Development of Efficient and Safe Lactoferrin Nanoparticles based MPT Microbicidal Drug Delivery Systems against HIV-1

DOCTOR OF PHILOSOPHY

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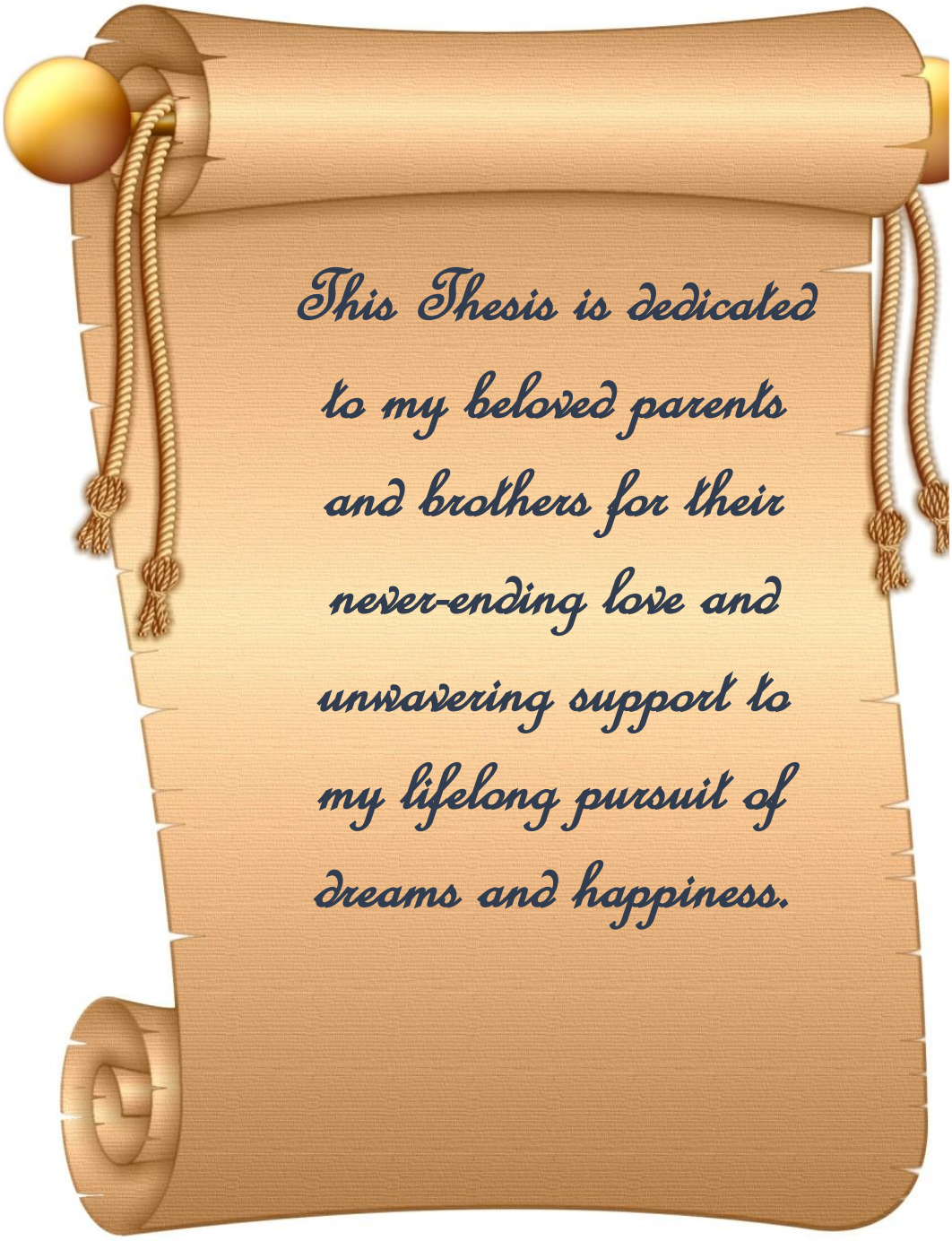
This Thesis is dedicated
to my beloved parents
and brothers for their
never-ending love and
unwavering support to
my lifelong pursuit of
dreams and happiness.

Development of Efficient and Safe Lactoferrin Nanoparticles based MPT Microbicidal Drug Delivery Systems against HIV-1

A thesis submitted to University of Hyderabad for the award of a Ph.D. Degree in Biotechnology

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(A central university established in 1974 by an act of Parliament)
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CERTIFICATE

This is to certify that thesis entitled **"Development of Efficient and Safe Lactoferrin Nanoparticles based MPT Microbicidal Drug Delivery Systems against HIV-1"** is a record of bonafide work done by **Ms. Samrajya Lakshmi.Y,** a research scholar for Ph.D. programme in the Department of Biotechnology and Bioinformatics, University of Hyderabad under my guidance and supervision. The thesis has not been submitted previously in part or full to this or any other university or institution for the award of any degree or diploma. I recommend her thesis for submission towards the partial fulfillment of Doctor of Philosophy degree in Biotechnology.

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Further, the student has passed the following courses towards fulfillment of coursework requirement for Ph.D .

Si.No	Course code	Name	Credits	Pass/Fail
1	BT801	Seminar	**1**	PASS
2	BT802	Research Ethics & Management	**2**	PASS
3	BT803	Biostatistics	**2**	PASS
4	BT804	Analytical Techniques	**3**	PASS
5	BT805	Lab Work	**4**	PASS

Supervisor **Head of Department** **Dean of School**

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DECLARATION

I, Samrajya Lakshmi.Y, hereby declare that the work presented in this thesis, entitled as **"Development of Efficient and Safe Lactoferrin Nanoparticles based MPT Microbicidal Drug Delivery Systems against HIV-1"** has been carried out by me under the supervision of Prof. Anand K. Kondapi, Department of Biotechnology and Bioinformatics. To the best of my knowledge this work has not been submitted for the award of any degree or diploma at any other University or Institution. I hereby agree that my thesis can be deposited in Shodganga/INFLIBNET. A report on plagiarism statistics from the University Librarian is enclosed.

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विद्या ददाति विनयं विनयाद्याति पात्रताम् ।

पात्रत्वाद्धनमाप्नोति धनाद्धर्म' ततः सुखम् ॥

Knowledge brings humility; from humility comes worthiness; with worthiness one attains wealth; with wealth one is able to perform his/her duties in a better way; and in performing his/her duties one attains happiness.

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Abbreviations

Antiretroviral	: ARV
Acquired immuno deficiency syndrome	: AIDS
Efavirenz	: EFV
Tenofovir	: TFV
Dapivirine	: DPV
Lactoferrin	: Lf
Soluble	: sol
Nanoparticles	: NPs
Maximum (or peak) concentration	: C_{max}
Half life	: $t_{1/2}$
Area under curve	: AUC
Area under mean curve	: AUMC
Human immunodeficiency virus	: HIV
50% Inhibitory concentration	: IC_{50}
Multipurpose prevention Technologies	: MPT
Non-nucleoside reverse transcriptase inhibitor	: NNRTI
Sexually transmitted infections	: STIs
Simulated vaginal fluid	: SVF
Field-emission scanning electron microscopy	: FE-SEM
Atomic force microscopy	: AFM

Transmission electron microscopy	: TEM
Phosphate buffer saline	: PBS
3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium)	: MTT
Vaginal and oral interventions to control the epidemic	: VOICE
Microgram	: µg
Microliter	: µl
Micromolar	: µM
Millimolar	: mM
Curcumin	: Cur
Curcumin loaded Lf NPs	: CNPs
EFV loaded Lf NPs	: ENPs
TFV loaded Lf NPs	: TNPs
DPV loaded Lf NPs	: DNPs
EFV & curcumin loaded Lf NPs	: ECNPs
TFV & curcumin loaded Lf NPs	: TCNPs
DPV & curcumin loaded Lf NPs	: DCNPs

CONTENTS

Chapter 1

Introduction

Introduction:

Human immunodeficiency virus (HIV), a lentivirus belongs to retroviridae family, causes destruction of the immune system leading to the onset of acquired immunodeficiency syndrome (AIDS). It is a condition in humans characterized by low immunity levels and life-threatening opportunistic infections. The major modes of HIV transmission are sexual contact, exposure to infected blood, infected needles, mother-to-child and others. High frequency of transmission of HIV has been reported through heterosexual route. Currently, more than 35 million people are HIV-1-positive. Over the past 25 years, there has been an extensive research conducted to understand the pathogenesis of the infection and the development of preventive methods and treatment strategies [1-3].

Exposure of the vaginal mucosal surface to HIV during sexual intercourse is the main reason for the highest rate of HIV transmission. Preventing the transmission is one of the important steps towards reduction in spreading of virus infection. This requires a special method devised to fight HIV at the surface. Intravaginal microbicide delivery systems provide a way and this could have considerable public health and economic impact especially in resource-poor countries. These microbicides work to block viral replication and provide a strong genetic barrier to the development of drug resistance in the virus. The mucous membrane of the vaginal epithelium has a large surface area, permeability and rich blood supply making it best suited for local and systemic delivery of drugs [4-6]. This delivery system is expected to maintain the acidic pH same as of normal vagina, should not alter the growth of normal vaginal flora (e.g. *Lactobacilli*) and should be nontoxic and provide protective environment to genital mucosa.

Overall ideologies for prioritizing microbicide development:

Safety:

Vaginal epithelium with its helpful microbiological flora acts as an innate barrier in preventing infections naturally. Since the use of the microbicides is preventive and is used by healthy population, it is important that they do not demonstrate any localized damage to the epithelium and to the vaginal microflora. Systemic toxicity

associated with dug dosage, frequency, duration should be avoided. In cases where the microbicide does not act as a contraceptive, it should not cause a negative impact on fertility and/or fetal aberrations which are a crucial concern for women.

Efficacy:

Any developed microbicide should certainly have a significant degree of efficacy. However, the level of efficacy for a microbicide remains the topic of considerable debate in different national programs.

Cost and availability:

Despite having effective biological interventions, availability at an affordable price impacts mass distribution that is required to achieve widespread use. Since the microbicide must be reasonably priced to at-risk populations, the production should be simple and accessible.

Compatibility and acceptability:

A microbicide must be acceptable during intercourse and compatible with other forms of contraceptives like condoms and cervical barriers. This is important as, a product that shows great efficacy in clinical trials may not be adopted by at-risk populations in the real world.

Appropriate drug delivery:

For any microbicide to be effective, sufficient drug levels must be sustained in the appropriate compartments of the genital tract or rectum during exposure to the virus. The formulations should be designed for sustained drug delivery, high drug levels in the genital tract and low drug levels in the systemic circulation.

Potential for resistance and impact on treatment:

With increased usage of ARV drugs as microbicides, it is important to ensure that, its usage should not cause drug resistance. This may limit the therapeutics availability to infected individuals at the period of drug use. To overcome this drug resistance, combination therapies with new delivery formulations are under development.

Antiquity of microbicide development:

An intravaginal drug delivery system developed in 1970, using a vaginal ring for the delivery of medroxyprogesterone acetate, a contraceptive, was first to be reported

[4]. Development of bioadhesive gels, microparticles and tablets, have emerged as controlled intravaginal microbicide delivery systems [7-17]**.** These microbicides, when inserted intravaginally prior to sexual intercourse would reduce the risk of transmission of STIs and HIV-1 [14, 18]. Development of controlled release formulations for an extended period of intravaginal delivery of microbicides is the current interest in this direction [19]. Major hurdles in the development of microbicide formulation are the localized and systemic toxicity of the antiretroviral agent, ineffectiveness of the ARV in vaginal flora and viral escape. An ongoing clinical trial employs conventional semi-solid aqueous gels and vaginal ring formulations to provide a single dose of a microbicidal agent [20-24]. Duration of drug release for these delivery systems is 6hr for vaginal gels [25, 26], 8 hr for vaginal tablets [27-29] and 71 days for vaginal rings [30]. Johnson and Masters showed that the microbicide distribution in the vaginal tissue varies considerably with the nature of the delivery system. Sufficient data is not available on the bioavailability of most intravaginal microbicide delivery systems after extended vaginal exposure [31, 32]. Nanomaterials based microbicide formulations like dendrimer-based microbicide formulation SPL2008 (VivaGel™) and gel containing dendrimer are under clinical trials [33-36]**.** Protein-conjugated noble metal NPs are also being developed as microbicidal formulation [37-39]. A study reported the increased effectiveness of the microbicide upon administering shortly before coitus, suggesting the importance of presence of high concentrations of the drug for preventing the virus invasion [41]. Thus, the current challenge is to provide high concentrations of the microbicidal compound in the vaginal compartment over a prolonged period of time [40].

Mechanisms of action:

The foremost action of microbicides is to control heterosexual transmission of HIV-1. Few microbicides act as contraceptives and also prevent other sexually transmitted infections [42-45]. Some biochemical/chemical spermicides, that show considerable activity against HIV-1 and other STI pathogens in vitro, have been assessed as topical microbicides. An effective microbicide, must be able to [20]:

- Support the natural defenses in the vagina to deactivate the virus
- Deactivate the virus while it is in the vagina
- Inhibit the virus attachment and fusion with the host cells in the Vagina/genital tract
- Inhibition of viral proliferation after entering a target cell [46]

Support the natural defenses in the vagina to deactivate the virus

Lactobacilli are the most colonizing bacteria and occur naturally in the vagina. They secrete a variety of anti-microbial compounds such as bacteriocins, bio-surfactants, hydrogen peroxide and lactic acid. These products act as a natural barrier to prevent STIs and other infections. Additionally, vaginal squamous epithelial cells act as the first line of defense against pathogens and are capable of producing anti-microbial peptides. These deactivate or recruit crucial immune cells. They also stimulate the secretion of cytokines which support the persistence of lymphocytes. IgA and IgG antibodies are also abundant in the secretions of the vagina that act against pathogens (Fig 1.1). Any microbicide could act or enhance the natural immune defenses of the vagina. A combination microbicide, consisting of physical and chemical barrier working alongside the natural immune defenses would protect the vagina effectively from infections. Any damage to the microbial flora leads to disruption of vaginal ecosystem leading to enhanced risk of HIV-1 infection. Lactic acid secreted by *Lactobacillus* contributes to the low acidic pH of the vagina. This is altered upon exposure to the semen which is alkaline in nature leading to the loss of vaginal barrier to the pathogens. Few microbicides act as a pH buffer, to prevent this phenomenon. This buffering ability enables the vagina to sustain a low pH by buffering the alkali in semen. Few other microbicides act as contraceptives that prevent unwanted pregnancies, STIs and other infections.

Deactivate the virus while it is in the vagina

Surfactants were the "first generation" microbicide candidates. These products with their detergent like properties were used to disrupt cell membranes or, in some cases, alter the cell's membrane structure to make it more vulnerable and liable to disruption. These surfactants target all cells like any host, commensal and pathogen.

Hence these have a broad spectrum of activity against several microorganisms, sperms, cell membranes etc. The most prominent of these surfactants was nonoxynol-9 (N-9) [47-49]. It was the first microbicide tested clinically, this clinical study involved 892 female sex workers in four countries. The rate of infection almost doubled that of the placebo group when the participants were using N-9 several times a day with a rate of ≥ 3.5 uses/day [47]. However, following studies have shown adverse effects such as inflammation, irritation at the site of application, tissue infiltration by immune cells and alterations in the vaginal flora caused by the regular usage of nonoxynol-9 for vaginal administration [50]. Two non-specific polyanionic candidates such as Carraguard® and cellulose sulfate have been evaluated in Phase III clinical studies. However, they failed in demonstrating efficacy [42, 43]. Some studies proved that, subsequent application and removal of above polyanionic compounds leads to significant increase in HIV-1 infection [51]. Other similar microbicides that are in the clinical evaluation include VivaGel® a dendrimer-based entry inhibitor, and ACIDFORM, another buffering agent [52, 53]. From the above studies, it can be concluded that there are no reliable methods available to confirm product quality during a study. The participant self- reporting mechanism that was used by some studies was shown to be problematic and unreliable [54].

Currently, HIV-1 specific ARVs grabbed major attention in the field of microbicide development. The HIV-1 life cycle offers several opportunities at various stages for the specific blocking of infection. In general, it is accepted that any microbicide should act prior to the integration of proviral DNA into the host cell DNA. The two major classes of ARV microbicides are entry and reverse transcriptase inhibitors (RTIs) [55]. As microbicides other classes of ARVs may also be suitable but, till today these have received less developmental attention.

Inhibit the virus attachment and fusion with the host cells in the vagina/genital tract:

HIV-1, gp120 is a surface glycoprotein which during the process of infection binds to CD4 receptors on target cells and facilitates the virus entry [56]. Several microbicides targeting this interaction have been developed. A novel protein derived

from cyanobacteria, cyanovirin-N has a potential binding affinity for the gp120 protein of mannose functional groups [57]. Another entry level inhibitor BMS-599793 is a potential microbicide candidate and has an effective profile. However, HIV gp120 H375, unique to subtype CRF01_AE shows robust resistance to this entry inhibitor, [58]. In addition to the CD4 receptors, HIV-1 binds to other chemokine receptors such as CCR5 or CXCR4, present on the target host cells. Blockage of co-receptors gives another opportunity for a microbicide intervention to prevent infection. Primarily, sexual transmission of HIV-1 occurs via CCR5 receptors [59], thus many microbicides developed, target these antagonists. The first CCR5 antagonist, Maraviroc (Pfizer, Inc., New York, NY, USA), was approved in 2007 by the US food and Drug Administration (FDA) for the treatment of HIV-1 infection [60, 61].

Inhibition of viral proliferation after entering a target cell:

Some ARV drugs, now being tested as microbicides, prevent the replication process. Reverse transcriptase (RT) inhibitors are mainly of two types; NRTIs (nucleoside RT inhibitors) and NNRTIs (non-nucleoside RT inhibitors).The NNRTIs act by terminating viral DNA chain elongation in the target cells (Fig 1.1). CAPRISA 004 study has reported some NNRTI-based drugs; DPV, TMC120, EFV, MIV 150, UC 781, and S-DABO etc. and NRTIs, mainly TFV in preclinical and clinical evaluation as microbicides. It was proved that 1% TFV gel declines HIV-1 infection by 39% and genital HSV infection by 51% in women [62]. Conversely, the VOICE and FACTS 001 trials proved that these microbicide gels were not efficient because of low adherence [63, 64]. The complications of adherence directly correlate with the product efficiency and perception, which differs among women of diverse age groups, the status of relationships and their economic standing [65].

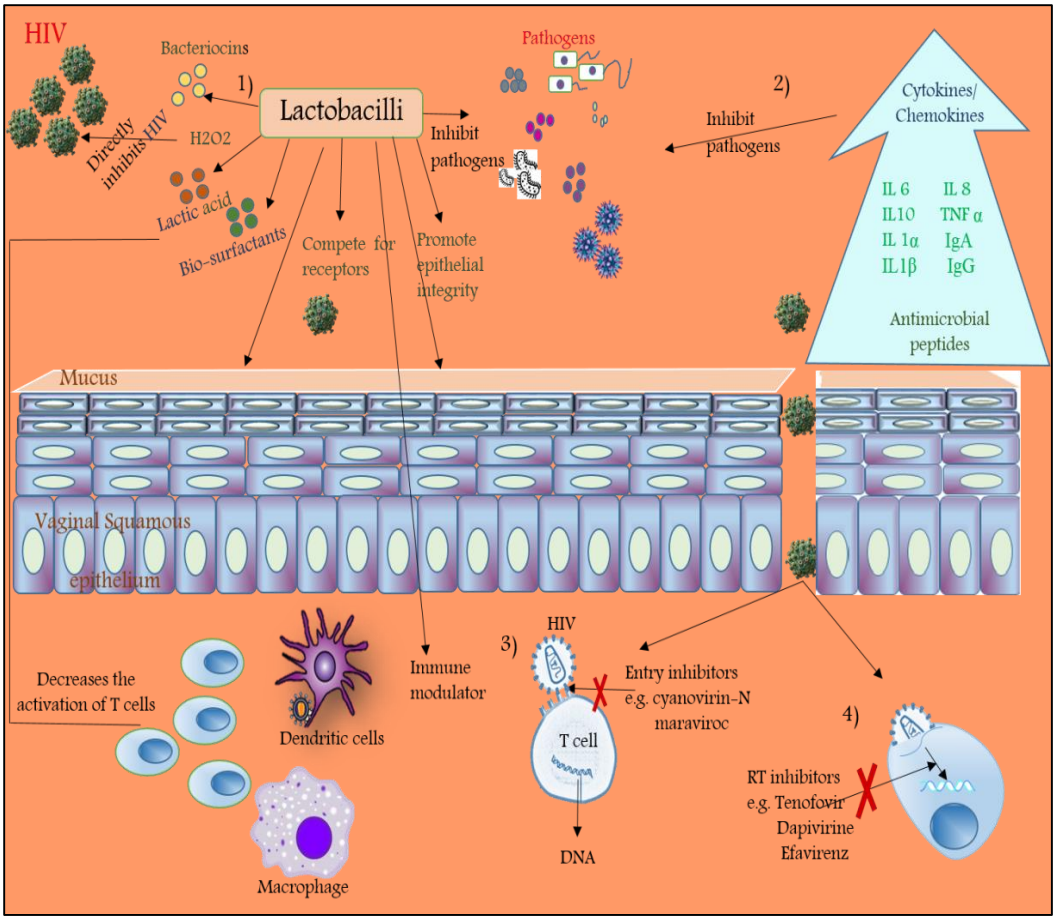


Fig 1.1: Transmission of HIV by squamous epithelium of the vagina and inhibition mechanism of HIV-1. The vaginal stratified squamous epithelium and ectocervix provides extensive protection against HIV-1. (Deposited HIV-1, on the mucosal layer must cross through the thick mucin layer). 1) It should cross vaginal microflora majorly consisting of *Lactobacilli* that showed multi functions against HIV-1 and other pathogens. 2) It should withstand innate immune defenses that include lysozyme, Lf, antimicrobial peptides and cytokines. Disruption of the vaginal epithelium may be caused by any physical abrasion, infection with pathogenic microbes or changes induced by hormonal imbalance. After virus entry in lumen 3) HIV-1 inhibition by Entry inhibitors and 4) HIV-1 inhibition by RT inhibitors.

FEM-PrEP and VOICE trials were unable to show the efficiency of oral PrEP in women. As the microbicide gels future is uncertain, their use for women is still

unclear. In the CAPRISA 008 study, it was mentioned that 1% TFV gel was effective to influence more women to use it. Apart from these, the results of trials testing ARV drugs which were delivered through vaginal rings are still awaiting. These vaginal rings are made to be worn continuously and can be changed every month to escape from the infection [66].

