any significant cytopathic/cytotoxic effects, and that they do have antiviral activities.

Our results confirm the results of previous studies that have shown that products or extracts derived from *Croton lechleri Müll.Arg. Uncaria tomentosa (Willd. ex Schult.) DC., Mangifera indica L.* and *Erythroxylum laurifolium* have activities against herpes infections. Both a medical preparation from *Croton lechleri Müll.Arg* called SP-303 and an extract from the stem bark of *Uncaria tomentosa (Willd. ex Schult.) DC.* were shown to interfere with HSV attachment and entry [179, 180]. Mangiferin, which is present in *Mangifera indica L.* and other plants, can inhibit HSV-2 replication post-entry [132]. Extracts from *Erythroxylum* species show activities against HSV-1, and they block both viral attachment and replication [181, 182]. Studies on *Equisetum giganteum L, Copaifera reticulata Ducke Tipuana cf tipu (Benth) Kuntze* and *Erythroxylum coca Lam* have shown broad activities against many different microorganisms (Gram-negative and Gram-positive bacteria, fungi, yeast and influenza virus) [121, 183-187], indicating that they can be used as bactericidal, microbicidal and antiseptic treatments. Thus, this research is a first step towards a more profound understanding of how these species might exert antiviral activity *in vitro*.

An interesting finding from the present research is that *Tipuana cf tipu (Benth) Kuntze* induces an antiviral immune response in PBMCs through the production of IFN-λ. IFN-λ, or type III IFN, shares many features with type I IFNs, including the ability to block viral replication. It has previously been shown that IFN-λ blocks HSV-2 replication *in vitro* and that mice that lack IFN-λ succumb more readily to HSV-2 infection [135].

IFN-λ production can be induced through several different pathways, most of which overlap with the pathways for type I IFNs, and they involve Toll-like receptors, RIG-1-like receptors, and cGas-STING [188]. We have not yet delineated the pathway through which *Tipuana cf tipu (Benth) Kuntze* induces IFN-λ but no other type of IFN, although it is tempting to speculate that it involves a pathway that involves the cellular mitochondria. Two independent observations point us in this direction. First, our data show that the *Tipuana cf tipu (Benth) Kuntze* extract induces mitochondrial ROS in PBMCs, which implies that the extract interacts with and/or affects the mitochondria. This is interesting as mitochondrial ROS promote IFN-λ production [189]. Second, the mitochondrial pathway to IFN-λ production is the only pathway known to us whereby IFN- λ can be induced independently of type I IFNs. This pathway involves the sensing of PAMPs (e.g., viruses) and/or DAMPs ('danger' molecules from dead cells) by RIG-I-like receptors. These receptors utilize mitochondrial signaling proteins (MAVS) to activate the transcription factors interferon regulatory genes 3 (IRF3) and IRF7 [189, 190], which leads to the induction of type I and/or type III IFN responses. Depending on the exact location of the MAVS, type I and type III IFNs are produced [189, 190]. Thus, it is possible that *Tipuana cf tipu (Benth) Kuntze* interacts with the mitochondria and induces IFN- λ directly *via* the MAVS pathway or indirectly through the generation of ROS.

Tipuana cf tipu (Benth) Kuntze extracts also promoted the transcription of IL-1β and CXCL10. The sensing of PAMPs and DAMPs by RIG-I like receptors proteins will activate not only IRF3 and IRF7, but also the transcription factor NF-κB [189, 190]. NF-κB induces the production of pro-inflammatory cytokines, such as IL-1β, IL-18 and IFN-γ, which enhance the antiviral response in the affected tissue. This inflammatory response will lead to the expression of additional cytokines and chemokines, including the chemotactic cytokine IFN-γ-inducible protein 10 (CXCL10), which can coordinate the activation and migration of cells to the site of infection [191]. Previous studies have noted that mice lacking CXCL10 are more susceptible to HSV and leishmaniasis [192, 193], and that the protective mechanism is due to the recruitment of CD8+ T cells and NK cells to the site of infection, as well as to the CNS [193]. Furthermore, the presence of the CXCL10/CXCR3 chemokine pathway can protect against the recurrence of HSV infection by developing T-cell immunity in locally infected tissues [194].