Combination of compounds /drugs as a microbicide:

Drug combinations for the development of microbicides are already being considered as next-generation products. Mainly 3 key reasons are present behind the development of combination based microbicides. 1) To increase the efficacy of microbicide, single drug based microbicide product would not be effective against the HIV. Self-mutating capacity of the virus leads to the development of resistance to a particular drug and it leads to the loss of efficacy. 2) The combination of ARV drugs leads to the synergistic or additive effect, allowing reduced concentrations of drugs to show effective activity to counter the virus. 3) Combination drugs would afford greater chances of protection by affecting different stages of infection and transmission process. For example, a combination product having a CCR5 blocker and an NRTI would be more likely to be effective than a microbicide having only a CCR5-blocking agent. All above reasons are supported by an experiential superiority of highly active anti-retroviral therapy (HAART) in the prevention of mother–child transmission (PMTCT) [67].

Numerous groups have developed various combinations as microbicides; some of the formulations have undergone different stages of clinical trials. At the same time, large numbers of formulations have failed due to safety, toxicity and efficacy issues. A major requirement of a microbicide is to release large concentrations of active drug in the vagina along with higher bioavailability.

Formulations and delivery methods:

Intravaginal drug delivery formulation vehicles predominantly comprise of gels and rings [68]. Some novel and/or less exploited dosage forms include foams, capsules, creams, films, sponges, pessaries, diaphragms, suppositories, tablets, and tampons.

All the above dosage forms except intravaginal rings (IVRs) require repeated need of dosing before or after intercourse due to their relatively low bioavailability and short therapeutically effective duration (hours to days). The concept of a microbicidal formulation was initiated based on a gel formulation where its application is dependent on coitus (i.e., dosed for each act of sexual intercourse) and many women may prefer this mode of delivery due to its significance. The major advantage of this approach is the direct delivery of drug before prospective viral exposure.

The disadvantages are associated with its acceptance. In advance, women may be able to apply the product discretely when she anticipates that she may have sex. But it is really impossible for many women and also it is difficult to ensure the availability of microbicide. As the experience shows that it is often difficult to make sure the compliance with microbicides upon coitus from the perspectives of clinical trials [69, 70]. In contrast to gels and other formulations, IVRs are having the ability of drug holding from weeks to month's duration, they can maintain controlled delivery of drug and desired vaginal drug concentrations consistently. Compare to gels, IVRs possess favorable user qualities such as coitus-independent and due to their long duration, it allows for sexual spontaneity. To date, their contribution to vaginal pharmacokinetics, bioavailability and bio distribution of drug is poorly understood. [71, 72].

Effects of seminal fluid:

During intercourse, seminal fluid (SF) influences the female genital tract to develop immune responses for the conceptus implantation into the uterus. Mice studies showed that SF triggers and develops the population of regulatory T cells in lymph nodes. These cells are later recruited into the uterus from the genital tract. [73, 74]. The large number of T cells within the genital tract suggests that, cell-mediated immunity has a significant function in the prevention of sexually transmitted infections [75, 76]. Cells involved in cell-mediated response such as macrophages, DCs, and T cells apparently express CCR5 and CXCR4 receptors for HIV and it can be envisioned that the increased number of target cells existing in the genital tract would intensify the opportunity for HIV transmission. However, due to an inverse

relationship between alloimmunization associated with SF exposure and susceptibility to HIV infection, it may be counter balanced [77]. Overall, it is evident that SF-mediated inflammatory response would impact on all physiological and pathophysiological events within the female genital tract [78].

Nano drug delivery methods:

In addition, to the above vaginal dosage formulations, highly advanced categories of the drug delivery have been suggested as strategies to enhance vaginal delivery. A number of nanocarriers are available such as polymeric NPs, liposomes, solid lipid NPs, dendrimers, complexes and nanocrystals. Nanocarriers organize a neat drug delivery system, due to their knack to cross the physiological barriers. They direct the drug to reach its target cell or intracellular partitions either passively or by ligand-mediated mechanisms. This is possibly due to their nanosize of 10–1000 nm range. The principal mechanisms of intracellular uptake of nanocarriers are endocytosis and receptor-mediated transport. They endeavor numerous advantages, such as the safe guarding the drugs against degradation, directing the drugs to precise sites of action, and delivery of biological molecules, such as proteins, peptides, and oligonucleotides. At present nanocarriers are being investigated for many therapeutic purposes to overcome typical drug delivery encounters, such as conformational and physicochemical stability, improved cellular uptake of low permeable drugs, decreased cellular and tissue clearance of drugs, sustained drug delivery and decline in immunogenic response. Few advantages offered by ARV loaded nanocarriers for the formulation of microbicides are the ability to modulate drug release [79], the ability to penetrate epithelial linings [80] and the specific drug targeting feasibility to HIV-1 target cells [81, 82]. Mallipeddi *et al.* have reported a thorough review on the use of nanotechnology in ARV drug delivery [83]. By utilizing nanotechnology, efforts have been made for encapsulating and characterizing the microbicide candidates such as PSC-RANTES, EFV, TFV and DPV [84-87]. An advanced drug delivery strategy provides the opportunities such as greater efficacy, greater stability, improved sustained drug release and targeting. Due to the sustained release aspect

of nano-formulations, it allows the user to apply the formulation independent of coitus, which might lead to an increased acceptance of a microbicide product.

Protein nanoparticles:

Proteins fall in a class of natural molecules, possess certain unique functionalities and potential applications in both biological and as well as in material fields [88]. Proteins interact with both solvent and drug, because of their amphiphilicity, hence they are considered as ideal materials for preparation of NPs [89]. NPs acquired from natural proteins are easily metabolized, biodegradable and are easily acquiescent to surface alterations to allow attachment of drugs and for targeting ligands. Different NPs have been synthesized from natural proteins such as BSA (bovine serum albumin), HSA (human serum albumin), apotransferrin and Lf etc. [90-93].

To overcome the existing microbicidal disadvantages through protein nano drug delivery system, recently our group established a triple combination of curcumin and ARV loaded Lf NPs as a microbicide [87]. Broad spectrum activity of curcumin towards antimicrobial, antibacterial, antiviral, antifungal, and antimalarial activities has been proved by several studies exclusively and in combination with other natural compounds. Curcumin's broad spectrum of action against HIV-1 has also been reported by various groups [94, 95]. Curcumin down regulates inflammatory responses in HIV-1 infected cells, thus inhibiting virus infection through blocking viral c-DNA synthesis. Even though, Curcumin has high efficacy and safety, due to its poor bioavailability it is not yet been employed as a therapeutic agent. It has been observed that Curcumin showed limited absorption, a great degree of metabolism and rapid systemic eradication [96]. In our studies, we have shown that curcumin bioavailability enhanced more than 3 fold by delivery through apotransferrin NPs [91]. In addition to anti HIV-1 activity curcumin acts as vaginal contraceptive [97] agent. It shows concentration-dependent inhibition of sperms. Mainly inhibits sperm mobility and complete blockage of sperms at a concentration of ≥250 μm [98]. Against this background, development of a microbicide formulation composed of protein NPs loaded with single or multiple ARV with a combination of curcumin would form safe microbicide with conception activity (Fig

1.2). This efficient nanotechnology-based delivery system can overcome these problems and improve bioavailability, absorption rate and the rate of metabolism.

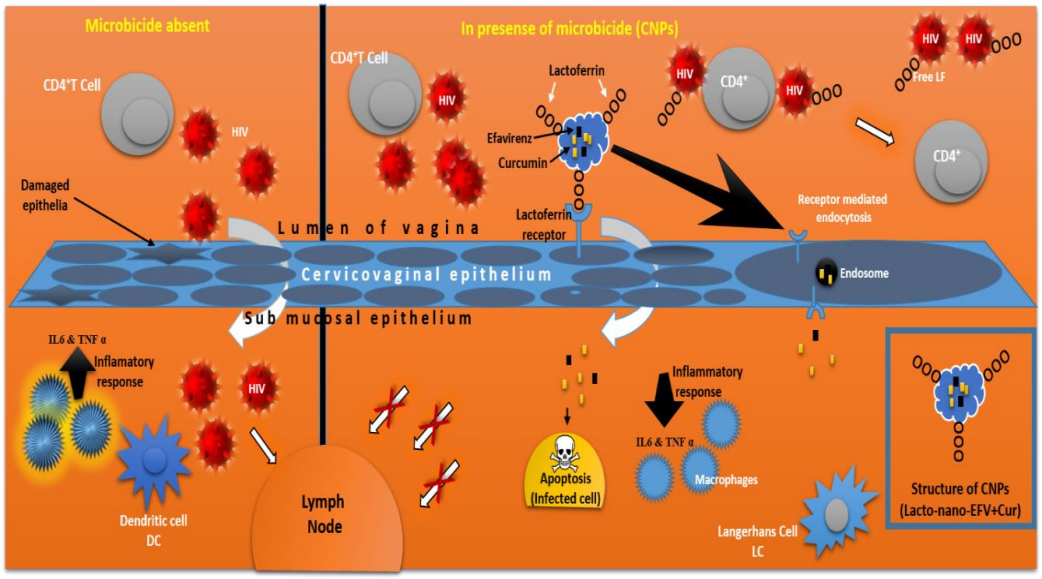


Fig 1.2: Mechanism of action of microbicidal formulations. In the absence of microbicide, HIV progression occurs through the damaged epithelium and leads to infection and high inflammatory response. But in the presence of microbicide (e.g. Combination microbicide- EFV, Curcumin and Lf (CNPs)), it acts as a barrier to prevent HIV infection. However, if HIV surpasses the barrier, it is inhibited by the drugs present in the microbicide. (Idea conceived from; http://www.scripps.edu/gallay/projects_hiv.html)

Lf is a member of transferrin family of iron-binding proteins. It is a glycoprotein having a molecular weight of 80 kDa, it is biodegradable, water soluble and is easily metabolized. Surface modification of such natural protein could be done very easily to facilitate required interaction of drugs and ligands [90]. It has several pleiotropic functions like as anti-bacterial, antifungal, anti-viral, anti-cancer, immunomodulatory and anti-inflammatory activities, and thus can act as the first line of defense to inhibit inflammation and infection [99-101]. It also plays a role in cell proliferation and differentiation. Lf belongs to a part of the innate immune system and indirectly takes part in specific immune reactions [102]. Lf stands as one of the first defense systems against microbial agents invading the organism mostly via

mucosal tissues due to its strategic and crucial position on the mucosal surface. Inhibition of growth and proliferation of a variety of infectious agents including both Gram-positive and negative bacteria, viruses, protozoa, and fungi by Lf was reported. Lf was reported to show the ability to kill certain DNA and RNA viruses [103, 104]. Its main contribution towards antiviral defense is by its binding to glycosaminoglycans of viral cell membranes. Thus Lf blocks viruses from invading cells and infection is ceased at an early stage. Such a mechanism has been demonstrated as being effective against the Herpes simplex virus, cytomegaloviruses, and the human immunodeficiency virus [105-109] respectively.

To prevent sexual transmission of HIV-1, ARVs in combination with curcumin and Lf as microbicides may lead to additive or synergistic effects that could enhance the ability of a vaginal or rectal product by showing triple action; anti HIV-1 activity, anti-inflammatory and contraceptive agent. All above features would lead to an improved barrier to infection, overcoming of resistance issues, and lessening of the required dose of each drug which could also reduce the potential for toxicity. The microbicides with further different anti-HIV compounds or ARV combinations also can lead to HIV-1 prevention.

Rationale of the study

The Clinical use of ARV drugs as microbicides is limited due to their low bioavailability, high tissue related toxicity, high inflammatory response and poor pharmacokinetic profile. By encapsulating these drugs in to the Lf NPs it is expected to increase the bioavailability, decrease toxicity and improve the pharmacokinetic profile as well as improve the anti-HIV activity. Further, it has been reported in studies that using several microbicidal formulations induces inflammation and damage in vaginal epithelium enhancing the risk of virus transmission [50, 51]. Semen is also reported to enhance a series of inflammatory responses in vagina [78].

Such an inflammatory condition would activate immune systems leading to exposure of susceptible immune cells enhancing the risk of virus infection. Further, such inflammatory responses may induce damage to the vaginal epithelium leading intracellular immune cell migration and risk of transmission. Hence including an anti-inflammatory agent in the combination would protect vaginal epithelium from damage, thus reducing the risk of infection. In this study curcumin is employed as an anti-inflammatory and spermicidal agent to protect vaginal epithelium from such damage, further acting as a contraceptive as well. Thus the technology developed in this thesis falls under the category of "Multipurpose prevention Technologies" (MPT).

Chapter 2

Materials and Methods

Materials and Methods:

Reagents:

Lf was purchased from Symbiotics, USA. The extra virgin olive oil used for the NPs preparation was a product of Leonardo, is an Italy-based oil-Company. Drugs such as curcumin, EFV, TFV and DPV were of HPLC grade. All the other chemicals and reagents used for this work were of analytical grade. TNF-alpha and IL-6 measurement kits were procured from BD bioscience with cat numbers 558535 and 550319 respectively.

Solvents: Methanol, DMSO, Acetonitrile, Glacial Acetic acid are HPLC grade.

Cell lines:

SUPT1: CD4 and CXCR4 positive T lymphoblast; HL2/3: contains steadily integrated copies of the HIV-1, HXB2/3gpt molecular clone and VK2: epithelial HPV-16 E6/E7 transformed.

Virus strain: HIV-1 subtype B molecular clone NL4-3 has been propagated in Sup T-1 cells. TCID-50 has been determined and used for infection experiments.

Rats:

Healthy Wistar female rats were used in this study, obtained from Sainath Agencies, Hyderabad, India. All animals were purchased after the approval from the Institute Animal Ethics Committee (IAEC), University of Hyderabad. Animals were caged for one week at the University of Hyderabad animal housing facility in 12 hr. light/12 hr. dark condition and given *ad libitum* access to water. The preferable weight of rats was in the range of 160 g to 240 g and of 6 months old.

Preparation of NPs:

Single drug loaded NPs:

CNPs, ENPs, TNPs and DNPs were prepared by a sol-oil chemistry method as previously described from our lab [110]. Briefly, curcumin (20 mg), EFV (10 mg), TFV (10 mg) and DPV (10 mg) were dissolved in 100 µl of DMSO individually and

add to 500 µl of Lf (40 mg) dissolved 1x PBS (ph-7.4) at 4 ºC and kept it for 1 hr incubation on ice with each drug respectively. Then the above mixture was added drop wise to 25 ml of olive oil and kept for 30 mins incubation. The sample was sonicated for 15 mins at 4 °C by an ultrasonic homogenizer having a narrow stepped titanium probe (model 300 V/T, Biologics Inc. Virginia, USA) with 30 sec long pulses per a gap of 1 min between the consecutive pulses and the sonication amplitude was 5 µm. The ensuing mixture was promptly frozen in liquid nitrogen for 10 min and was then and incubated for 4 hrs on ice. NPs were recovered by ultracentrifugation at 8000 rpm and at 4 ºC (HERMLE Z 36 HK) and pellet washed thrice with Diethyl ether to remove excess oil and dispersed in PBS.

Combination drug loaded NPs:

ECNPs, TCNPs and DCNPs were prepared by a sol-oil chemistry method as previously described from our lab [110]. Briefly, curcumin (20 mg) & EFV (10 mg), curcumin (20 mg) & TFV (10 mg) and curcumin (20 mg) & DPV (10 mg) were dissolved in 100 µl of DMSO to 500 µl of Lf (40 mg) dissolved 1x PBS (ph-7.4) at 4 ºC and kept it for 1 hr incubation on ice. Then the same above methodology was followed for this combination NPs.

Microscopic analysis:

FE-SEM analysis:

Structure and morphology of the NPs were analyzed by using a Field emission scanning electron microscope (FE-SEM, Philips FEI-XL 30 ESEM; FEI, Hillsboro, OR, USA) operated at 20 KV. The gold palladium coated particles were used for FE-SEM analysis. All instructions were followed in accordance to the manufacturer.

AFM analysis:

NPs were visualized through AFM (AFM; SPM-400). All particles were spin coated on a piece of glass slide before visualizing through microscope. All instructions were followed in accordance to the manufacturer.

TEM analysis:

NPs were fixed on the 200 mesh type-B carbon coated copper grid (TED PELLA Inc.). NPs was stained with 2% Uranyl acetate prior to analysis. All instructions were followed in accordance to the manufacturer.

DLS analysis (particles size distribution and zeta potential analysis) of NPs:

NPs suspension were optimally diluted in PBS and samples were then transferred to 2 ml quartz cuvette and analyzed for size distribution. For measurement of zeta potential 200 µl of sample was transferred to specially designed cuvette with electrode. NPs were characterized for hydrodynamic diameter, polydispersity index (PDI) and zeta potential (ζ) measurement by dynamic light scattering (DLS), methods using NPs analyzer, SZ-100 (Horiba Scientific).

Encapsulation efficiency:

Encapsulation efficiency (EE) is defined as the ratio of actual and initial amount of drugs encapsulated in the NPs. Generally EE is measured in percentage. The procedure for calculation of EE% is as follows. Freshly prepared NPs has been incubated with 1 ml of PBS (pH5) at room temperature at rocking condition for 30 min. After 30 min, 100 µl of 30% silver nitrate was added to the solution to precipitate the protein. Then drug/s was/were extracted by adding 1 ml of HPLC grade methanol into it. Further the mixture was centrifuged at 12000 rpm for 15 min and supernatant was evaluated for the presence of drugs. All experiments were performed in triplicate. One standard curve was made using different known concentration of drug/s measured using HPLC. Finally the EE% was calculate using the below mentioned formula.

$$\mathbf{EncapsulationEfficiency(\%)=(M_{total}-M_{lost})/\ Mtotal\times 100}$$

$\mathbf{M_{total}}$ is the total amount of drug entrapped during preparation of NPs and $\mathbf{M_{lost}}$ is the amount of drug unavailable after release from NPs.

FT-IR analysis:

Chemical stability of drugs were determined in nano form compared to the native form of the drug by FTIR study, a pinch of lyophilized samples were directly kept

under the IR probe and scanned from 500 cm^{-1} to 4000 cm^{-1}. Analysis of spectra was done by using OMNIC series software. Base line correction has been done after obtaining spectra.

pH- dependent drug release studies:

The NPs pellet was suspended in 1 ml of 1X PBS (of different pH range 1-9) and vaginal simulated fluid; kept for incubation on a rocker at room temperature for 2 hrs. Then 300 µl of 30% silver nitrate was added after that drugs were extracted by adding 1 ml of HPLC grade methanol; centrifuged at 12000 rpm for 15 min. supernatant was filtered using 0.2-micron syringe filter and analyzed by using HPLC (Alliance Separations Module e-2695 Waters) with 2487 dual detector by using Empower 3 software.

In-vitro studies:

Cell culture conditions:

SUPT1 cells in RPMI, HL2/3 cells were maintained in DMEM, all the mediums were supplemented with 10% fetal bovine serum (FBS) and VK2 cells were maintained in Keratinocyte-serum free media with 0.1 ng/ml human recombinant EGF, 0.05 mg/ml bovine pituitary extract and additional $CaCl_2$, 44.1 mg/ml (final concentration 0.4 mM), 2 mM l-glutamine at 37 ^oC under a 5% CO_2 atmosphere. For each cell line, 70% confluent cell culture flask was trypsinized and were seeded cells in a 96-well plate at a density of (0.2×10^5/well) in the appropriate complete media.

Nanoparticle localization assay:

SUPT1 cells, HL2/3 cells and VK2 cells were seeded in six well plate (Corning Life sciences) and treated with 10 mM curcumin of soluble form and equivalent nano form later the cells were incubated with the time points of 1, 2, 4 and 8 hrs. After incubation, then cells were washed thrice with PBS (pH 7.4) and observed by using laser confocal microscope to analyze the extent of intracellular Curcumin retaining the intrinsic fluorescence of curcumin (Ex 458 nm and Em 530 nm).

Cell viability assay:

SUPT1 cells (0.2×10^5/well) were seeded in a 96-well plate and incubated at 37 ºC for 4hrs whereas VK2 and HL2/3 cells for 24 hrs in a 5% CO_2 incubator (Forma Scientific, Marietta, OH, USA). These cells were treated with increasing concentrations of ARVs and curcumin in both the forms (soluble & nano) and incubated for 16 hrs. The cells were pellet down at 1200 rpm for 10 min and re-suspended in a new medium for SUPT1 cells and for epithelial cell lines fresh media was added after discarding previous media. To this, 20 µl of 5 mg/ml MTT (Sigma-Aldrich) was added and incubated for 4 hrs. The cells were then pelleted down at 1200 rpm for 20 mins, the medium was removed, and the precipitate was dissolved in DMSO and read in an ELISA microplate reader at 570 nm.

***Lactobacillus* viability assay**:

To evaluate the effect of ECNPs on the growth of *Lactobacillus,* viability test was performed according to standard protocol [111]. The Bacterial density of 0.06 (OD at 670 nm) or 108 CFU/ml (100 µl) was seeded into a sterile 96 well plate and incubated with 100 µl of 1 mg/ml of ECNPs at 37 °C. Media without ECNPs was considered as negative control and triton X (1%) as a positive control. After 4 and 48 hr of incubation, 20 µl of MTS reagent was added and absorbance was measured at 490 nm. The percentage viability was calculated using the formula; viability percentage = (absorbance of Test sample/absorbance of Control sample)/100. Where absorbance of Test and Control samples is represented by the quantity of formazan reduced by viable cells.

***Lactobacillus* safety analysis by disc diffusion test:**

Firstly, *Lactobacillus* from Spore lac cultured in nutrient broth, Bacterial cultures were first grown in nutrient broth at 37 °C for 18-24 hrs, incubated till turbidity became equivalent to McFarland 0.5 turbidity standard was obtained. The inoculates of the particular bacteria were streaked on the Muller Hinton Agar (MHA) plates using a sterile inoculation loop, in order to ensure a uniform thick lawn of growth following incubation. The sterile disks of 6 mm in diameter were placed on Muller Hinton agar

plates. The drugs (both soluble and nano forms in single and combination forms) were placed on disc with increased concentrations of 1 mg, 2 mg & 5 mg and one disc with negative control. Inoculated plates were incubated at 37 °C for 24 hrs. These studies were performed in triplicate for each drug. The diameter of the inhibition zone around each well was taken as a measure of antibacterial activity.

Sperms viability assay:

Sperms were isolated by teasing cauda from albino rat; sperms (0.2×10^5/well) were seeded in a 96-well plate. These sperms were treated with increasing concentrations of TFV and curcumin in both soluble and NPs forms, incubated at 37 ºC for 1 hr in a 5% CO_2 incubator (Forma Scientific, Marietta, OH, USA). The cells were pellet down at 1200 rpm for 10 min and re-suspended in a new medium. Later 20 µl of MTT (5 mg/ml) was added and incubated for 2 hrs, cells were centrifuged at 1200 rpm for 20 mins, and DMSO was added to the pellet. Finally, read at 570 nm in ELISA microplate reader and results were analyzed.

Anti-HIV assay:

Anti-HIV activity of soluble drugs was analyzed individually and in combination using HIV-1_{NL4-3} clone. Initially, the individual drug-loaded NPs have been tested for their anti-HIV activity. For individual drug anti-HIV assay, 0.1 million of SUPT1 cells having 100% viability were infected with HIV-1_{NL4-3} for 12 hrs in RPMI 1640 media containing 0.1% fetal bovine serum. After 12 hrs, cells were washed carefully to remove any unattached virus particles then re-suspended in fresh media with 10% fetal bovine serum. After 96 hrs, by using p24 antigen capture ELISA assay level of p24 protein in cell supernatant was evaluated. Different concentrations of all drugs have been tested and IC_{50} (50% inhibitory concentration) was calculated for each drug in the soluble and in nano formulation. Further, for the combination of drugs in both forms (soluble & nano) anti-HIV assay was performed (Two combination drugs were mixed in accordance with their IC_{50} values in various ratios such as 1:1, 0.5:0.5, 0.25:0.25, 0.10:0.10 and 0.05:0.05).

In-vivo studies:

Animal experimental protocol:

Three different experiments were conducted in *In-vivo* analysis; Local pharmacokinetics, Time course analysis and Dose-dependent toxicity& bio availability studies.

Local Pharmacokinetic studies in Rats:

The local PK assessment of the vaginal formulations in rats was analyzed. The PK analysis involved in the evaluation of ECNPs, TCNPs and DCNPs were administered as a soluble form and equivalent nano form. Randomly 54 rats were selected for each drug combination, grouped into two groups having 27 rats each. One group was treated with a soluble form of drugs for different time points (0.5, 1, 1.5, 2, 2.5, 4, 6, 12, and 24 hrs post-dose) (Fig 2.1) with three rats for each time point. Likewise, another group was treated with equivalent nano forms of above drugs for same time points. After each time point, vaginal lavage was collected from all rats and the collected samples were analyzed for the levels of curcumin, EFV, TFV and DPV.

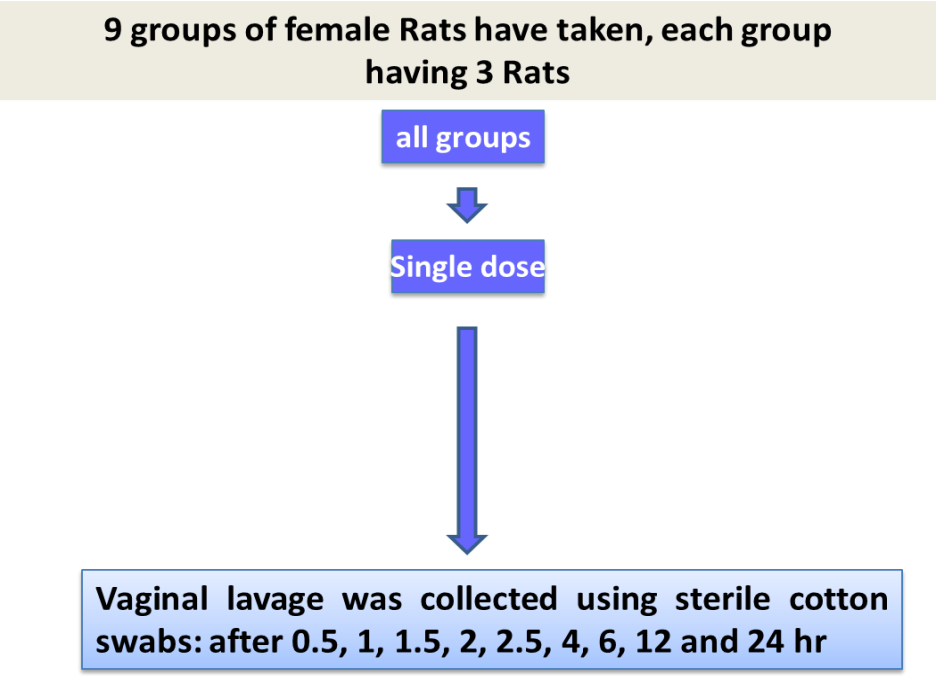


Fig 2.1: Animal study design for local pharmacokinetic analysis.

Drug release kinetic assay (Time course analysis):

This experiment was conducted to know the time of drugs availability at the site of application. On the basis of T_{max} acquired from PK study, the time course study was performed with a lag period of 1 hr (for soluble curcumin), 1 hr (for soluble EFV), 1.5 hr (for soluble TFV), 2 hrs (for nano form of curcumin and EFV), and 2.5 hrs (nano form of TFV and DPV) rats were administered with a single dose of soluble (Curcumin + TFV) and TCNPs**.** For any microbicide, drugs should be present at the site of application for a long time to show its microbicidal and virucidal action. The soluble forms of (Curcumin -20 mg + EFV-10 mg), (Curcumin-20 mg + TFV-10 mg) and (Curcumin-20 mg + DPV-10 mg) and an equivalent amount of ECNPs, TCNPs and DCNPs was topically applied with single dose through bend 18 SWG (standard wire gauge) needle. From T_{max} of respective drug, at different time points of post-application of single dose such as 1, 1.5, 2, 2.5, 4, 8, 12, 16 and 24 hrs vaginal lavage was collected using sterile cotton swab (Fig 2.2); drugs present in swabs was extracted in HPLC grade methanol and estimated individually through HPLC.

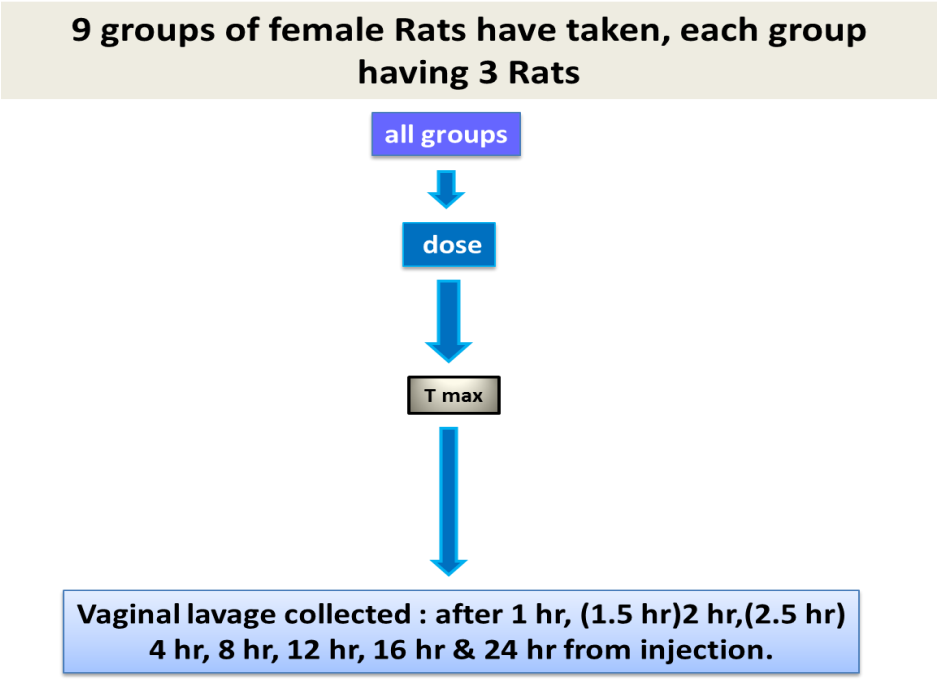


Fig 2.2: Animal study design for time course analysis.

Dose-dependent toxicity &bio availability studies:

These studies were done by three different doses that are: -Single dose, Double dose and Triple dose. All three doses were administered through the vaginal orifice as a topical administration of both the formulations such as the soluble forms of curcumin, EFV, TFV and DPV individually and in a soluble combination and the equivalent amount of nano formulation of both single and combination drugs with above mentioned concentrations. This concentration ratio of a formulation is best in encapsulation efficiencies when compared to the other ratios as we explained in E.E% analysis.

Three groups of rats were taken each having 18 rats, each group again sub divided into three groups of 6 each. These groups were used for dosage study that is a single dose, double dose and triple dose. For single dose 3 rats were administered with soluble curcumin (20 mg) and another 3 were administered with equivalent CNPs. Then for double dose like same above two doses were given with 2 hrs interval time and for the third dose, three doses were given with 2 hrs gap between each dose. Likewise, all groups were administered with soluble EFV (10 mg), soluble TFV (10

mg), soluble DPV (10 mg) and equivalent amount of single drug nano forms and the combination of soluble (Curcumin-20 mg + EFV-10 mg), soluble (Curcumin-20 mg + TFV-10 mg), soluble (Curcumin-20 mg + DPV-10 mg) and their equivalent amount of combination nanoforms.

After finishing 2 hrs of each dose, vaginal lavage samples were collected through sterilized cotton swabs from the animals and those rats were quietus using mild diethyl ether and final sacrifice by cervical dislocation. Through heart puncture, blood and later vaginal tissue were collected from each rat (Fig 2.3).

Drugs present in swabs were extracted with HPLC grade methanol and vaginal tissues were homogenized in PBS by a homogenizer, drugs were extracted with HPLC grade methanol by silver nitrate precipitation method. For histopathology studies through Hematoxylin and Eosin tissue staining, a small part of vaginal tissues were saved in 10% formalin. A major part of vaginal tissue was homogenized in PBS through the homogenizer. After final processing of vaginal tissues, blood plasma and vaginal lavage; drugs accumulation were estimated by using HPLC. For all the experiments animals were grouped as triplicates. Three rats were used for control treated with phosphate buffer saline (PBS).

Measuring systemic and vaginal proinflammatory cytokine response:

Vaginal tissues were homogenized in PBS using REMI homogenizer. Cytokine (TNF-alpha and IL-6) levels were estimated in plasma and in the supernatant obtained after the homogenization of vaginal tissue by using measurement kits developed from BD bioscience with CAT numbers 558535 and 550319 respectively as per suggested protocol. For all the experiments, animals were grouped as triplicates. Three rats were used for control treated with phosphate buffer saline (PBS).

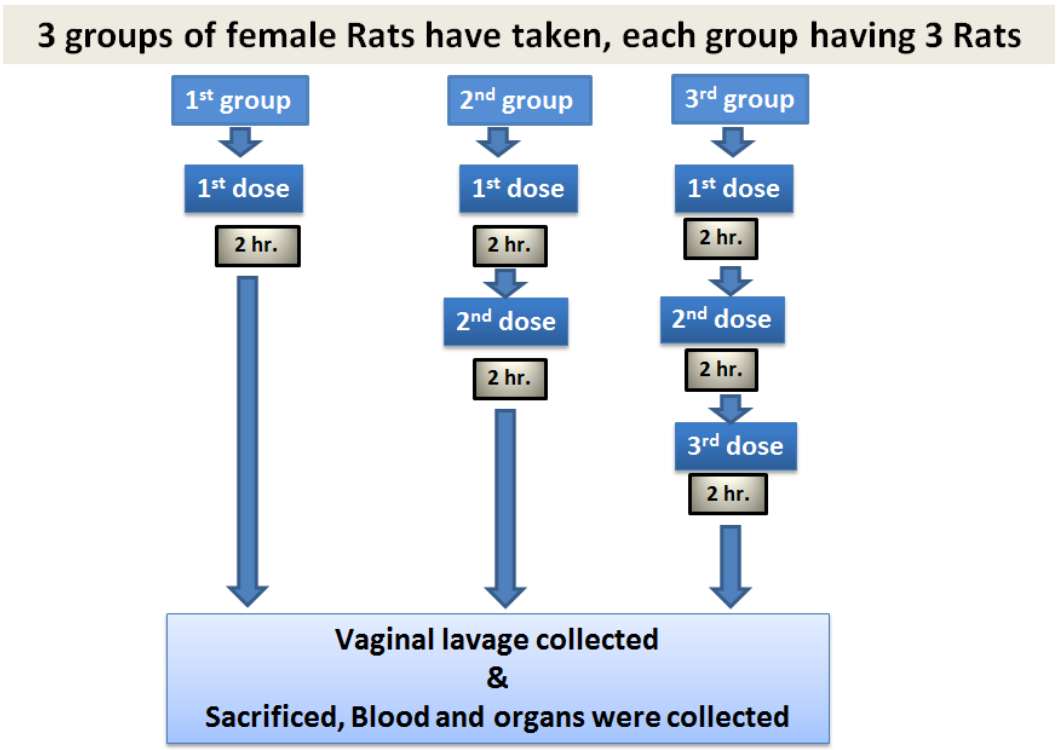


Fig 2.3: Animal study design for dose-dependent toxicity &bioavailability studies.

Mobile phase chromatographic condition:

Drugs concentrations from samples were analyzed using Waters HPLC. The HPLC condition for all drugs are indicate below in Table 2.1.

Table 2.1. HPLC conditions for all drugs.

Drugs	*Mobile phase*	*Wavelength*	*Flow rate*
Curcumin	**Acetonitrile :5% glacial Acetic acid(75:25)**	**420 nm**	**1 ml/min**
EFV	**Acetonitrile:0.1% formic acid (75:25)**	**247 nm**	**1 ml/min**
TFV	**Acetonitrile: water (75:25)**	**259 nm**	**1 ml/min**
DPV	**Acetonitrile : 0.1% glacial acetic acid (80:20)**	**245 nm**	**1 ml/min**

Histopathology study:

The Histopathology study was done only for combination form of drugs either in soluble form or nanoformulation. Doses of soluble drugs combination and nano combination were given as described in doses schedules (*n=3*). Negative control animals were treated with PBS, positive control animals were treated with 10 mg/kg of nonoxynol-9 (N-9). Multiple doses were repeated up to three doses at time gap of 2 hrs. After the completion of time points, animals were sacrificed under standard protocol; a small part of vaginal tissue was removed and fixed in 10% formalin following by Hematoxylin and Eosin staining.

Statistical Analysis

All *in-vitro* and *in-vivo* studies were carried out in triplicate. Data were represented as a mean ± standard deviation. The significance of the difference between two groups was analyzed by using student's t-test and between multiple groups were analyzed by one-way ANOVA. The level of significance was stated as *** $P<0.0005$, **$P<0.005$ and *$P<0.05$.

Chapter 3

Development of Curcumin and Efavirenz (EFV) Loaded Lactoferrin Nanoparticles as a Microbicidal Formulation.

Introduction:

The Multipurpose prevention technologies (MPT) are currently the most promising and intricate products under development which can prevent both, the transmission of STIs like HIV/AIDS and unwanted pregnancy [112]. The three types of MPT currently available include male and female condoms, female diaphragms and microbicides (chemical barrier) [113]. Our current study focuses on the vaginal microbicide based MPT. Various combinations of microbicides have been reported by several research groups [114,115]. Anti-retroviral (ARV) drugs based microbicides have shown improved neutralization and thus are clinically more significant [116].

EFV is an ARV medication used to treat and prevent HIV/AIDS. EFV is generally administered in combination with other ARVs. It is a non-nucleoside reverse transcriptase inhibitor (NNRTI) that works by blocking the function of reverse transcriptase. Both nucleoside and non-nucleoside RTIs inhibit the activity of same target, i.e. reverse transcriptase enzyme, an essential viral enzyme which transcribes viral RNA into DNA. NNRTIs and NRTIs inhibit reverse transcriptase using different mechanisms of action. Unlike nucleoside RTIs, which bind at the enzyme's active site but NNRTIs act by binding to a different site away from the active site called as NNRTI pocket through allosteric interaction. This mechanism is used as part of highly active antiretroviral therapy (HAART) and as a microbicide for the treatment of HIV-1.

Turmeric has been used as the key spice ingredient in most parts of Asian subcontinent since centuries. Curcumin is an active principle of unique herbal compound turmeric. It exhibits many pleiotropic effects such as anti-HIV [117], anti-inflammatory [118], anti-oxidant [119], vaginal contraceptive [97], etc. Curcumin shows a concentration-dependent inhibition of sperm motility. It completely blocks sperm motility at a concentration of $\geq$ 250 μM [98]. However, its hydrophobic nature and low bioavailability placed limitations on its use in conventional therapeutic applications [120]. Nanoparticle mediated drug formulation is one of the approaches being investigated to overcome these limitations. The therapeutic ability and the degree of safety of drugs can be considerably enhanced through targeted

delivery by nanocarriers [110, 83]. Many protein-based NPs have been already developed for therapeutic uses to treat cancer, AIDS and Parkinson's [121,122]. The NPs can be easily prepared in relatively mild conditions without the use of any toxic chemicals [122–124]. Proteins are an ideal vehicle for drug transportation in nano-form because their amphiphilic nature helps in cooperating with drugs as well as solvent [125]. Curcumin loaded-apotransferrin NPs have been shown to inhibit HIV-1 by prevention of viral infection and down-regulation of host inflammatory responses [76]. Natural proteins available readily in nature such as Lf are generally water soluble, can be easily metabolized and are also biodegradable. Surface modification of such natural proteins can be done very easily to facilitate the required interaction of drugs and ligands [75]. Lf protein has several pleiotropic functions like immune modulation, anti-viral, and anti-cancer, and thus can act as the first line of defense to inhibit inflammation and infection [99-102].

The objective of our study is to develop a triple-combination topical formulation that can simultaneously action HIV, HIV-mediated inflammation, other viral and bacterial infections with contraceptive action, based on the principle of multipurpose prevention technologies (MPT). This is a triple combination of broad spectrum Lf (as vehicle) and curcumin as preventive and protective agent and EFV as therapeutic agent against HIV. The principle steps in realizing the objectives of this studies are (1) preparation and characterization of Lf NPs loaded with curcumin and EFV; (2) Studying bioavailability and pharmacokinetic profile of curcumin and EFV in vaginal lavage upon topical application of nanoformulation, and (3) Evaluation of safety of nanoformulation in terms of inflammation.

Results:

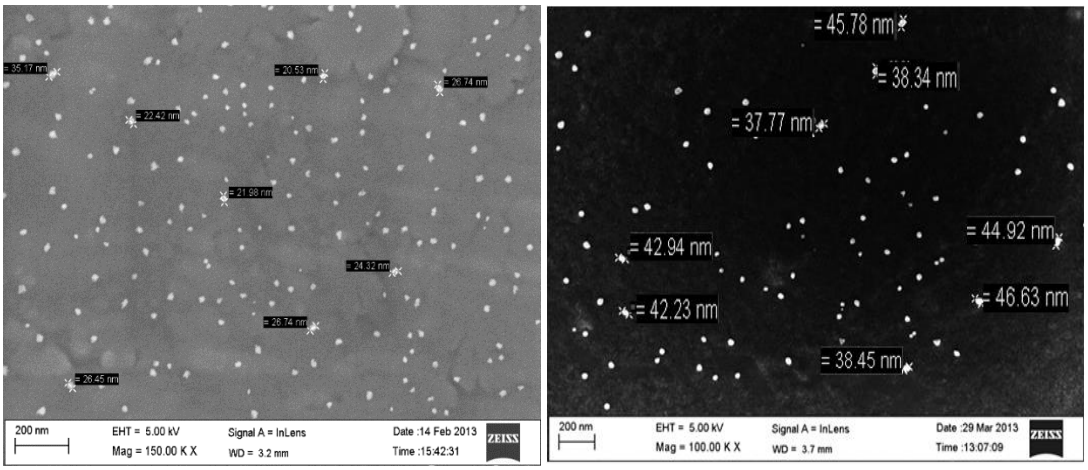
Preparation and characterization of EFV and Curcumin loaded Lf nanoparticle:

Curcumin loaded Lf NPs (Lacto-curcumin-nano), EFV loaded Lf NPs (Lacto-EFV-nano) and EFV plus curcumin loaded Lf NPs (ECNPs) were prepared using sol-oil chemistry as described in materials and methods section. The NPs prepared were characterized through Field Emission Scanning Electron Microscope (FE-SEM), Atomic-force microscopy (AFM), Transmission electron microscopy (TEM) and Dynamic light scattering (DLS).

Results presented in Fig 3.1-3.3 and 3.4 respectively, shows that NPs were uniformly dispersed spherical particles with size in the range of 40–70 nm. AFM images provide a three-dimensional surface profile reveal a particular type of projection which may help in binding with the receptor.

DLS analysis of blank or drug/s loaded NPs has showed the hydrodynamic size in a range of 40 nm and 91–125 nm respectively (Fig 3.4). Increased apparent size in DLS is due to the surface water shell that contribute in DLS measurements. The zetapotential of freshly prepared blank or drug/s loaded NPS were found to be in a range of -21 to -25 mv (Fig-3.5) respectively indicating their stability.

Lf NPs: **CNPs:**



ENPs: **ECNPs:**

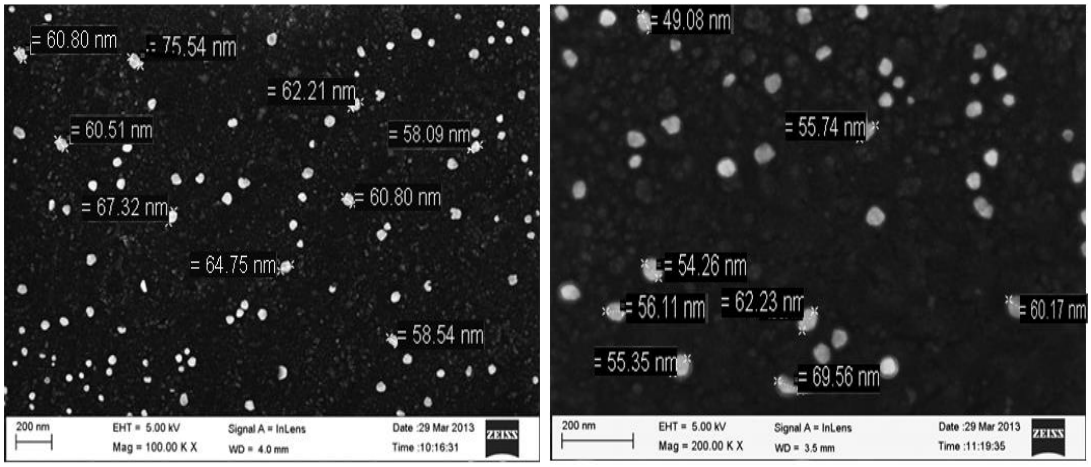
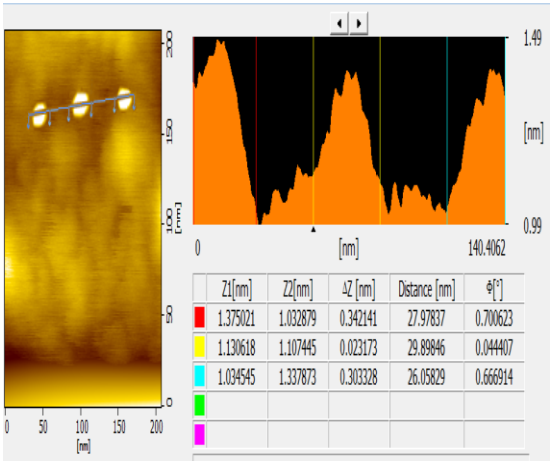
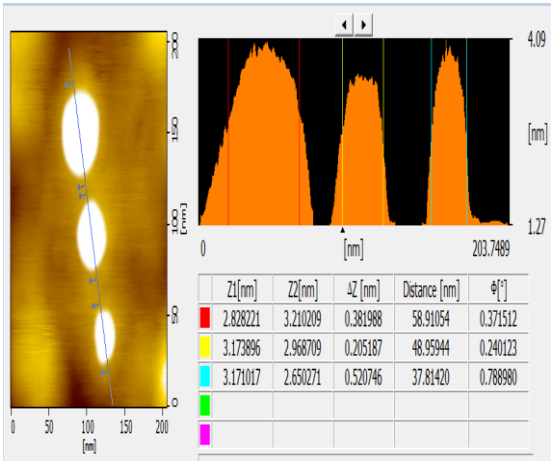


Fig 3.1: FE-SEM analysis of NPs: Lf NPs, CNPs, ENPs and ECNPs were analyzed by using the Field Emission Scanning Electron Microscope. The range of diameter for Lf NP was found to be 20-35 nm and for drug loaded Lf NPs was found to be 40-75 nm.