The ability to induce IFNs is not unique to *Tipuana cf tipu (Benth) Kuntze*. *Bupleurum kaoi*, which is a plant used in oriental medicine, can prevent Coxsackie B virus infection of fibroblasts *via* the induction of type I IFNs [195]. *Cimicifuga foetida*, which is a medicinal herb used in China, blocks

human respiratory syncytial virus infection of epithelial cells *via* the induction of IFN-β [195, 196].

Our findings, while preliminary, suggest that *Tipuana cf tipu (Benth) Kuntze* and *Erythroxylum coca Lam* could be further evaluated *in vivo* as therapeutic treatments for genital herpes. Taken together, our results suggest that the medicinal plants used by the Tacanas in the Amazonas region can be used as prophylactic microbicides because they target the binding and entry of HSV-2.

4.3. Anti-inflammatory activity

Inflammasome activation

In interview-based reports on traditional Tacana medicine, it is evident that the majority of the medicinal plants utilized by the native population are used to treat inflammation in different parts of the body. Therefore, we investigated in **Paper III** whether extracts from *Tipuana cf tipu (Benth) Kuntze*, *Mangifera indica L.* and *Erythroxylum coca* could influence the inflammatory response in human PBMCs. We posed the following questions; a) Can either of these plants induce an inflammatory response similar to the antiviral response observed above; and b) Can either of these plants block inflammation provoked by LPS. To answer the first question, we exposed cells to plant extracts for 3 hours and measured the inflammasome-mediated release of IL-1β and IL-18, as well as the upregulation of mediators that promote the inflammasome-mediated response. None of the extracts induced the release of IL-1β or IL-18. While *Tipuana cf tipu (Benth) Kuntze* induced weak upregulation of IL-1β transcription, it had no effect on the levels of mRNA for IL-18 or the inflammasome-component/receptor NLRP3 (Fig. 15). Another endogenous pathway for the activation of the inflammasome is the liberation of damage-associated molecules (DAMPs) that can affect the mitochondria, and release subsequently the mitochondrial ROS. We show that the extract of *Tipuana cf tipu (Benth) Kuntze* induces a modest but significant release of mitochondrial ROS (see Fig. 2 B in **Paper III**).

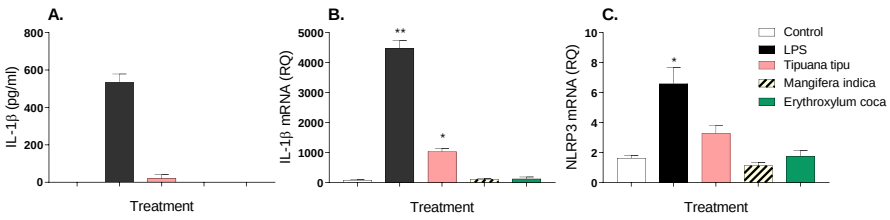

Figure 14. *PBMC were stimulated with extracts from* Tipuana cf tipu (Benth) Kuntze, Mangifera indica L. *and* Erythroxylum coca, *and LPS endotoxin as positive control for 3 hours. (A) protein levels of IL-1β were measured in supernatants. (B) mRNA levels of IL-1beta and (C) NLRP3, are expressed as relative quantification (RQ) that was calculated by the target mRNA expression versus GAPDH mRNA ratio. Data is expressed as mean +/- SEM for n=3. ** = p<0.01, * = p<0.05 using matched ordinary one-way ANOVA with Dunnetts´s post-test compared to control untreated cells.*

LPS inhibition

To answer the second question (if plant extracts can block inflammation provoked by LPS), we pretreated PBMCs with plant extracts (or control medium) for 30 minutes prior to LPS exposure, and measured the levels of released IL-1 β after 3 hours of incubation. We show that all three of the plant extracts tested (*Tipuana cf tipu (Benth) Kuntze*, *Mangifera indica L.* and *Erythroxylum coca)* can reduce by 50% the LPS-induced release of IL-1β in PBMCs pre-exposed to the plant extracts (Fig. 15).