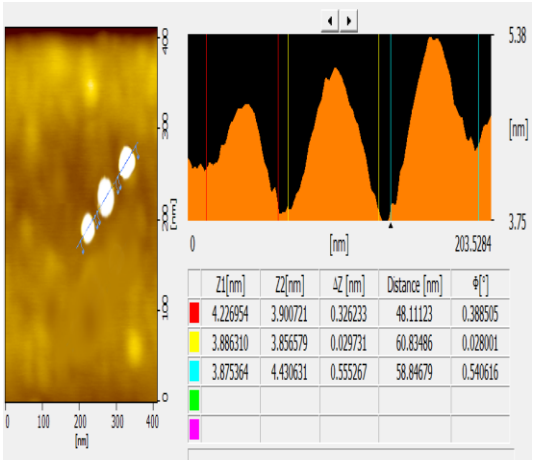
Lf NPs:



CNPs:



ENPs:



ECNPs:

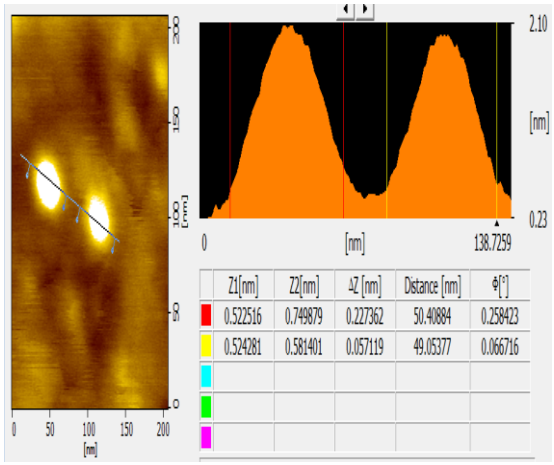
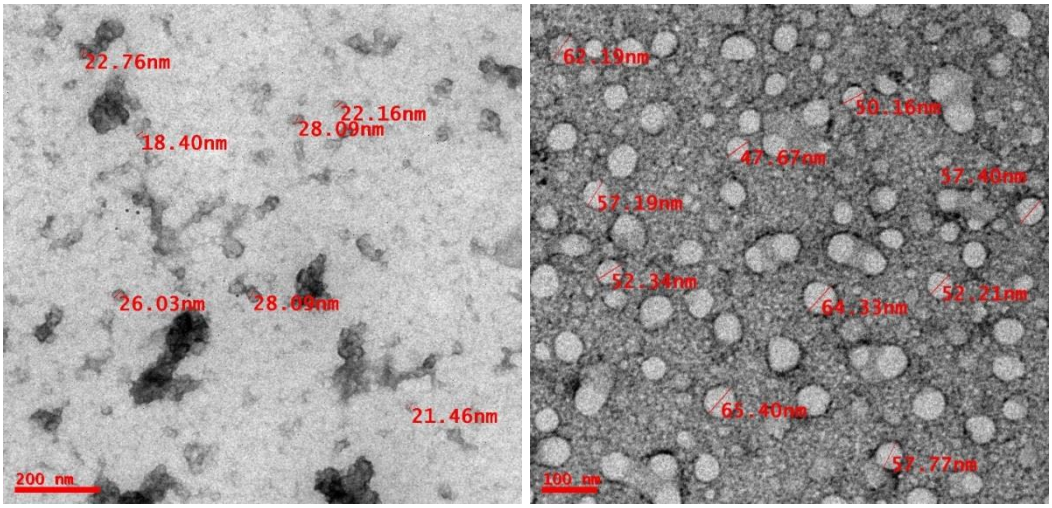


Fig 3.2: AFM analysis: Lf NPs, CNPs, ENPs and ECNPs were analyzed by using the Atomic Force Microscope. The average diameter for Lf NP was found to be 26 nm and for drug loaded Lf NPs was found to be 40-60 nm.

Lf NPs: **CNPs:**



ENPs: **ECNPs:**

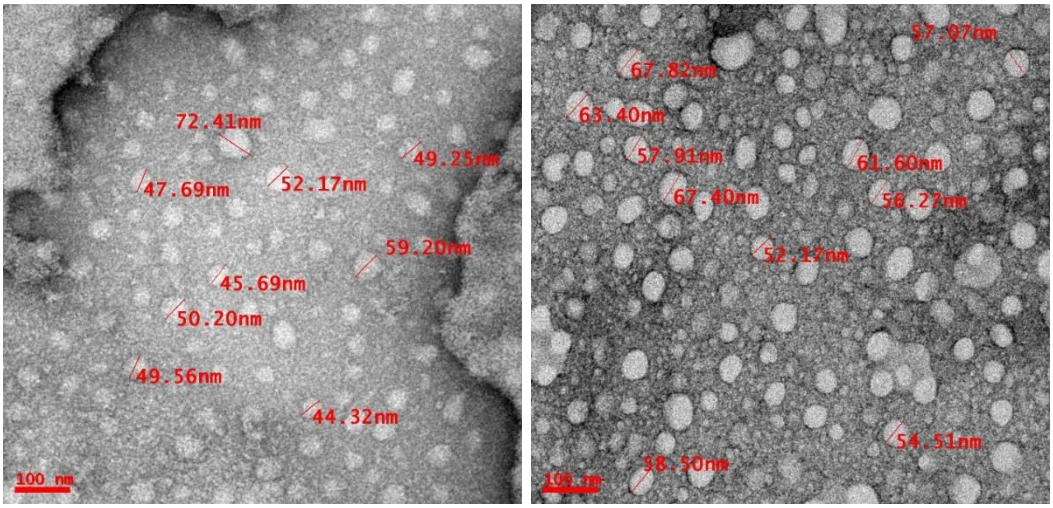
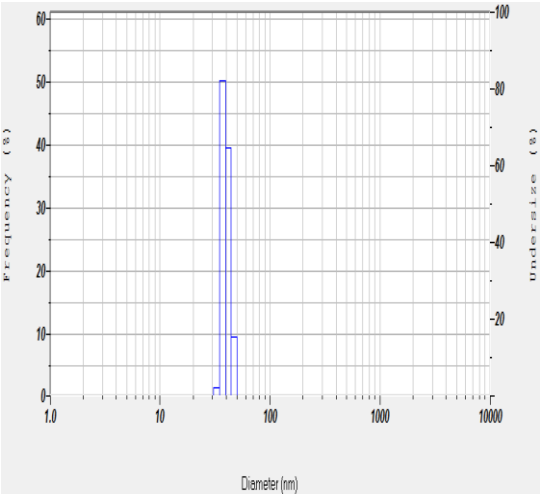
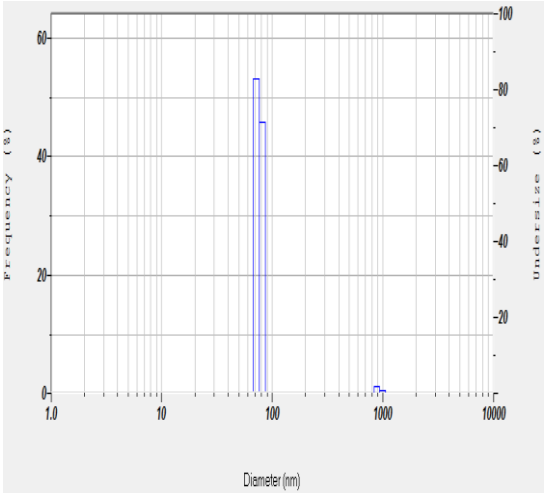


Fig 3.3: TEM analysis: Lf NPs, CNPs, ENPs and ECNPs were analyzed by using the Transmission Electron Microscope. The range of diameter for Lf NP was found to be 20-28 nm and for drug loaded Lf NPs was found to be 44-72 nm.

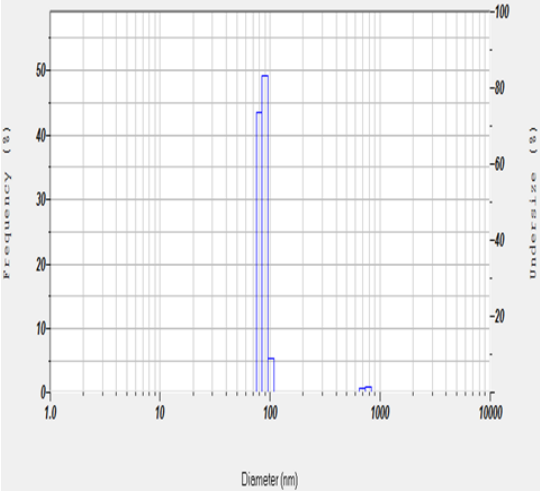
Lf NPs:



CNPs:



ENPs:



ECNPs:

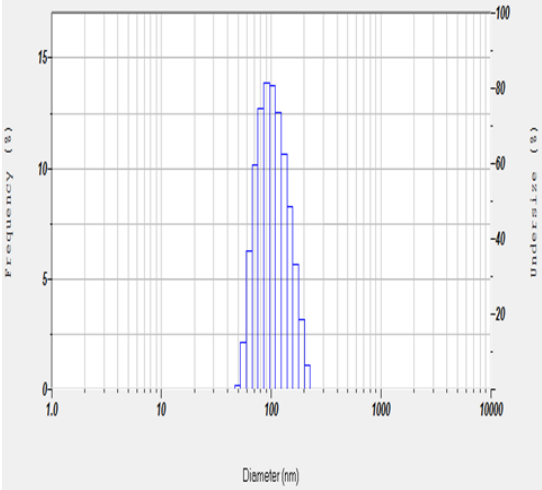
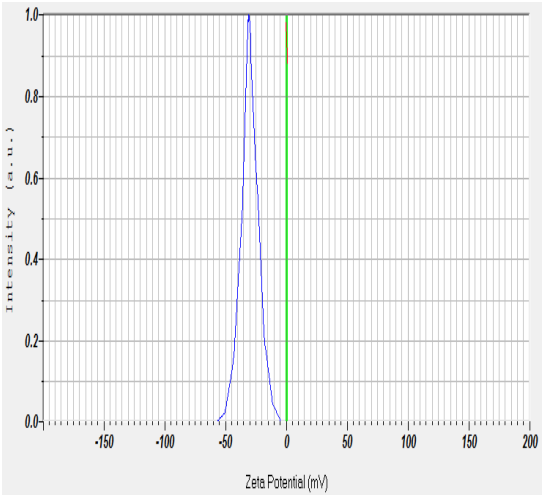
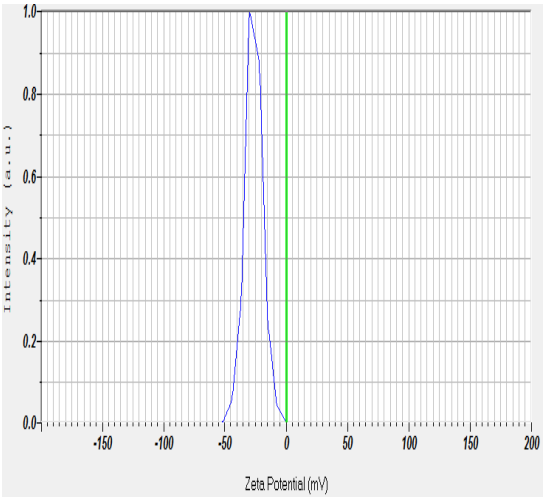


Fig 3.4: DLS analysis: DLS analysis was performed for the measurement of hydrodynamic radii. A mean size of 45 nm for Lf NPs and 109 to 113 nm for drug loaded NPs were observed.

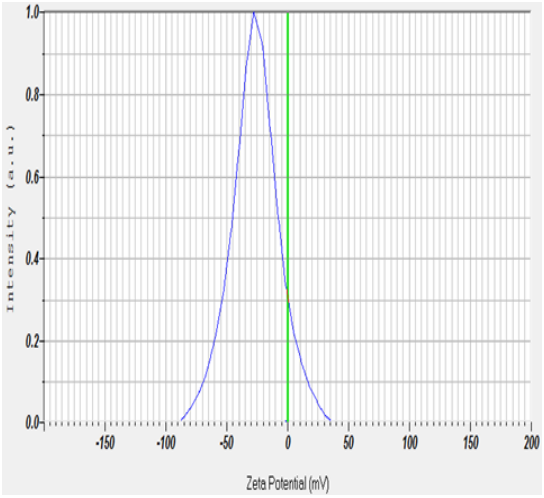
Lf NPs:



CNPs:



ENPs:



ECNPs:

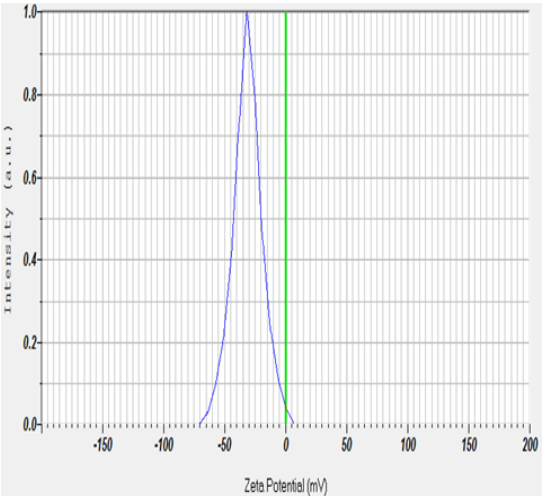
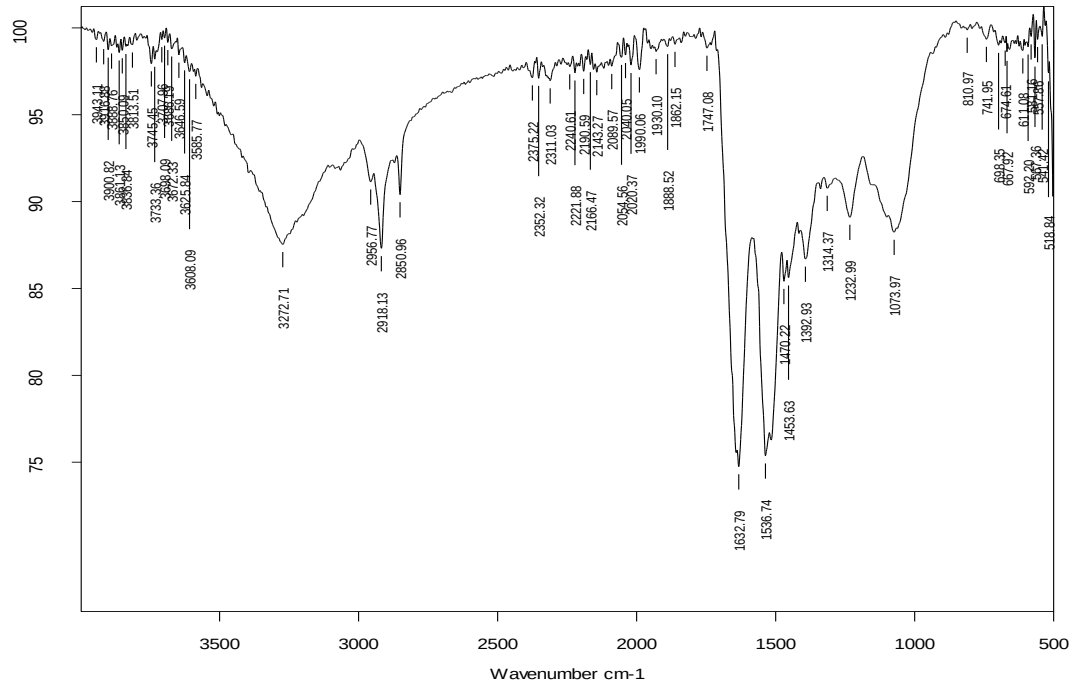


Fig 3.5: Zeta potential analysis of NPs: Zeta Potential or surface charge was analyzed by using Zetasizer. A negative surface charge of -23 mV for Lf NPs and -21 to -25 mV for drug loaded NPs were observed.

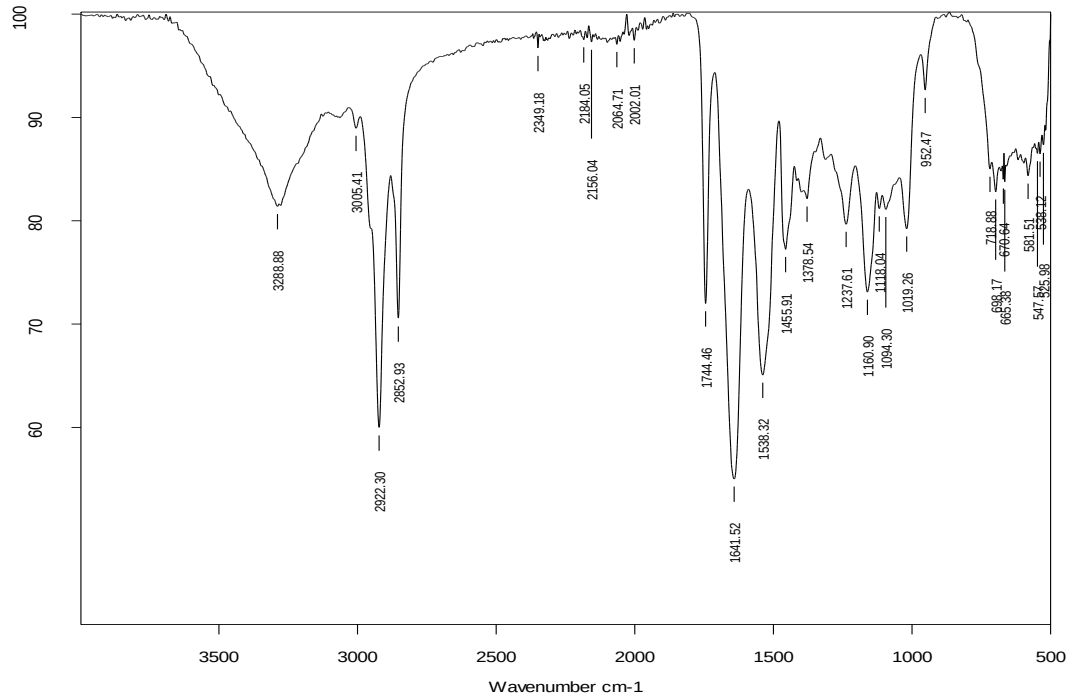
FT-IR spectral analysis:

FT-IR spectral data showed the stability of Lf, EFV, curcumin and EFV plus curcumin combination which remained conserved in their nanoformulation (Fig 3.6). In Lf, the characteristic amide I band was situated at 1632.79 (soluble) & 1641.52 (nano) cm^{-1}, the amide II was sensed at 1536.74 (soluble) & 1538.32 (nano) cm^{-1}. In curcumin a very strong combined peak of C=O and C=C was found at 1626.20 (soluble) & 1626.50 (nano) cm^{-1}, one broad peak at 3273.57 (soluble) & 3275.89 (nano) cm^{-1} and one sharp peak at 3507.82 cm^{-1} (soluble) & 3501.99 cm^{-1} specifying the existence of –OH. A; peak at 1600.74 (soluble) & 1601.62 cm^{-1} corresponds to the characteristic aromatic ring, another peak at 1504.46 (soluble) & 1505.84 (nano) cm^{-1} indicates the presence of C=O group, peak at 1271.17 (soluble) & 1273.00 (nano) cm^{-1}, corresponds to enol C=O group and C-O –C peak was found at 1024.62 (soluble) & 1025.13 (nano) cm^{-1}. In EFV, the C-O peak was found 1073.45 (soluble) & 1073.47 (nano) cm^{-1}, C=O band at 1744.04 (soluble) & 1744.95 (nano) cm^{-1}, C-F stretch present at 1396.75 (soluble) & 1395.75 (nano) cm^{-1}, CH2 stretch at 1195.06 (soluble) & 1195.43 (nano) cm^{-1} and the featured alkyne group was at 2248.72 (soluble) & 2249.26 (nano) cm^{-1}.

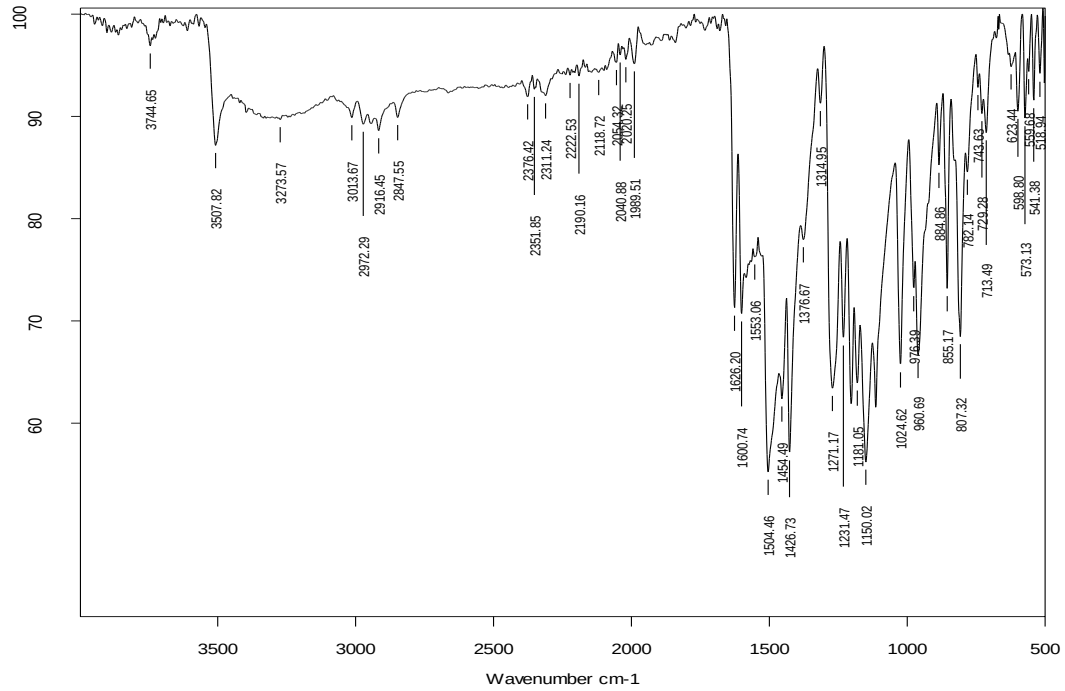
A. Pure Lf:



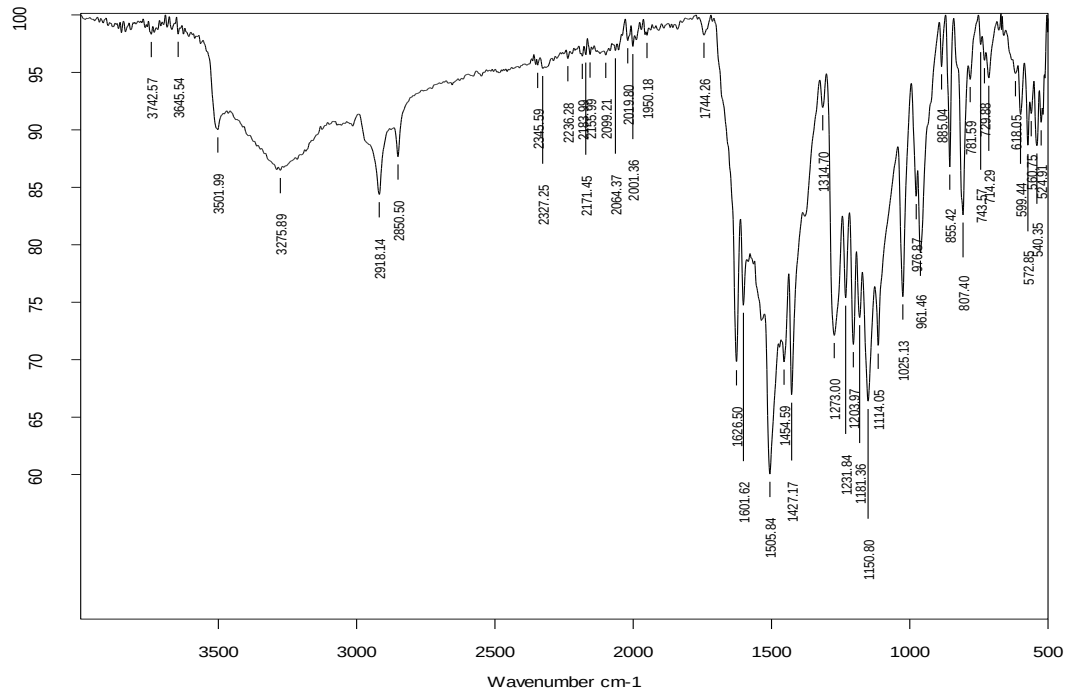
B. Nano Lf:



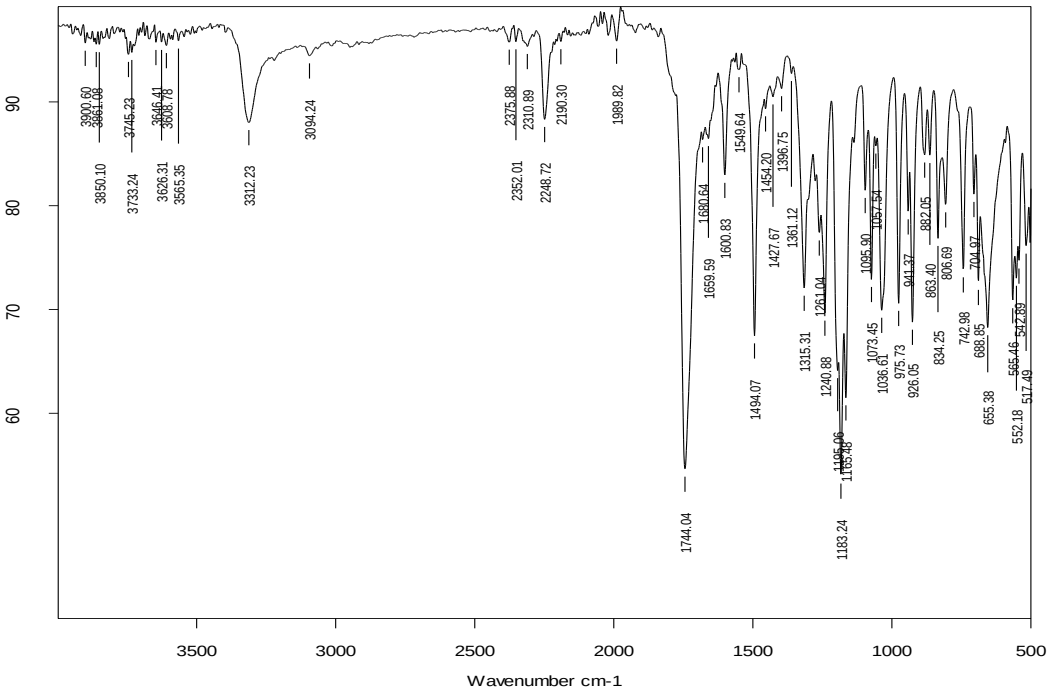
C. Soluble Curcumin:



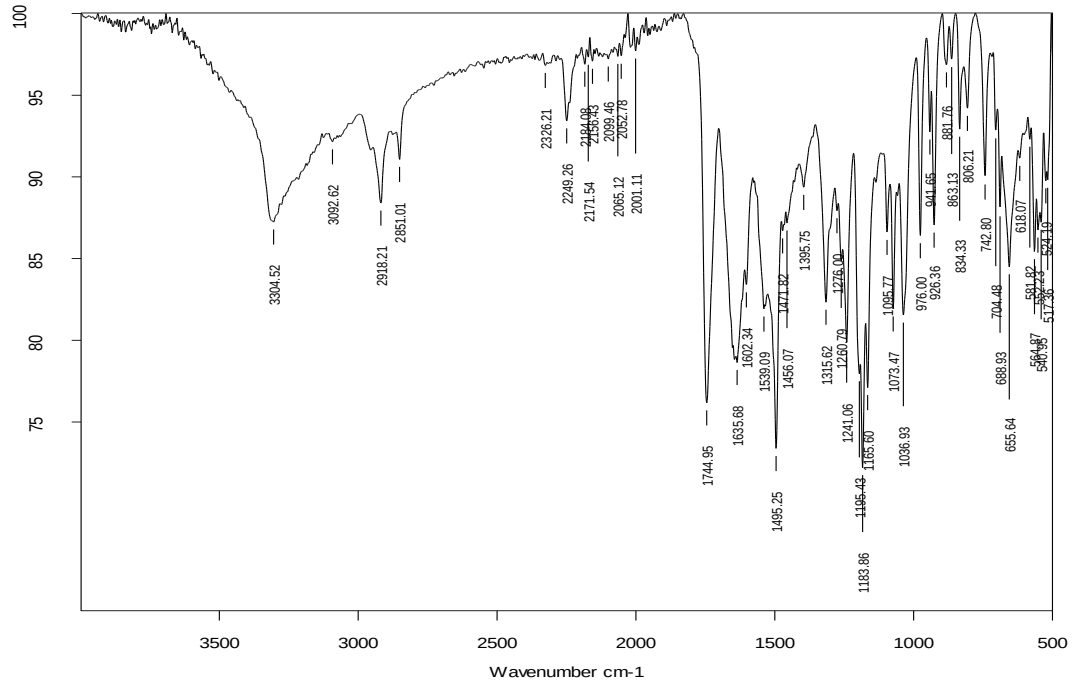
D. CNPs:



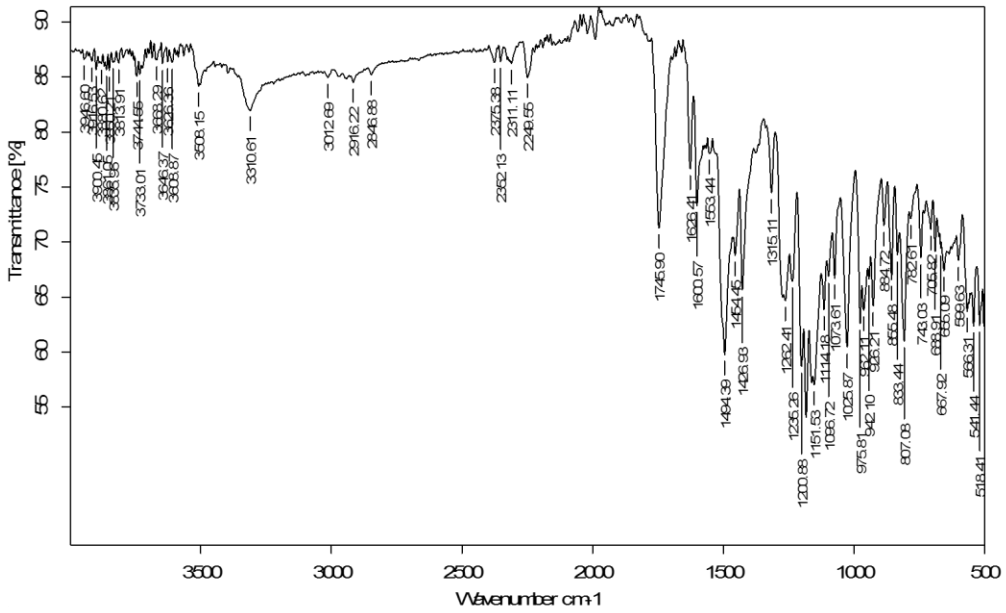
E. Soluble EFV:



F. ENPs:



G. Soluble Curcumin and EFV:



H. ECNPs:

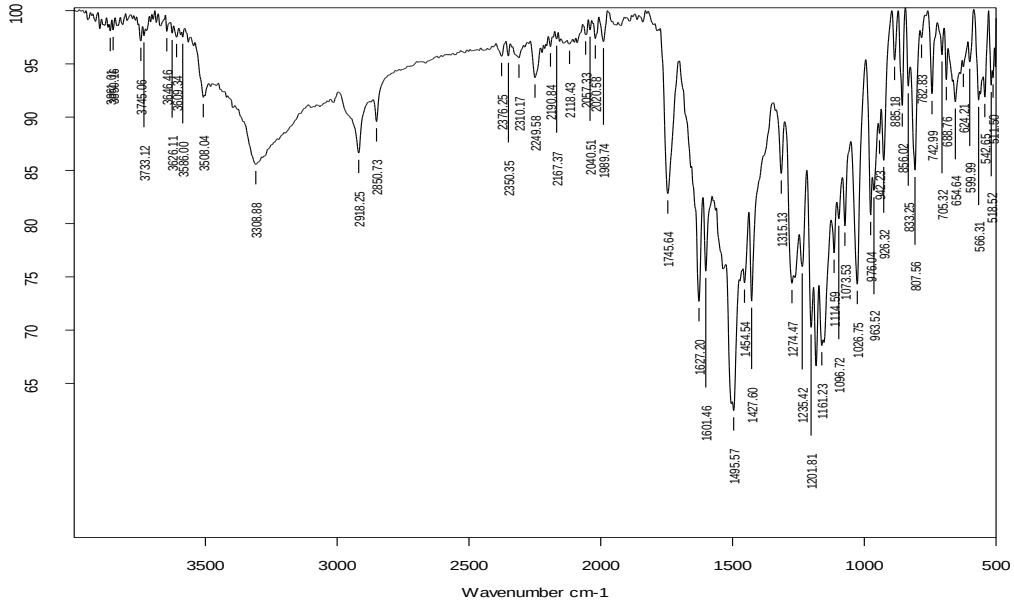


Fig 3.6: FT IR analysis: NPs were lyophilized prior to scanning. The lyophilized NPs and soluble drug/protein in powder form, both were scanned and data was collected using OPUS software. IR spectra run for samples: Lf, Nano Lf, curcumin, EFV and combination of curcumin and EFV. These results suggest that there is no change in the functional groups of drug and protein.

Assessment of loading efficiency:

Loading efficiency of Lacto-curcumin-nano, Lacto-EFV-nano and ECNPs were assessed. ECNPs were prepared at four different concentrations of EFV by keeping the concentration of Lf and curcumin constant (Table 3.1). Maximum loading was observed in formulation ratio II for ECNPs (63% ± 1.9 of curcumin. and 61.5% ± 1.6 of EFV), IIA for Lacto-curcumin-nano (59% ± 1.34) and IIIB for Lacto-EFV-nano (58.4% ± 1.79). This suggests that maximum amount of drugs has been entrapped in protein. It has also been observed that combination of EFV and curcumin are synergistic in loading of one drug to the other.

Table 3.1: Encapsulation efficiency (EE) of drug(s) loaded Lf NPs.

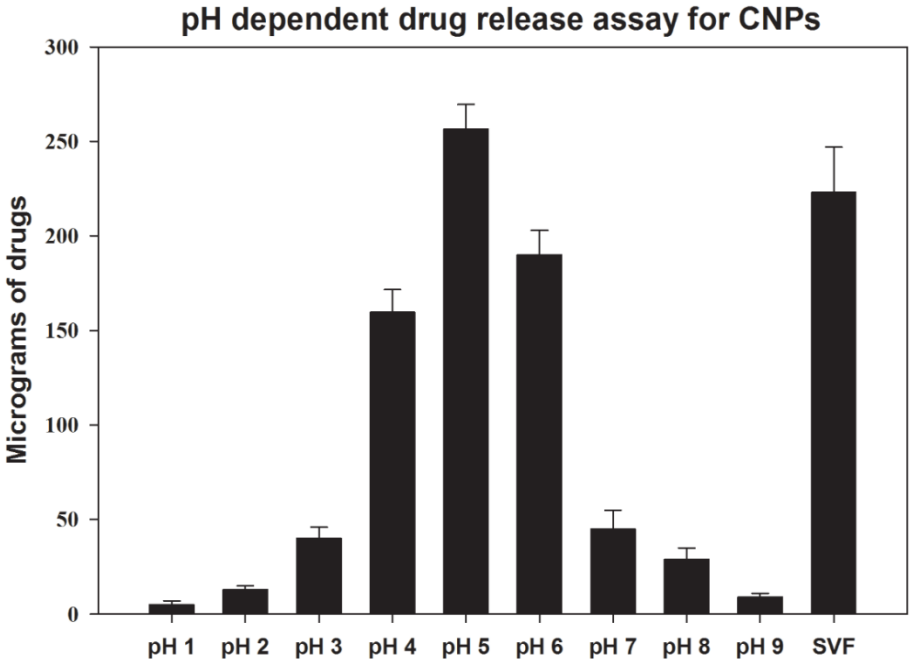
Formulations	Lf concentration (mg)	Curcumin concentration (mg)	EFV concentration(mg)	EE of Curcumin	EE of EFV
EE of ECNPs					
I	40	20	5	47% ± 2.5	49% ± 2.4
II	40	20	10	63% ± 1.9	61.5% ± 1.6
III	40	20	15	57.4% ± 3.2	51.7% ± 2.7
IV	40	20	20	48% ± 2.8	53% ± 3.7
EE of CNPs					
IA	40	5	0	38% ± 1.45	NA
IIA	40	10	0	59% ± 1.34	NA
IIIA	40	15	0	52% ± 2.2	NA
IVA	40	20	0	49.5% ± 2.5	NA
EE of ENPs					
IB	40	0	5	NA	41% ± 1.73
IIB	40	0	10	NA	47.6% ± 2.8
IIIB	40	0	15	NA	58.4% ± 1.79
IVB	40	0	20	NA	57.83% ± 2

NA: Not applicable

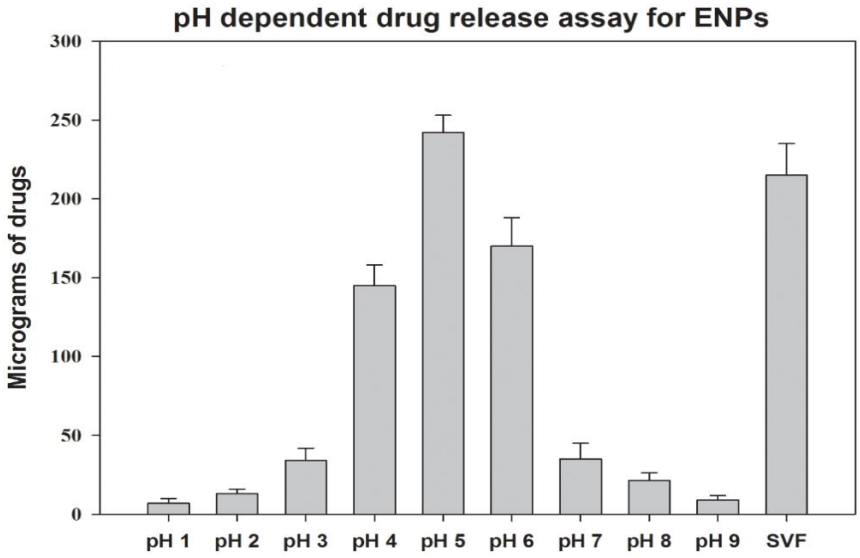
pH dependent release of drugs from NPs:

ECNPs were incubated at different pH conditions to mimic the in vivo environment of rat vagina; 300 μg of drug loaded NPs were incubated with different pH values (1–9) of PBS and simulated vaginal fluid (SVF). Results showed that ECNPs are more sensitive at pH 5 and 6 with maximum drug release observed at pH 5 (Fig 3.7). All three types of NPs either in single or combination form showed more than 80% of drug release at pH 5. At pH below 4 and above 6, only 10% of drug release was found. Thus suggests that NPs slowly release drugs in the vaginal lavage in the pH range of 4 to 4.5. Furthermore, higher concentrations of curcumin and EFV will be released at ≥ pH 4.5, a condition where higher virus infectivity was detected in vaginal lavage [126]. In addition, bacterial vaginosis related-pH increase was seen which allows virus shedding [127] and under these pH conditions ECNPs release higher concentrations of curcumin and EFV thus promoting higher viral neutralizing environment.

A. CNPs:



B. ENPs:



C. ECNPs:

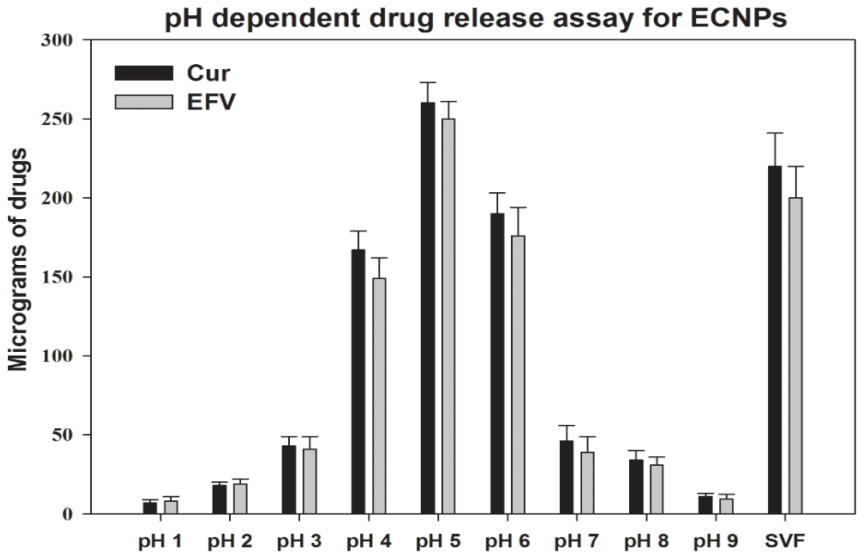


Fig 3.7: pH dependent release of NPs: pH dependent drug release assay for CNPs, ENPs and ECNPs 300 µg of particles were incubated in the buffers of different pH, and simulated vaginal fluid. The release of curcumin, EFV was found to be maximum at pH-5 followed by pH-6 and pH-4. Optimum drug relese was observed at SVF, Each data points were repeated in triplicate (n=3) and presented asMean ± standard deviation (S.D).

Stability studies of NPs:

The stability of ECNPs in PBS (phosphate buffer saline, pH 7.4) suspension form was analyzed for at least 20 days at 4 °C and 25 °C. Data presented in Table 3.2 shows that all the four parameters were found to be quite steady at both temperatures. An average negative charge of -25 mV and a PDI of 0.4 indicates the high stability and homogenous colloidal solution property of NPs. The loading efficiency and size distribution of ECNPs were found to be reasonably constant.