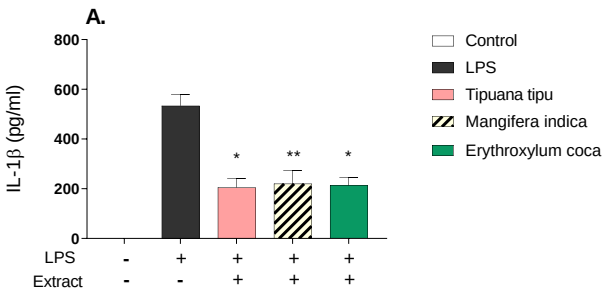

Figure 15*. Plant extracts inhibit LPS-induced IL-1β production. (A) PBMC cells were treated for 30 minutes with 100 μg/mL of plant extracts from* Tipuana cf tipu (Benth) Kuntze, Mangifera indica L. *and* Erythroxylum coca *prior challenge with 100 ng/mL of LPS endotoxin. After 3hours of culture, protein levels of IL-1β were measured in supernatants. Data is expressed as mean +/- SEM for n[7]=3. ** = p<0.01 and * = p<0.05 using matched ordinary one-way ANOVA with Dunnetts´s post-test compared to LPS.*

Discussion: Anti-inflammatory activity

Many medicinal plants have a high content of polyphenols, which are believed to be the principal constituents responsible for the antioxidant and anti-inflammatory properties. Polyphenols such as Quercetin, Feluric acid, and Luteolin have all been proven to block inflammasome activation and inflammatory reactions [197, 198]. Mangiferin from *Mangifera sp* and Baicalin, which is a flavone from *Scutellaria* species, not only modulate the NLRP3 inflammasome, but they also modulate NF-κB, which controls the production of pro-inflammatory cytokines [199, 200]. Food plant constituents and secondary products, such as lectins, can act as "danger signals" to induce mitochondrial damage and subsequent NLRP3 inflammasome activation [201]. Our results show that extracts of *Mangifera indica L.* and *Erythroxylum coca Lam* do not have the ability to activate the inflammasome. However, *Tipuana cf tipu (Benth) Kuntze* can weakly prime the transcription of IL-1β, although it does not promote the release of the cleaved active form of IL-1β This modulation may be attributed to the presence of mitochondrial ROS [202].

We confirm that the *Tipuana cf tipu (Benth) Kuntze* extract contains high concentrations of flavonoids and of terpenoids, particularly catechin and epicatechin, which are the units of tannins and potentially confer antioxidant and anti-inflammatory activities [203, 204]. Methanolic extracts of the leaves of *Tipuana cf tipu (Benth) Kuntze* have antioxidant and anti-inflammatory activities in paw-induced edema [204, 205]. On the other hand, extracts of the stem and branches of *Tipuana cf tipu (Benth) Kuntze* contain a cytotoxic cinnamylphenol, called Macharistol, which may be cytotoxic for cancer cells [206, 207]. This is interesting from the perspectives that the extract that we used was derived from the stem bark, and that the natives use the stem bark to treat not only gastritis and wounds, but also cancer [117]. One unexpected finding is that extracts of *Tipuana cf tipu (Benth) Kuntze* inhibit the release of IL-1β in LPS-stimulated mononuclear cells.

The anti-inflammatory and antioxidant activities of *Mangifera indica L.* are most likely attributed to mangiferin which is also found in *Pueraria tuberosa* [200]. Mangiferin can reduce the production of pro-inflammatory cytokines in LPS-stimulated mouse macrophages or in mice that suffer from dextran sodium sulfate-induced colitis, by targeting MAP kinases and the NF-κB signaling pathway [208, 209]. It can inhibit the function of the NLRP3 inflammasome by blocking the formation of the NLRP3 inflammasome complex [200, 210]. Mangiferin has also been found to suppress ROS production and apoptosis induced by high levels of glucose in endothelial cells,

and this is ascribed to its role as a novel activator of Nrf2 factor, which regulates oxidative stress [211]. This part of the study confirms that the crude extract of the leaves of *Mangifera indica L* inhibits significantly LPS-induced inflammation, also in primary human cells.

Studies on *Erythroxylum* species have uncovered analgesic and anti-inflammatory activities that reduce the pain levels and paw edema in *in vivo* models [212, 213]. The use of these extracts also provides protection against the toxic effects of venoms, in terms of reducing coagulation, proteolysis or edema [214]. *Erythroxylum coca Lam* is widely used for treating acute mountain sickness or altitude sickness pain and inflammatory disorders, although the majority of these studies focused on its high content of alkaloids and their analgesic activities [215, 216]. Nonetheless, there are few reports in the literature on *in vitro* studies that elucidate the inflammation pathway involved in this activity. Apart from their content of alkaloids, these extracts also contain high levels of sugars, phenolic compounds (such as polyphenols), and organic acids, which have been implicated as the active compounds in the studies of the other *Erythroxylum* species mentioned above. Our results confirm that *Erythroxylum coca Lam*, which is a species that is broadly used by indigenous women in Bolivia, strongly suppresses inflammatory LPS-induced production of IL-1β *in vitro*.