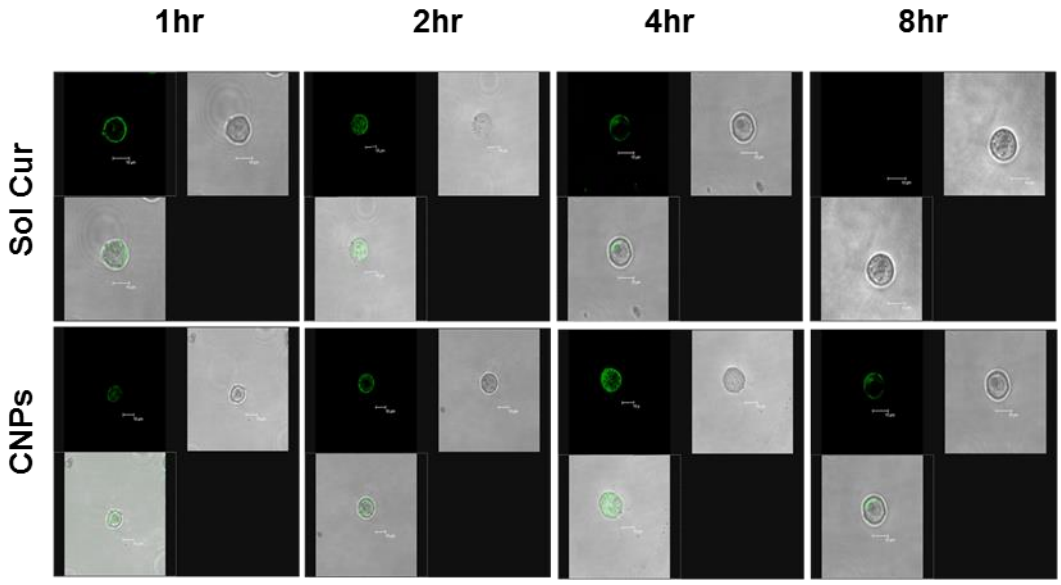
Table 3.2: Stability profile of ECNPs

	Size[a] (nm)		ζ potential[b] (mV)		PDI[c]		Encapsulation Efficiency %			
Days	4 °C	25 °C	4 °C	25 °C	4 °C	25 °C	4 °C		25 °C	
							Cur	EFV	Cur	EFV
0	98±5.6	98±5.6	-19±3.2	-19±3.2	0.435	0.435	63%±1.9	61.5%±1.6	63%±1.9	61.5%±1.6
1	105±6.4	108±5.7	-21±4.1	-22±1.9	0.351	0.361	62%±2.1	60%±5.9	62.81%±2.4	61.27%±3.1
2	110±7.3	114±6.4	-24±3.8	-22±2.3	0.473	0.483	64%±3.2	62%±4.8	62.3%±3.30	60.3%±2.9
4	105±8.5	118±6.9	-20±4.6	-21±1.8	0.483	0.495	63%±2.3	62%±8.4	61.0%±2.70	60.75%±2.1
6	116±9.5	121±5.1	-21±2.3	-25±2.7	0.535	0.497	61%±5.4	60%±4.6	61.87%±3.1	60.61%±1.8
8	109±5.7	109±6.7	-17±1.9	-28±2.3	0.364	0.457	64%±4.9	62%±6.8	62.6%±2.55	60.83%±3.2
10	121±9.6	110±6.1	-24±2.8	-25±2.9	0.472	0.532	59%±7.4	60%±4.8	62.31%±1.9	59.7%±3.6
12	110±7.5	103±7.4	-25±1.7	-19±1.9	0.385	0.452	61%±5.1	60%±5.3	61.3%±2.25	59.57%±1.9
14	117±8.7	119±5.8	-27±3.1	-23±2.8	0.518	0.583	62%±4.9	60%±5.4	60.7%±2.56	59.85%±2.1
16	112±4.8	115±4.0	-30±2.8	-27±1.3	0.485	0.517	63%±3.9	62%±4.7	60.33%±1.7	59.18%±3.5
18	119±7.3	123±9.1	-26±1.5	-24±2.8	0.392	0.428	64%±3.2	61%±5.0	60.65%±3.6	58.97%±2.7
20	120±5.9	116±6.1	-19±2.8	-22±1.9	0.373	0.486	65%±6.1	63%±5.1	60.5%±2.25	58.77%±1.8

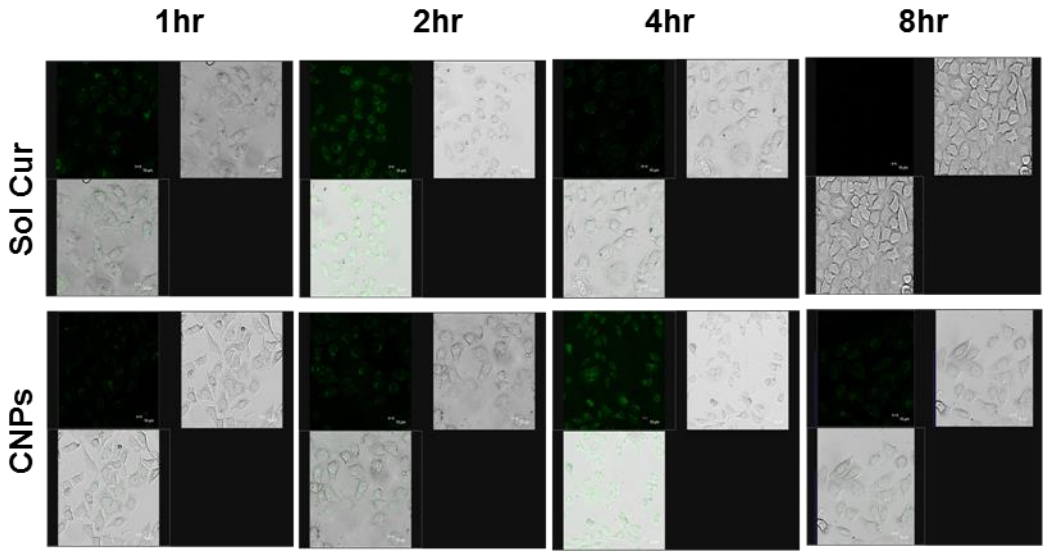
Cellular Localization assay:

NPs showed improved cellular retention in SUP-T1 cells, HL2/3 and VK2 cells. Cells were incubated with 10 mM soluble curcumin & CNPs for different time points of 1, 2, 4 and 8 hr, later examined by confocal microscopy. Soluble curcumin was taken up quickly by the cells, maximum at 2 hr, but was quickly eradicated out by 4 hr (Fig 3.8). CNPs, in contrast both were taken up and released very slowly with peak at 4 hr and, 50% of the drug still present at 8 hr. Further, the results confirmed that CNPs exhibit a time-dependent intracellular localization of curcumin (in SUP-T1 HL2/3 and VK2 cells) that is steady for almost the complete 8 hr. Contradictory to CNPs, soluble curcumin was taken up hastily and evanished from the cells rapidly. The steady and constant uptake of CNPs is a characteristic of receptor mediated transport. These results infer that cells could retain curcumin for a longer time, followed by treatment with CNPs than soluble curcumin.

A. SUP-T1 cells:



B. HL2/3 cells:



C. VK2 cells:

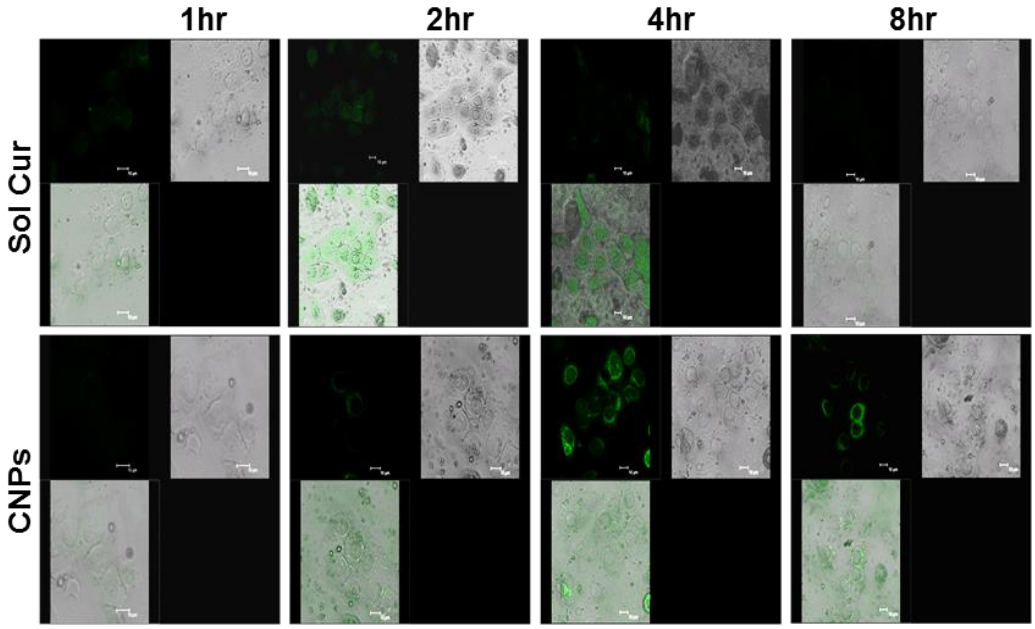


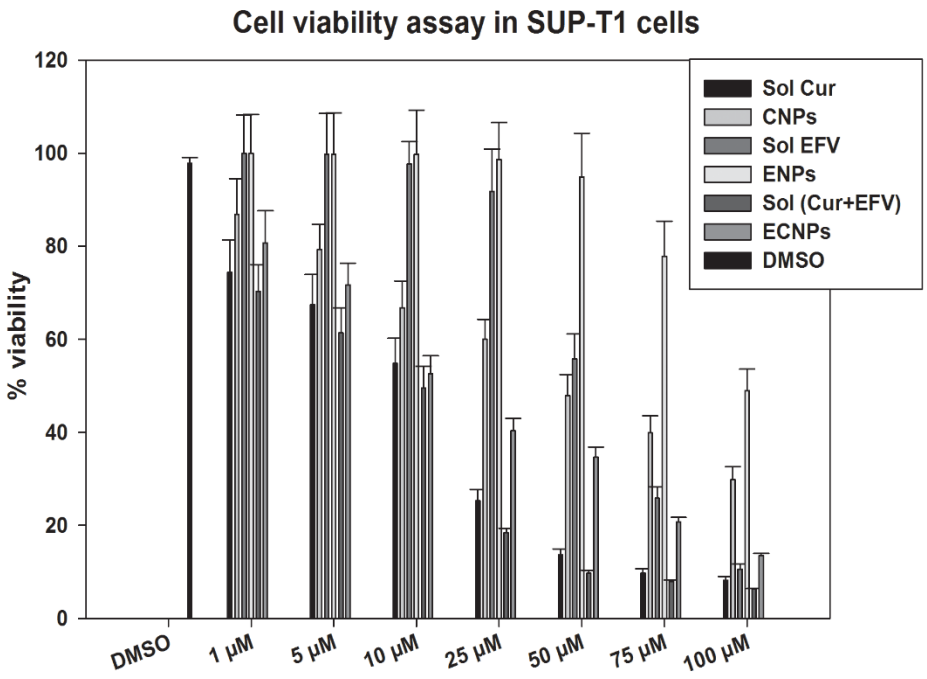
Fig 3.8: Cellular localization assay: CNPs exhibit increased cellular retention in SUP-T1, HL2/3 and VK2 cells. Cells were incubated with 10 mM soluble curcumin and equivalent nano curcumin and checked by confocal microscopy at given time points as 1, 2, 4 and 8 hr. Each panel contains three images: fluorescence, bright field and merged.

Cell viability assay:

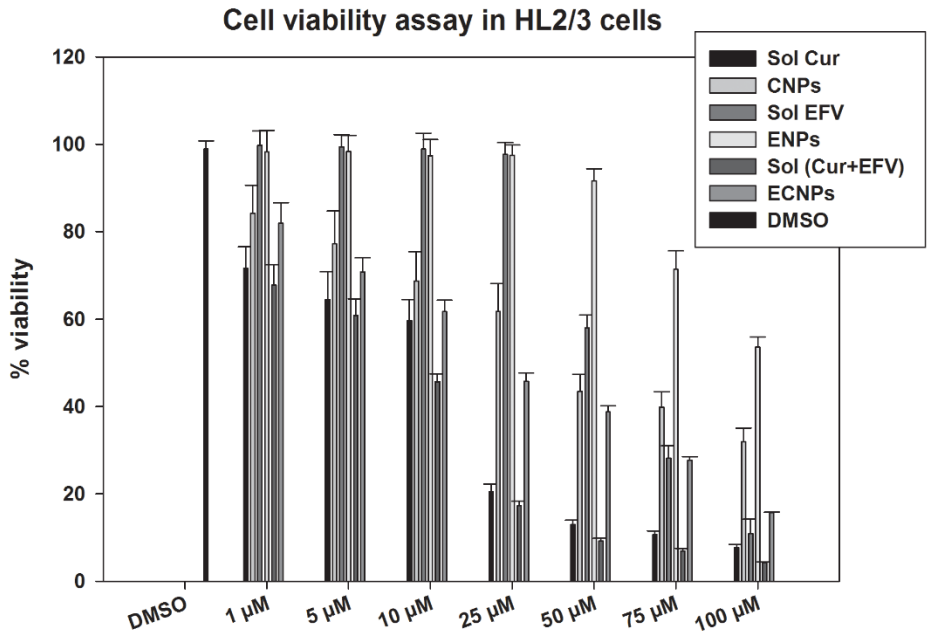
SUP-T1 cells, HL2/3 and VK2 cells (Fig 3.9) were incubated with a series of increasing concentrations (1, 5, 10, 25, 50 and 100 μM) of both soluble and nano formulations and cell viability was evaluated by MTT assay. Compared to soluble curcumin, CNPs were found to be less toxic at all concentrations. Soluble curcumin was highly cytotoxic even at higher concentration while CNPs at the same concentration was comparatively less cytotoxic.

The lower cytotoxic properties of CNPs highlights the perception that direct administration of soluble curcumin is lethal to cells at 10 μM concentrations and above. Both soluble EFV and ENPs formulations were found non-toxic at all concentrations. In combination drug treatment, soluble combination form showed more toxicity when compared to ECNPs.

A. SUPT1 cells:



B. HL2/3 cells:



C. VK2 cells:

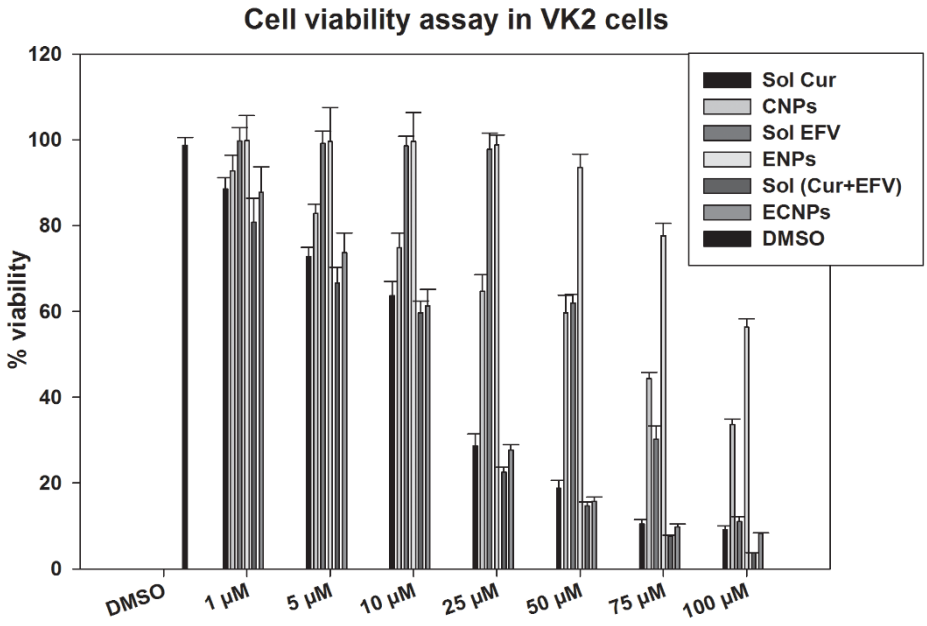


Fig 3.9: cell viability assay: SUP-T1, HL2/3 and VK2 cells were treated with a series of concentrations (1, 5, 10, 25, 50, 75 and 100 µM) of soluble curcumin, soluble EFV, soluble (Curcumin+EFV), CNPs, ENPs and ECNPs. Later the cells viability were tested a by MTT reagent and results were plotted.

Viability assay profile of *Lactobacillus:*

Lactobacillus is the most colonizing bacteria in the vagina, and is generally considered as a gatekeeper of the vaginal ecosystem. Any microbicide could be considered as safe until it is non-toxic to the growth of *Lactobacillus*. *Lactobacillus* naturally produces hydrogen peroxide which provide natural barrier for HIV transmission. From Fig 3.10 it is clear that there is no difference in the viability of bacteria between the media control and ECNPs, which confirms that ECNPs could be a safe microbicide.

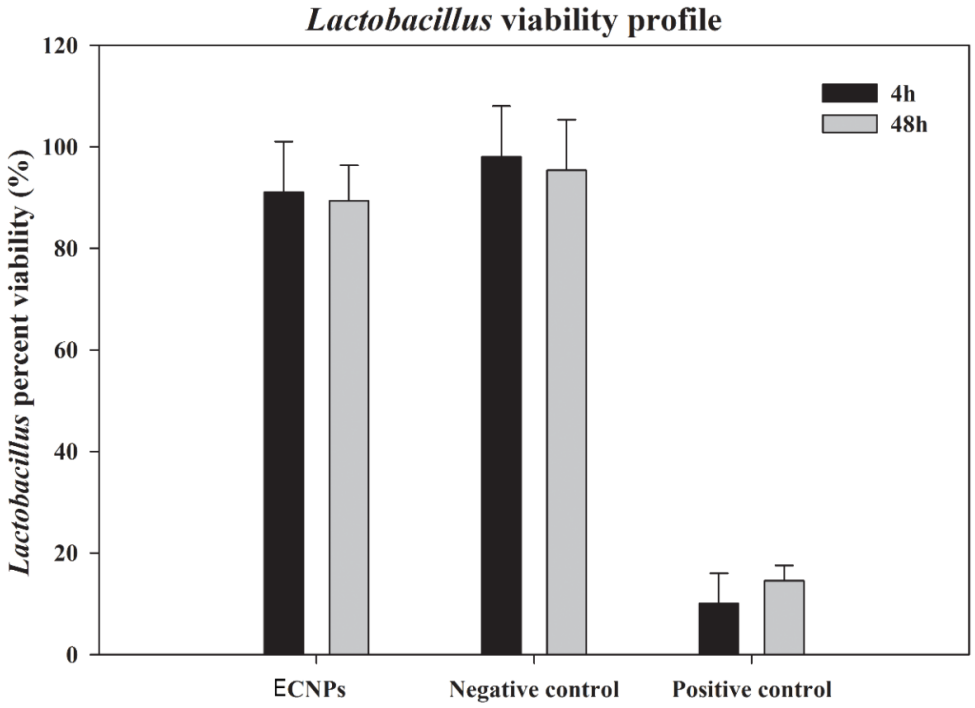


Fig 3.10: *Lactobacillus* viability profile: *Lactobacillus* percentage viability was tested when treated with ECNPs, at 4 hr and 48 hr, media without ECNPs and 1% triton X served as negative and positive control respectively.

Sperms viability assay:

Microbicide having contraceptive property is an additional asset to any microbicidal formulation that to act as a multipurpose microbicide against HIV. Sperms (Fig 3.11) were incubated with increasing concentrations (10, 50, 100, 200, 300, 400, 500 and 600 μM) of the two formulations and viability percentage was estimated by MTT assay. Both soluble curcumin and CNPs and combination formulations (ECNPs) were found to be non-toxic at very low concentrations. Greater than 200 μM concentration of curcumin was showed nearly 80% cytotoxic to sperms in both forms. This data suggests that these formulations can also act as a contraceptive due to its spermicidal activity of curcumin. Both soluble EFV and ENPs formulations were found non-toxic when compared to curcumin. In combination drug treatment, soluble combination and nano form showed similar results as like curcumin.

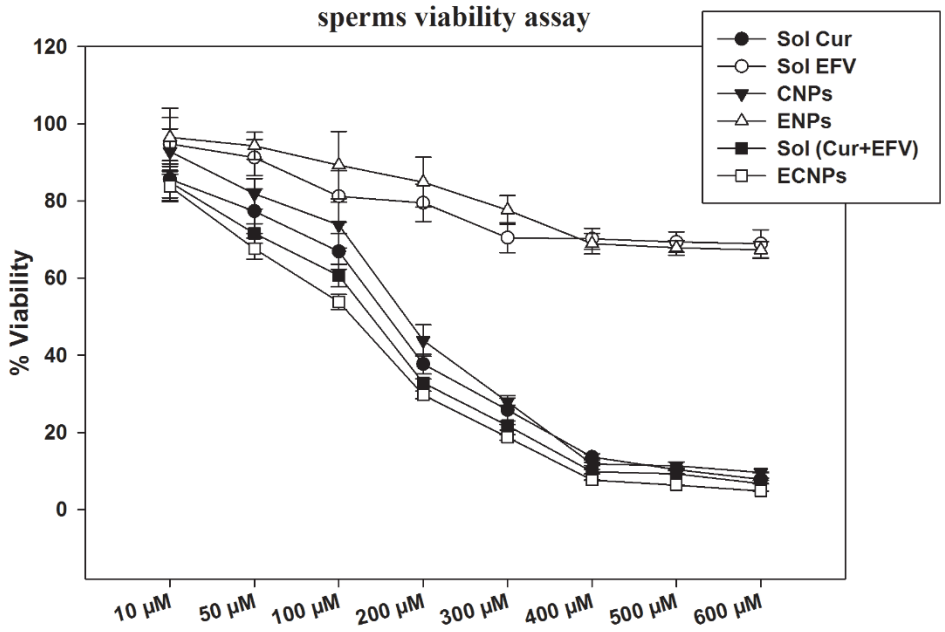


Fig 3.11: Sperms viability assay: Sperms were treated with increasing concentration (10, 50, 100, 200, 300, 400, 500 and 600 μM) of soluble curcumin, soluble EFV, soluble (Curcumin+EFV), CNPs, ENPs and ECNPs. Later the sperms viability were tested a by MTT reagent and results werd plotted.

Anti-HIV activity of NPs:

The *in-vitro* anti HIV-1 activity of ECNPs was evaluated as individual and in combination for soluble and nano formulation. The individual IC_{50} for soluble Curcumin (5.1 μM) and CNPs (1.75 μM), soluble EFV (2.56 nM) & ENPs (1.1 nM). Further, the anti-HIV activity of ECNPs have been found to be improved over its soluble formulation when used at 1:1, 0.5:0.5 and 0.25:0.25 while no significant advantage is observed at 0.1:0.1 and 0.05:0.05. The percent HIV-1 replication vs. drug combination ratio have been plotted (Fig-3.12). The Anti-HIV activity in a combination of Curcumin + EFV are found to be improved or intact even after 50 % reduction in dose. The antiviral activity was improved in combination nano form, so reducing the dose frequency and prevent unwanted side effects and toxicities.

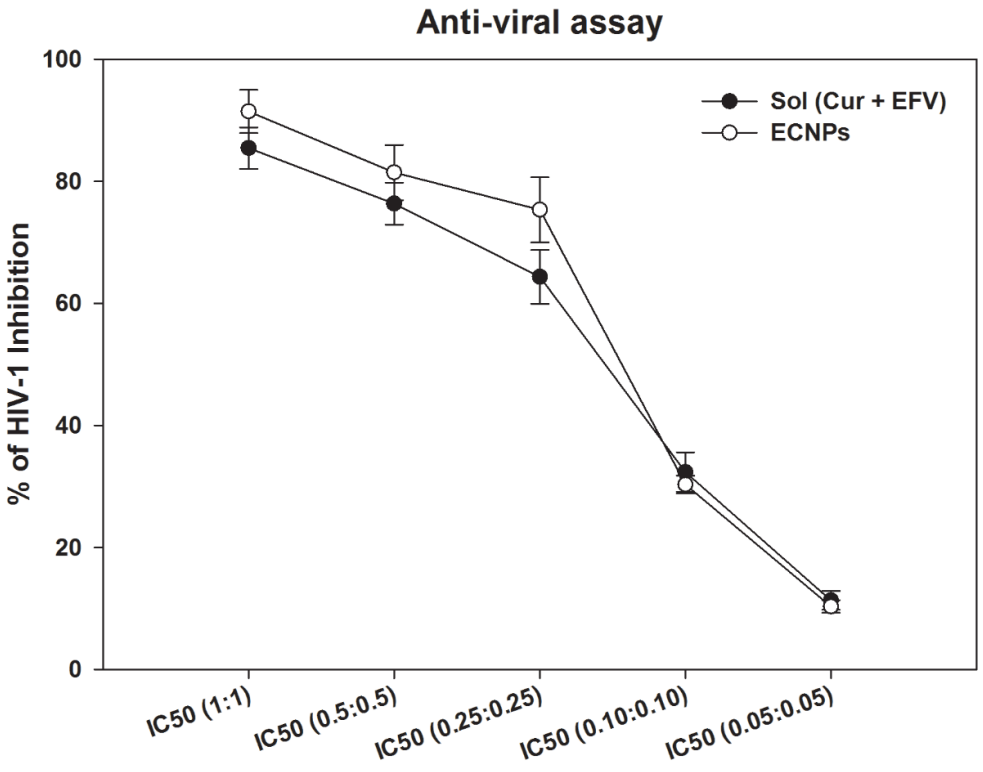


Fig 3.12: Anti-viral assay: Various different IC_{50} ratios of curcumin and EFV drugs were mixed in soluble and nano form. Later the antivral assay were performed and plotted.

Pharmacokinetic (PK) studies result in vaginal lavage:

Curcumin and EFV levels were measure in vaginal lavage after administration of single dose of combination drugs either in soluble or nanoform. Drug levels were observed at nine different time points up to 24 hr (Fig 3.13). We found enhanced PK profile (Table 3.3) when drugs were delivered via NPs. The lavage PK shows more than 3-fold increase in AUMC both in the case of EFV as well as curcumin. Similarly there was more than 2-fold increase in T_{max} and $t_{1/2}$. AUC values were also seen to be increased by 50% when NPs were used as delivery system. This PK profile suggest the higher bioavailability of drugs when given through Lf NPs.

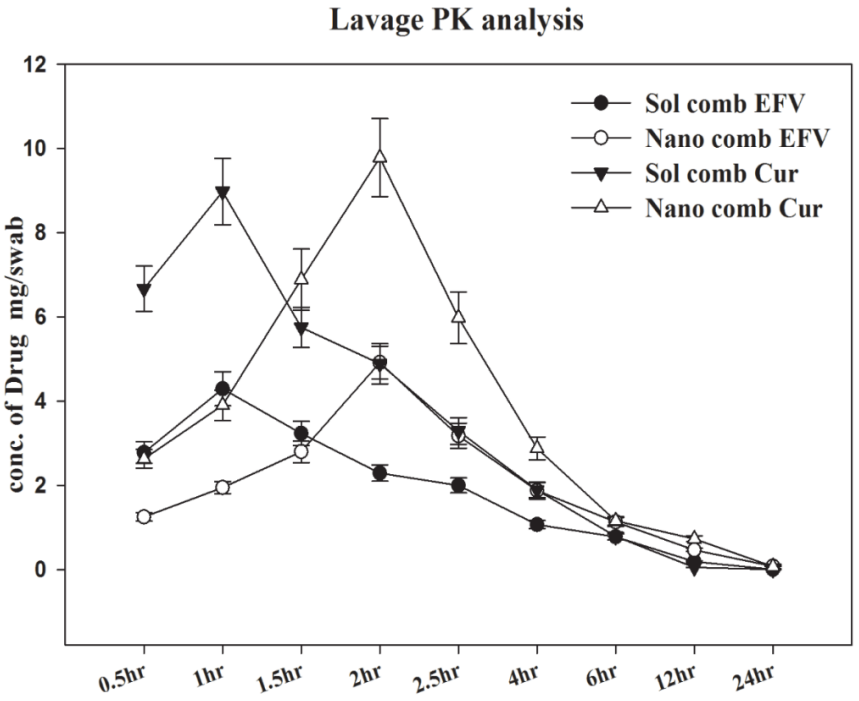


Fig 3.13: Pharmacokinetics: 20 mg of soluble curcumin plus 10 mg of soluble EFV and an equivalent amount of ECNPs were applied as single dose in vagina for indicated time points. Lavages were collected after these time points and curcumin and EFV concentration were calculated separately. Abbreviation: Sol comb curcumin and sol comb EFV represents curcumin and EFV drugs released from soluble form (Curcumin+EFV). Nano comb curcumin and Nano comb EFV represents curcumin and EFV drugs released from ECNPs.

Table 3.3: Pharmacokinetic profile in vaginal lavage

Parameters	Units	EFV		Curcumin	
		Nano	Soluble	Nano	Soluble
AUC	(h)*(mg/ml)	20.4164	13.7581	32.4251	21.9066
AUMC	(h)^2*(mg/ml)	132.482	52.448	172.922	56.496
C_{max}	mg/ml	4.9126	4.2897	9.7794	8.9723
T_{max}	hr	2	1	2	1
$t_{1/2}$	hr	4.63222	2.14916	3.97347	1.8513

Values in the parenthesis designate the concentration of drugs in milligrams per swab.

Pharmacokinetic parameters.

AUC: The integral of the concentration-time curve (after a single dose or in steady state).

AUMC: Partial area under the moment curve between t start and t end.

C_{max}: The peak plasma concentration of a drug after vaginal administration.

T_{max}: Time to reach C_{max}.

$t_{1/2}$: The time required for the concentration of the drug to reach half of its original value.

Evaluation of drug release kinetics of ECNPs:

To investigate the extent of availability of drugs over a period of long time, the time-dependent release study was performed. The time-dependence was studied individually for the two drugs curcumin and EFV delivered in ECNPs; the results are shown in in Fig 3.14. The figure represents that the concentration of curcumin and EFV in vaginal lavage at various time point, after one (soluble drugs combination) and two (ECNPs) hours of lag phase of drug administration. The results suggest high concentration in the initial stage for both the soluble and nano form, which got decreased later exponentially. The nano formulations of drugs even after 2 hr of lag phase showed sustained and significant release of drug up to 12 hr whereas both the drugs in the soluble form, showed fast reduction (at 4 hr) in the concentration. We have indeed detected the presence EFV and curcumin up to 8–12 hr, when drugs were given in nanoformulation but in case of the soluble form, the drugs were eliminated in 4–6 hrs.

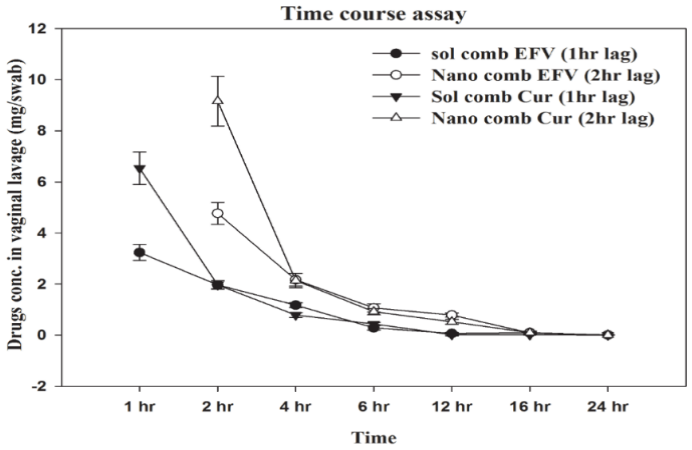


Fig 3.14: Drug release Kinetics: 20 mg of soluble curcumin plus 10 mg of soluble EFV and an equivalent amount of ECNPs were applied at a lag period of 1 hr (for soluble EFV + curcumin) and 2hr (for ECNPs). Lavages were collected after above mentioned time points and curcumin and EFV concentration were calculated separately. Abbreviation: Sol comb Cur and sol comb EFV represents curcumin and EFV drugs released from soluble (Curcumin+EFV). Nano comb curcumin and Nano comb EFV represents curcumin and EFV drugs released from ECNPs.

Dose dependent toxicity and bioavailability studies:

EFV and Curcumin concentration in vaginal lavage:

Assessment of EFV and curcumin in vaginal lavage. The fixed doses mentioned in dose schedule were administered intravaginally and the drugs estimated in lavage are plotted (Fig 3.15). Results suggest that the nano formulation of drugs either in single form or combination showed 1.8 (1stdose) to 2.2 (3rddose) fold more availability of drugs at the topical site in a dose-dependent manner.

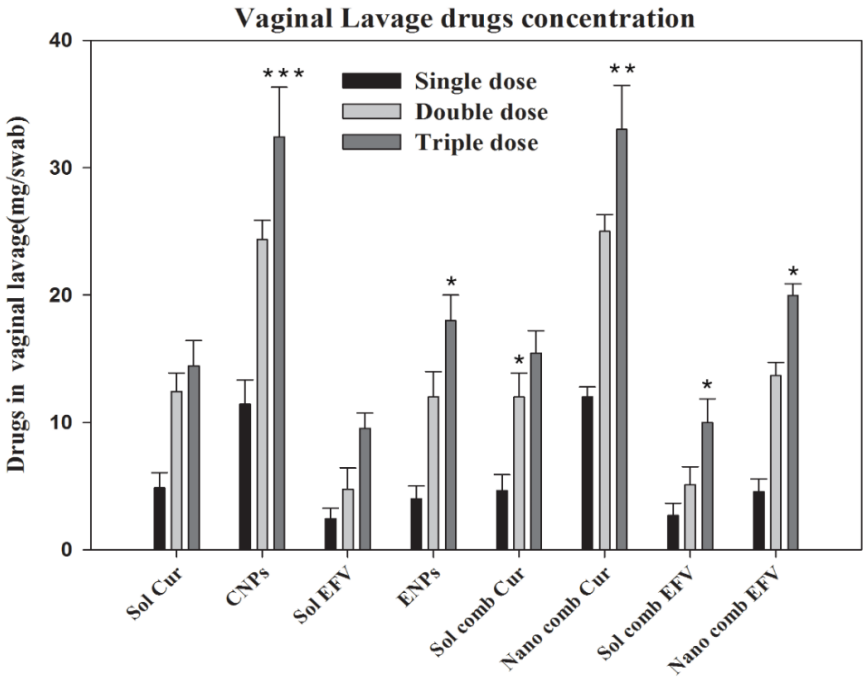
EFV and Curcumin concentration in vaginal tissue:

Absorption of drugs in local cervical-vaginal tissue has been estimated and represented in Fig 3.15. EFV and curcumin were found in very low concentrations in the range of micrograms, 1000-fold less than vaginal lavage. Curcumin NPs and EFV NPs didn't show appreciable difference in exposure of drugs as compared to soluble counterpart. But in the case of ECNPs, we found there was 1.2 (1stdose) to 1.8 (3rddose) fold lower drug concentrations as compared to soluble drugs in combination.

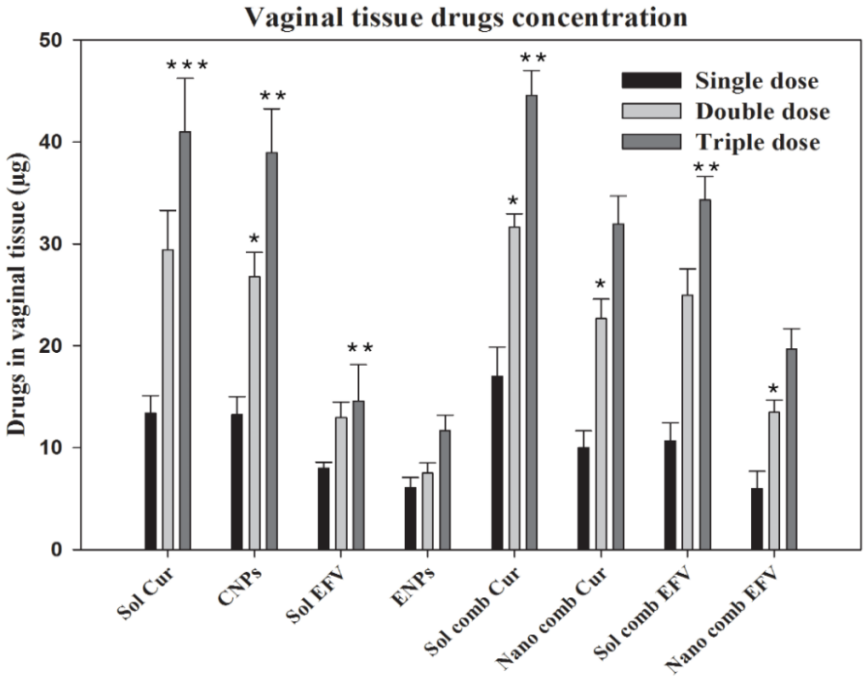
Systemic bioavailability of EFV and Curcumin:

Fig 3.15 represents the nature of accumulation of drugs in the systemic circulation. We found that the soluble form of drugs either in single or combination showed 1.5 (1stdose) to 1.7 (3rddose) fold more accumulation in plasma as compared to its nano form; leading to higher organ-associated toxicity in the case of soluble formulation. In the comparison study between the ECNPs and its soluble combination form at the third dose, curcumin and EFV were found to be approx. 30 ng/ml & 20 ng/ml and 44 ng/ml & 35 ng/ml respectively, suggest that at higher doses also the NPs could me marked as safe.

A. Vaginal lavage:



B. Vaginal tissue:



C. Systemic Bioavailability:

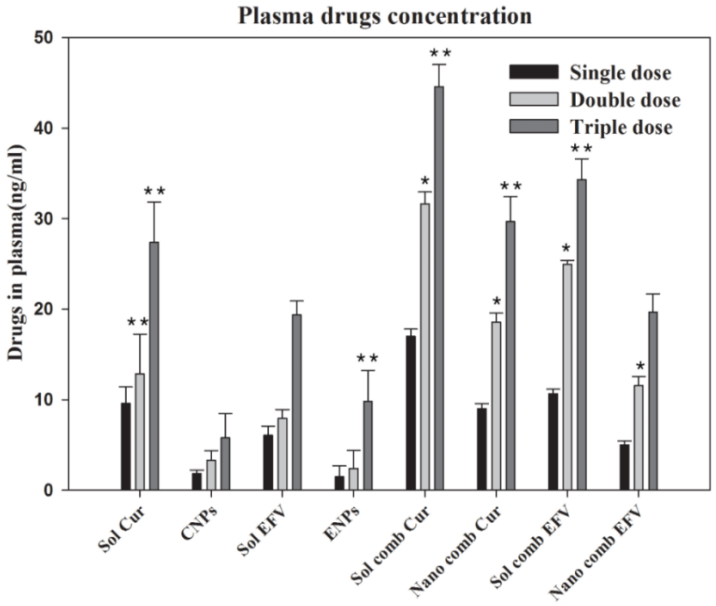


Fig 3.15: Bio availability studies in rats: Dose dependent toxicity study of drugs in single form or combination form either in soluble (EFV + Curcumin) and ECNPs were performed. The concentration of drugs in vaginal lavage, vaginal tissue and plasma were analyzed and plotted. Sample data were recorded as Mean ± S.D, n=3. The difference between groups was calculated using one-way analysis of variance and the value of significance expressed as ***P<0.0005, **P< 0.005, *P< 0.05.

Histopathological analysis:

Histopathological studies of cervicovaginal epithelia showed that ECNPs causes a reduced amount of toxicity in a dose dependent manner. The integrity of epithelia was found heavily damaged when the combination of soluble drugs were used. In the case of ECNPs, the integrity of tissue was found to be same as that of control or very lesser extent. Tissue section pictures revealed that even at higher dose, ECNPs caused very less amount of tissue damage and at the same time soluble drug combination shows higher tissue damage. Negative and positive controls were treated with saline and nonoxynol-9 respectively (Fig 3.16).

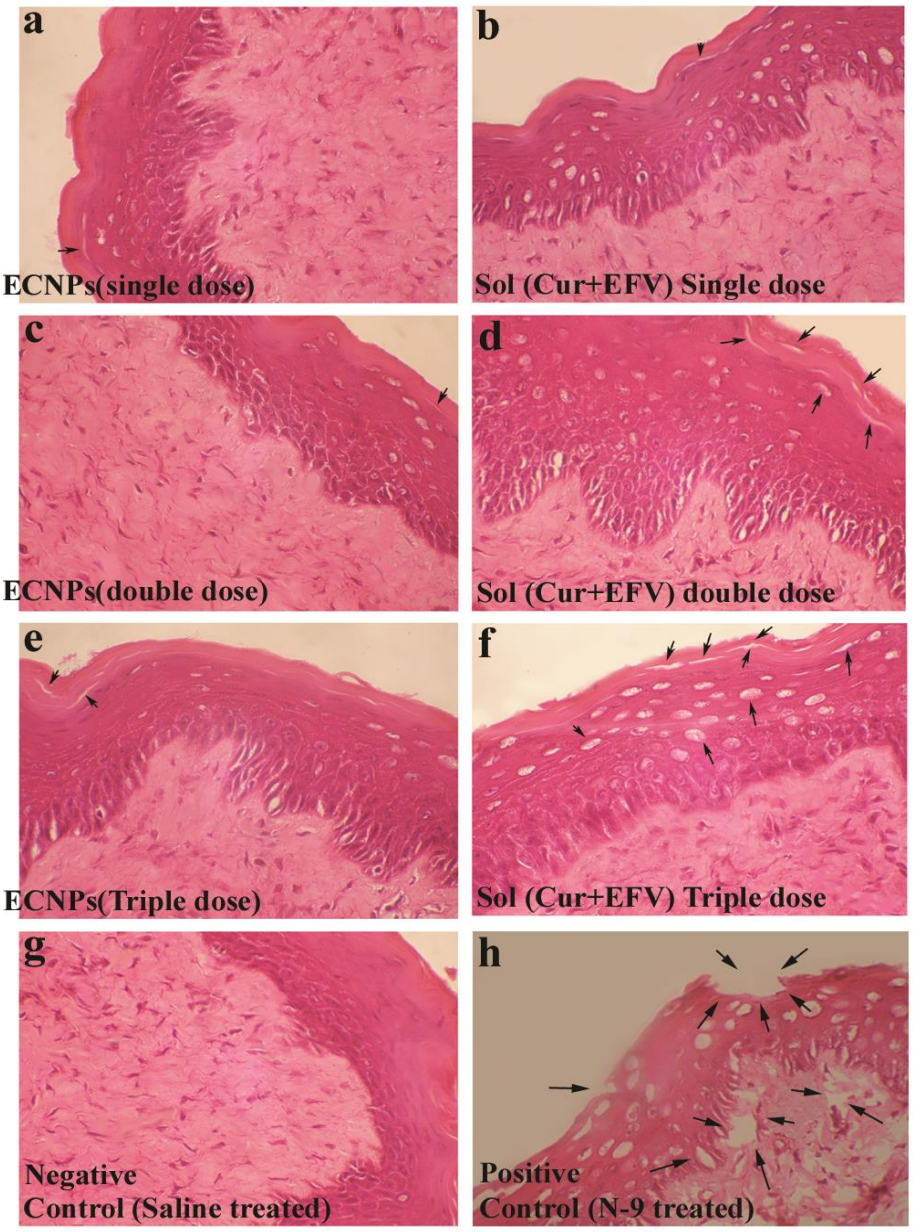


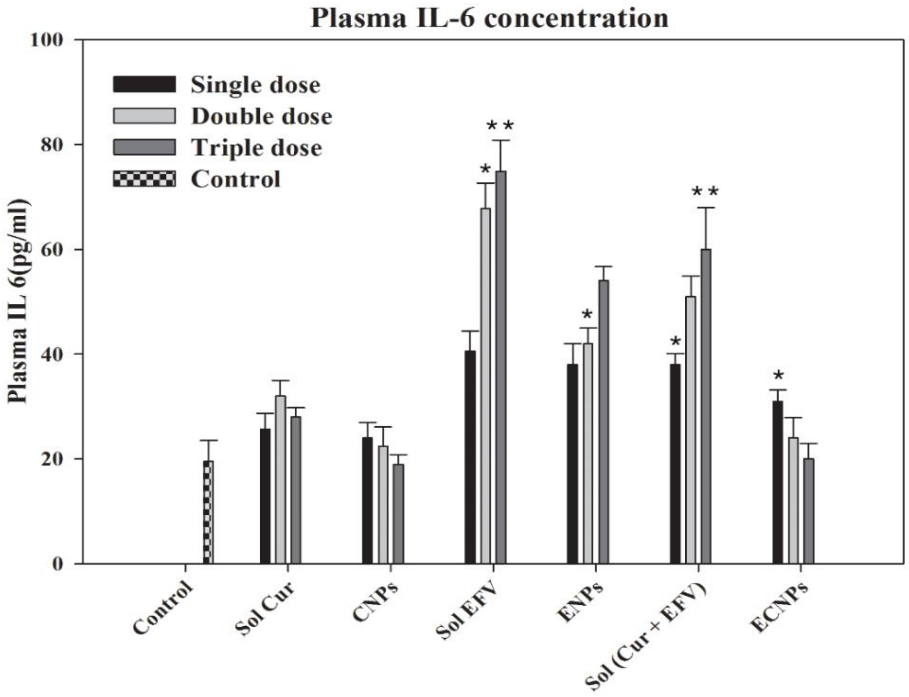
Fig 3.16: Histopathology: Right panel represent the vaginal epithelia treated with soluble (curcumin + EFV) for single, double and triple dose respectively and positive control. Left panel represent the vaginal tissue integrity when applied topically with ECNPs for single, double and triple dose respectively and negative control. Damaged site or lesions were shown through a printing arrow. Negative and positive controls were treated with PBS (pH-7.4) and nonoxynol-9 (10 mg/kg) respectively.

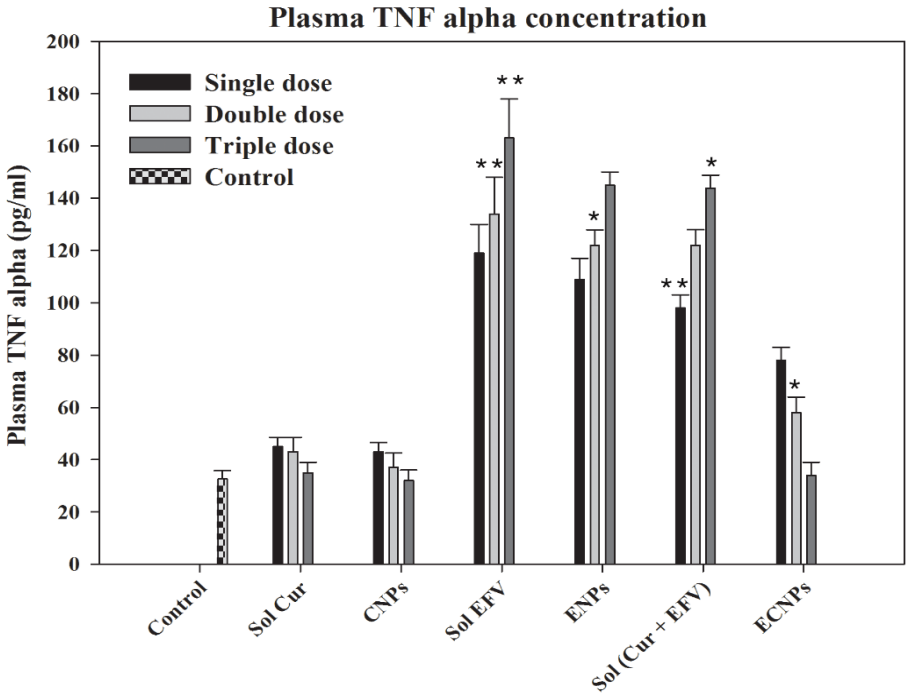
Systemic and vaginal Proinflammatory cytokine response:

The proinflammatory cytokine markers; interlukine-6 (IL-6) and tumor necrosis factor alpha (TNF-alpha) levels were estimated in isolated plasma and vaginal tissue. Fig 3.17 represents the IL-6 level in vaginal tissue and plasma respectively. Soluble EFV and ENPs showed at least three-fold increase in the level of IL-6. When EFV was encapsulated into Lf together with curcumin, the IL-6 levels significantly decreased by 2.5 fold as compared to its soluble formulation. At the third dose of ECNPs, we couldn't detect any inflammation, in contrast to soluble (Curcumin + EFV) which leads to more than three-fold increase of IL-6, both in the vagina as well as in blood.