5. CONCLUSION

Prevalence of viral STIs in women in rural La Paz, Bolivia

There is an unusually high seroprevalence of HSV-2 infection in women who live in the villages and towns of the Department of La Paz in Bolivia. Both HPV and HBV infections are common in these areas, and we note that the pattern of high-risk HPV types differs both from that found around the world and from the HPV types included in the HPV vaccine that was introduced in Bolivia in 2017. Therefore, there remains a need to improve and implement appropriate prevention and monitoring strategies for STIs, especially HSV-2 that is a risk-factor for the acquisition of HIV and for the development of cervical cancer.

Antiviral and Immunomodulatory activities of plant extracts

We show that hydroethanolic extracts of *Equisetum giganteum, Copaifera reticulata Ducke, Tipuana cf tipu (Benth)* and *Erythroxylum coca* have potent antiviral activities against HSV-2, comparable to those of previously identified plants, *Croton lechleri Müll. Arg*., *Uncaria tomentosa (Willd. ex Schult.) DC*. and *Kuntze*, *Mangifera indica L.* All plant extracts blocked the binding and entry of HSV-2 into cells. *Equisetum giganteum L, Copaifera reticulate Ducke, Croton lechleri Müll.Arg*. and *Uncaria tomentosa (Willd. ex Schult.) DC*. also protected against genital HSV-2 infection in mice. The treated animals showed no clinical signs of disease and no viral replication in the genital tract or in the spinal cord.

Unlike the other plant extracts, *Tipuana cf tipu (Benth) Kuntze* and *Mangifera indica L.* contained substances that could block HSV-2 replication after viral entry into cells and thus hold promise as therapeutic remedies. *Tipuana cf tipu (Benth) Kuntze* could augment the antiviral immune response through the induction of IFN-λ implying that it holds immunomodulatory properties. Several of the plant extracts, *Tipuana cf tipu (Benth) Kuntze, Mangifera indica L* and *Erythroxylum coca Lam* had anti-inflammatory properties as they blocked LPS-induced inflammasome activation and subsequent release of IL-1β.

These studies indicate that plants used by the Tacana tribe could be explored further for the development of topical antiviral microbicides and anti-inflammatory treatments.

6. FUTURE PERSPECTIVES

Our study highlight the burden of viral STIs in women in rural Bolivia and emphasizes the need for better preventive measures and also for better and regular surveillance of these infections throughout Bolivia. Overall, more studies should be performed to assess the prevalence of infectious diseases in the Bolivian population, in particular among the indigenous populations in rural setting where access to health-care and sanitary conditions are limited as well as access to education. It is particularly important to continuously monitor HIV infection in rural settings and to take active measures to prevent the spread of HIV into these remote areas where risk-factors for HIV-infection are abundant: High prevalence of HSV-2, high rate of male infidelity, women's economic dependency on their husbands and a high rate violence against women.

Cervical cancer is 20 times more common in Bolivia compared to e.g. in Sweden. We found that although many women are infected with HR-HPV, HPV16 and HPV18 infections are less common than in other areas of the world. Instead other HR-HPV strains prevailed. Thus, we need to characterize the pattern of HR-HPV in women with cervical neoplasia to understand which HR-HPV that are associated with cervical cancers in Bolivian women. This knowledge is fundamental for the HPV vaccine program that has been introduced in Bolivia and will answer if an appropriate HPV vaccine has been implemented.

Medicinal plants are attracting more and more attention as alternative treatment options for a variety of human diseases, including infections, inflammatory diseases and cancer. The key challenge for future research is to purify all components in these plants and identify and characterize those with medicinal activity. This requires high throughput technology and considerable amounts of plant material. Such a project, although costly, could have strong implications for the development of new treatments and thus improve our future health, and could (if successful) lead to considerable economic benefits for the Bolivian society. To maintain the ancient knowledge of medicinal plants is thus a priority, and should be performed in a systematic fashion.

ACKNOWLEDGEMENT

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