TNF-alpha is a major cytokine which stimulates the inflammatory response and leads to many autoimmune diseases and other clinical problems. In the case of ECNPs and Lacto-curcumin-nano at the third dose, the TNF-alpha level was found significantly decreased. These results suggest that nanoformulation provides an effective anti-inflammatory environment along with anti-HIV-1 drug EFV in vagina for protection from infection.

A. Plasma:


Plasma IL-6 concentration
Plasma IL 6(pg/ml)
Single dose
Double dose
Triple dose
Control
0
20
40
60
80
100
Control
Sol Cur
CNPs
Sol EFV
ENPs
Sol (Cur + EFV)
ECNPs


Plasma TNF alpha concentration
Plasma TNF alpha (pg/ml)
Single dose
Double dose
Triple dose
Control
0
20
40
60
80
100
120
140
160
180
200
Control
Sol Cur
CNPs
Sol EFV
ENPs
Sol (Cur + EFV)
ECNPs

B. Vaginal tissue:

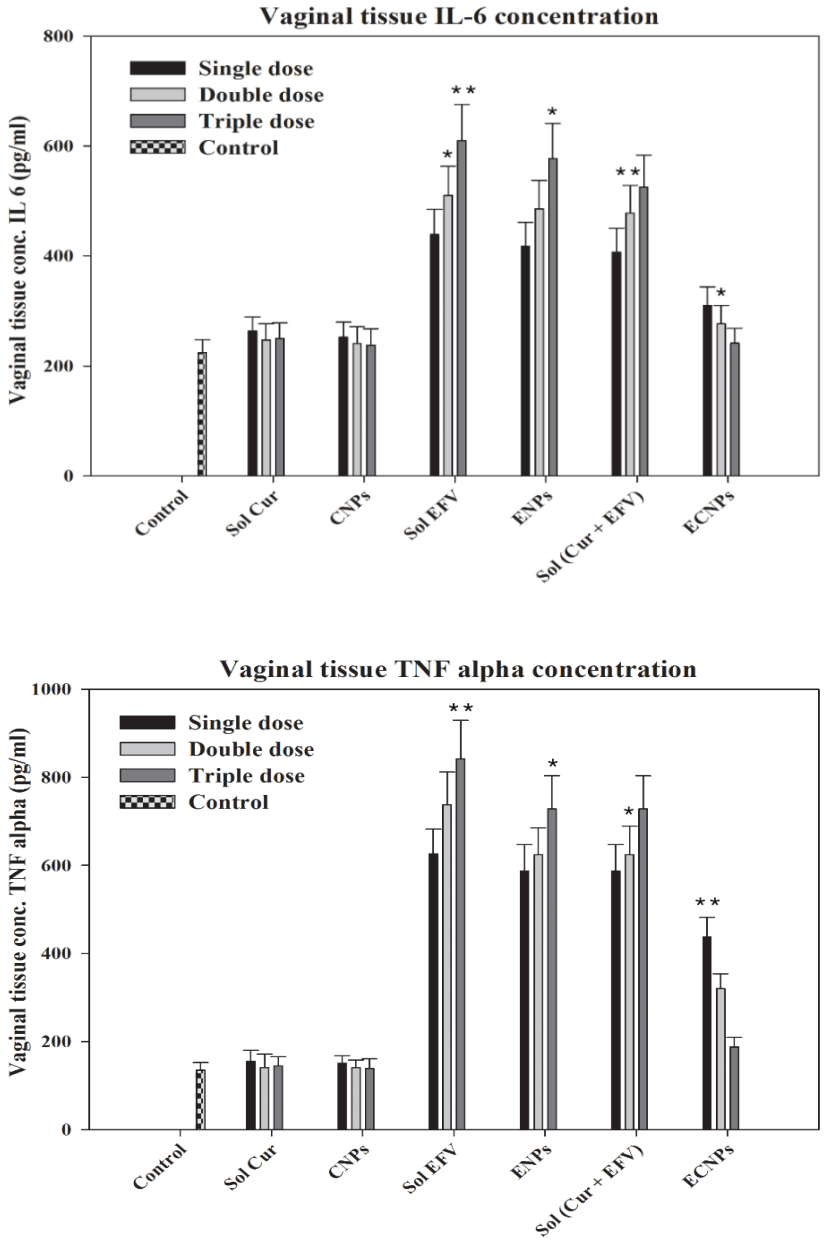


Fig 3.17: Proinflammatory cytokine response: Soluble EFV, Soluble curcumin, Soluble (EFV + Curcumin), CNPs, ENPs and ECNPs were applied topically in vagina. IL-6 and TNF alpha levels in plasma and vaginal tissue were analyzed and plotted. . Sample data were recorded as Mean ± S.D, n=3. The difference between groups was calculated using one-way analysis of variance and the value of significance expressed as ***P<0.0005, **P< 0.005, *P< 0.05.

Discussion:

The microbicide based MPT is one of the important inventions against HIV, MPT reported possess several limitations [128-131]. According to our study, Protein NPs formulation made of a combination of drugs would be one of the multipurpose prevention technologies (MPT) in against HIV. Using the simple and fast approach sol-oil method, EFV and Curcumin loaded with Lf were developed without undergoing any chemical modifications [132]. According to this method, a solid-liquid interface is produced from small molecules over macromolecular assemblies. Sonication followed by low temperature exposure prevents protein aggregation at this solid-liquid interface. Precipitated particles can be stored at room temperature even for a very longer time period. This nanoparticle suspension becomes visible as a group of uniformly dispersed particles at room temperature (Ex. Colloid). This methodology offers a chance to generate various materials with a novel, well defined properties and reasonably at low cost. Producing highly pure particles and uniform nanostructure based on assessment through FE-SEM and AFM analysis were the major advantages of this method. It was found that the DLS size measurement were greater than their SEM or AFM sizes in both types of nanoformulation, either drug/s loaded or blank. The reason for the difference is fairly rational because light scattering method was used to measure the size which could be affected by water shell interface of nanoparticle and their surface charge may lead to increase in size. ECNPs having polydispersity index (PDI) of 0.435 were found to be a homogeneous mixture. It was found that ECNPs in buffer were more stable, up to 20 days, as the stability parameters were found to be constant which shows the reliable characteristics of Lf NPs. Based on FT-IR data, the stability of nanoforms of EFV and curcumin was confirmed by the presence of conserved major characteristics bands. A constant or little shift in the peaks may be observed in case of ECNPs functional groups due to the presence of dipole moment in the molecules, as the bond nature is electrostatic. The results of the study showed that protein can be encapsulated with more than one drug. Cooperation among drugs in loading by synergistic action and molar equivalences were observed. Hence patients with HIV-oba1 infection can acquires resistance to different drugs combinations and in some

cases it is patient-dependent thus it created the necessity for the design and development of various drug regimens with multi-targeting capabilities Encapsulating curcumin and EFV into Lf could resolve the major issue of solubility limitation as they have poor aqueous solubility and the ECNPs were designed based on its triple action comprising of EFV, curcumin and Lf. Apart from the anti-inflammatory activity of curcumin, it also helps in HIV-1 replication inhibition by binding to the enzymes integrase and protease [133, 134] hence providing added specificity to formulation. As the Lf blocks virus entry, EFV targets the Reverse transcriptase enzyme. Also, Lf acts as an immune modulator [101] and show a wide range of anti-microbial activities [100]. This formulation would work as a characteristic broad spectrum HIV microbicide. The major observation in ECNPs is their antiviral activity was enhanced when compared to their individual soluble and nanoforms. Here the maximum activity that is up to 98% was obtained even during curcumin and EFV individual concentration was reduced to half. It gives an advantage to use the combination of drugs with their nanoform there by a reduction in dosage leading to the reduction of non-target side effects and toxicities. There is an increase in failure of HIV treatment due to emergence of drug resistance [135]. An enhanced PK profile of drugs was shown by vaginal lavage bioavailability studies. When delivered via NPs the AUC (area under the curve) of EFV and curcumin were found to be increased by more than 50%, thus showing nanoform of drugs gets long time exposure to the vagina thereby giving a long lasting protection against HIV as compared to soluble formulation. Similarly a 2-fold increase was shown by the other PK parameters T_{max} and $t_{1/2}$, suggesting an enhanced stability of NPs encapsulated with drugs. Further, ECNPs showed minimum burst effect suggesting in the time course study suggesting that the drugs release was slow and steady. Better performance indicated by results of 2 hr. time lag (for nano drugs) as compared with one hour time period (for soluble drugs). The drug releases of ECNPs were found up to 8–12 hr whereas 70% of drugs were eliminated rapidly in soluble combination from the application site. This difference could be due to the adsorption of NPs on the surface of vaginal epithelium and then followed by steady release of drugs into lavage [136]. Also in the human vaginal secretion, Lf is found in its natural form with

premenstrual concentration of3.8–11.4 μg/mg which increases up to 62.9–218 μg/mg [137] after menopause. Lf present in ECNPs may escape its recognition in the system owing to its abundance in vaginal secretions, thus provides less or no chance of inflammatory response. Furthermore, inflammatory responses induced by EFV was down regulated by curcumin, thus it may be one of the reasons for low inflammatory response in contrast to single dose and multiple dose application of soluble combination. Hence in the ECNPs topical application, the presence of Lf together with curcumin neutralized the major inflammation caused by EFV. EFV will get an in obstructive environment where in the lavage it will exhibit maximum antiviral properties [138]. In the systemic circulation presence of lower concentrations of drugs was observed viz. drugs of nanogram concentrations, which was 10^{-6} fold lesser than that noticed in lavage and 10^{-3} fold lesser than that in vaginal tissue. EFV may not show any major toxicity in these concentrations. In contrast there was a significant amount of drug was found in the plasma and in tissue when soluble drugs are employed which indicates the risk of using soluble EFV for the vaginal application. ECNPs exhibit low toxicity to the *Lactobacillus*, thus if ECNPs are used it nullifies the threat vaginal microflora, a key marker for the use of microbicide with safety in concern. Compared to the soluble counterpart, ECNPs delivered more load of drugs at all the dosage levels. An important feature and critical requirement of a microbicide is to ensure sustained release and controlled delivery of the drug in the vaginal environment without altering the vaginal epithelium which could be achieved by the ECNPs. Previous studies showed the toxicity of local cervicovaginal epithelial tissue caused by that intravaginal application of microbicide which damages tissue and leading to the loss of its integrity [139]. Higher viral exposure and viral invasion was observed at the damaged tissue and leads to the infection. In conclusion, Lf encapsulated with curcumin and EFV NPs forms a control release formulation with higher bioavailability and lower toxicity.

Conclusion

Hence in this study, the drugs curcumin and EFV were successfully encapsulated using sol-oil method and in the vaginal route it was used as a controlled delivery system as a microbicide. The vaginal pH is favorable for drug release was shown by EE% and drugs release assay. Less toxicity to *Lactobacillus* and vaginal epithelial tissue was shown by ECNPs and active drug entities were released in the vaginal lavage. In summary, Lf loaded curcumin plus EFV NPs work as a most effective and efficient multi-protective microbicide formulation.

Chapter 4

Development of Curcumin and Tenofovir (TFV) Loaded Lactoferrin Nanoparticles as a Microbicidal Formulation.

Introduction:

TFV is a nucleotide analog belong to reverse-transcriptase inhibitor (NtRTI) family. It particularly inhibits viral reverse transcriptase, a vital enzyme in retroviruses such as HIV and hepatitis B virus. TFV deficiencies a hydroxyl group on the 3' carbon of its deoxyribose sugar, preventing the formation of the 5' to 3' phosphodiester linkage essential for DNA chain prolongation. Once incorporated into an amplifying DNA strand, TFV leads premature cessation of DNA synthesis, reverse transcription and preventing viral replication.

A variety of microbicidal formulations have been propounded and explored as a means to administer as vaginal microbicides [140]. Various types of delivery systems are being assessed for these molecules, including gels, films, dissolving tablets, fiber meshes and intravaginal rings [116, 141-144]. Among all the microbicides TFV gel and other vaginal gels are extensively reported [145-147]. But from these formulations, gel leakage and the low availability of drug concentrations at the site of HIV-1 transmission was observed. All these issues lead to reduce the overall effectiveness of a microbicide product [148, 149]. For small hydrophobic molecules like impoverished ARVs, a variety of NPs systems has sustained drug delivery for greater than one day and NPs can release a wide range of low molecular weight drugs. Some examples of these NPs, made with a variety of biodegradable and biocompatible polymers are PLGA, PSA and PLA. Current traditional NPs are found to be mucoadhesive which are beneficial for mucosal drug delivery [150-153]. For developing of long-acting ARV drug combinations as nano carrier systems provide an innovative approach and have been explored earlier for use in HIV treatment and prevention [154-157]. For controlled release of drugs, different carrier systems combined with the adaptability of the drugs which can be encapsulated are available. They have the capacity to motivate the use of nano carrier systems for ARV drug delivery in different systems. Nanocarriers also have the capacity to increase the activity and reduce cytotoxicity of several ARV drugs [158-160]. Against HIV, various combinations of microbicides have been reported by several research groups. Few of those formulations have tried for different stages of clinical trials; among all, a large number of formulations have not succeeded due to safety, efficacy

and toxicity issues [31,161,162]. This efficient nanotechnology-based delivery system can overcome these problems and improve bioavailability, absorption rate and the rate of metabolism.

The NPs have shown greater stability at vaginal fluid barriers, they can improve intracellular drug delivery to HIV-susceptible cells and provide favorable toxicity profile, as well as of their ability to influence the PK of TFV as compared to the free drug administered as a suspension. The studies reported herein, three different NPs were designed; 1) CNPs 2) TNPs and 3) TCNPs, to assess the *in-vitro* cell viability studies, *Lactobacillus* safety analysis, spermicidal activity, anti-HIV assay and *in-vivo* local pharmacokinetics (PK), time course analysis of the NPs and Dose-dependent toxicity & Bioavailability studies of the NPs, in comparison to soluble form of TFV and curcumin.

Results:

Physicochemical testing & characterization of NP:

Microscopic analysis (FE-SEM, TEM&AFM analysis):

Nanoparticles were analyzed using FE-SEM, TEM and AFM, the results revealed that the size of a vehicle, which is Lf protein alone was found to be nearly 22-30 nm and spherical in shape where as in drug loaded NPs size was increased to almost double. For 40-55 nm, TFV 45-55 nm and for TCNPs 65-75 nm size and spherical in shape were observed (Fig 4.1-4.3).

TNPs: **TCNPs:**

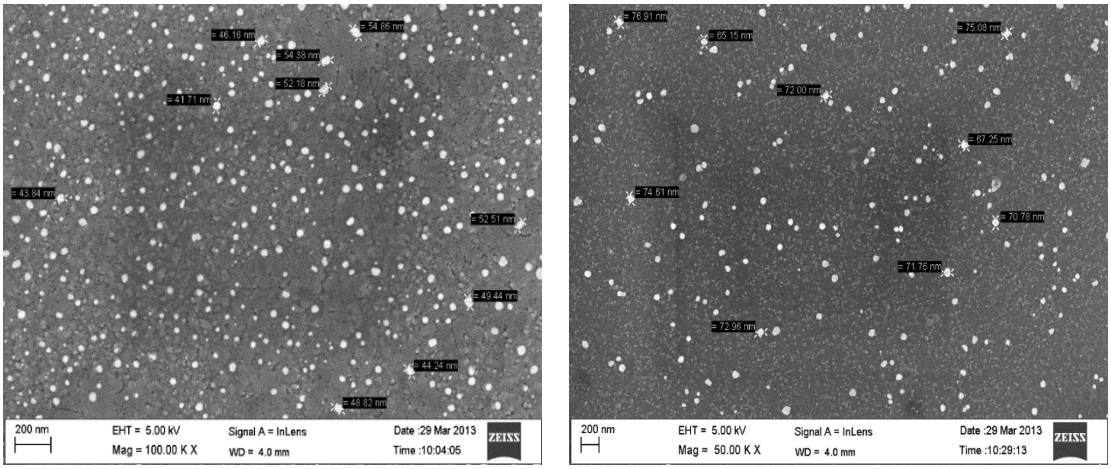


Fig 4.1: FE-SEM analysis of NPs: TNPs and TCNPs were analyzed by using the Field Emission Scanning Electron Microscope. The range of diameter for TNPs was found to be 40-55 nm and for TCNPs is 65-76 nm.

TNPs: **TCNPs:**

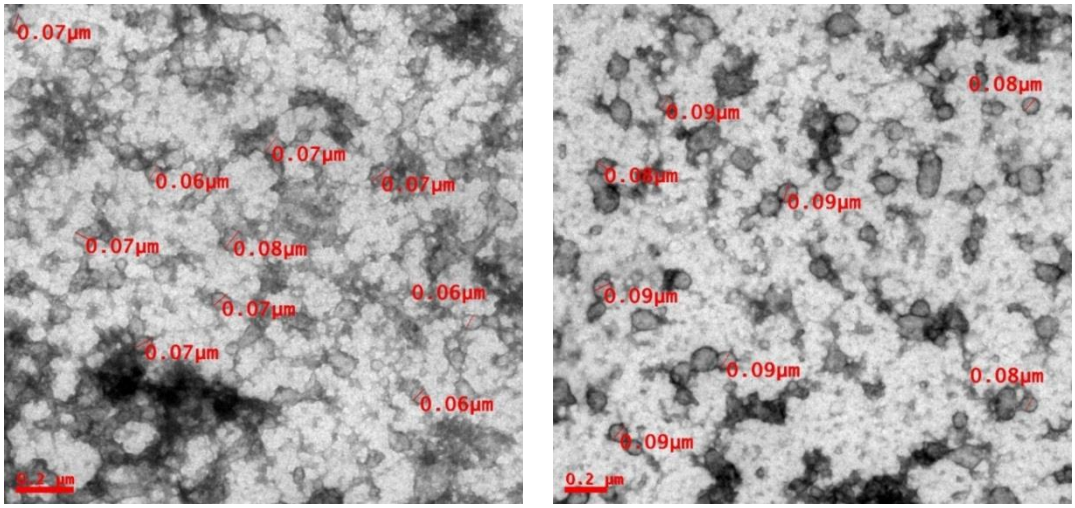


Fig 4.2: TEM analysis: TNPs and TCNPs were analyzed by using the Transmission Electron Microscope. The range of diameter for TNPs was found to be 60-70 nm and for TCNPs was found to be 80-90 nm.

TNPs: **TCNPs:**

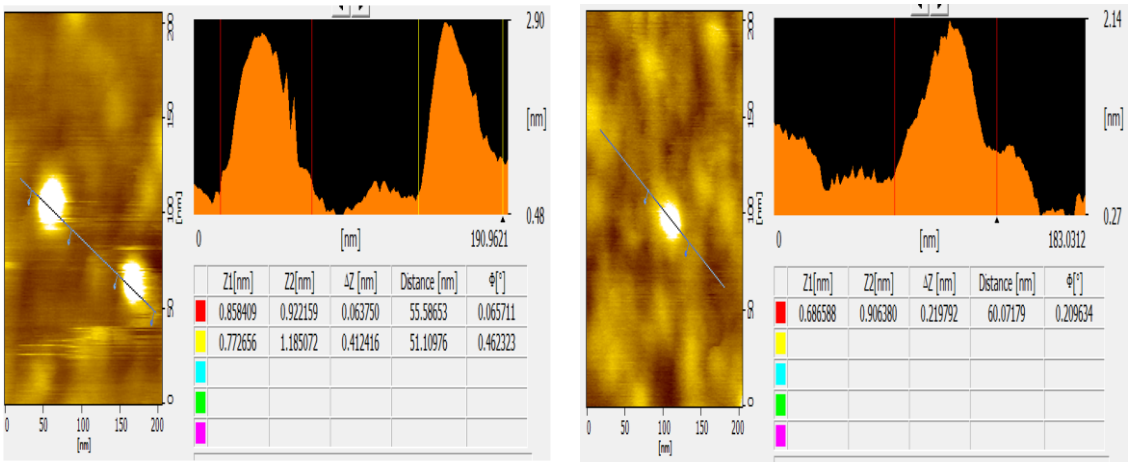


Fig 4.3: AFM analysis: TNPs and TCNPs were also analyzed by using the Atomic Force Microscope. The average diameter for TNPs was found to be ≈55 nm and for TCNPs was found to be 60 nm.

DLS analysis of NPs:

DLS was performed to determine the hydrodynamic size of NPs as follows; the size of Lf alone, CNPs, TNPs and TCNPs are 45 nm and 107 nm, 103 nm and 119 nm respectively (Fig 4.4). These results showed that increased in particle size when compared to microscopic analysis data of FE-SEM, AFM and TEM; this may be due to the hydrodynamic shell present around the particle. Zeta potential and PDI values of prepared NPs were found in the range of -19 to -27 and 0.3 to 0.45 respectively.

TNPs: **TCNPs:**

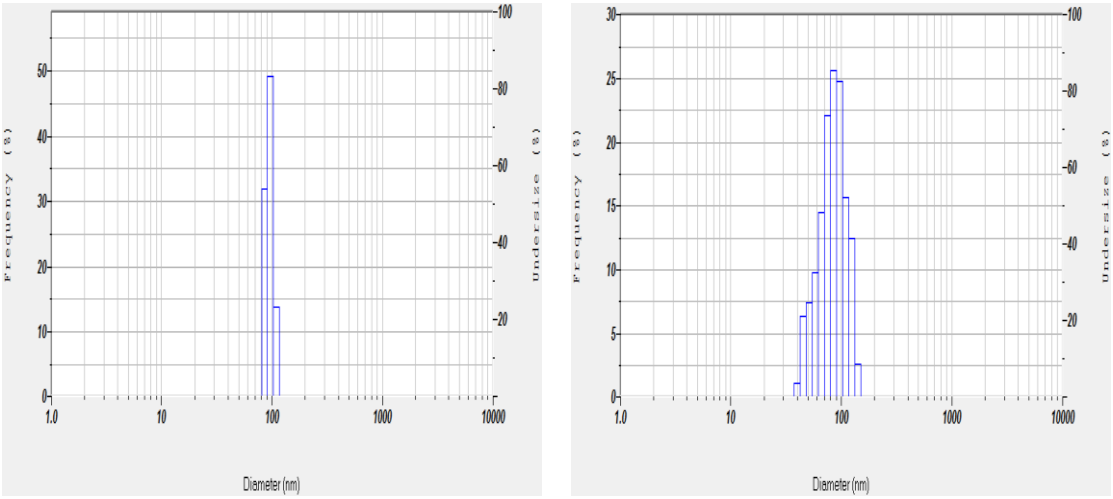


Fig 4.4: DLS analysis: DLS analysis was performed for the measurement of hydrodynamic radii. A mean size of TNPs and TCNPs 108nm and 120nm for drug loaded NPs were observed.

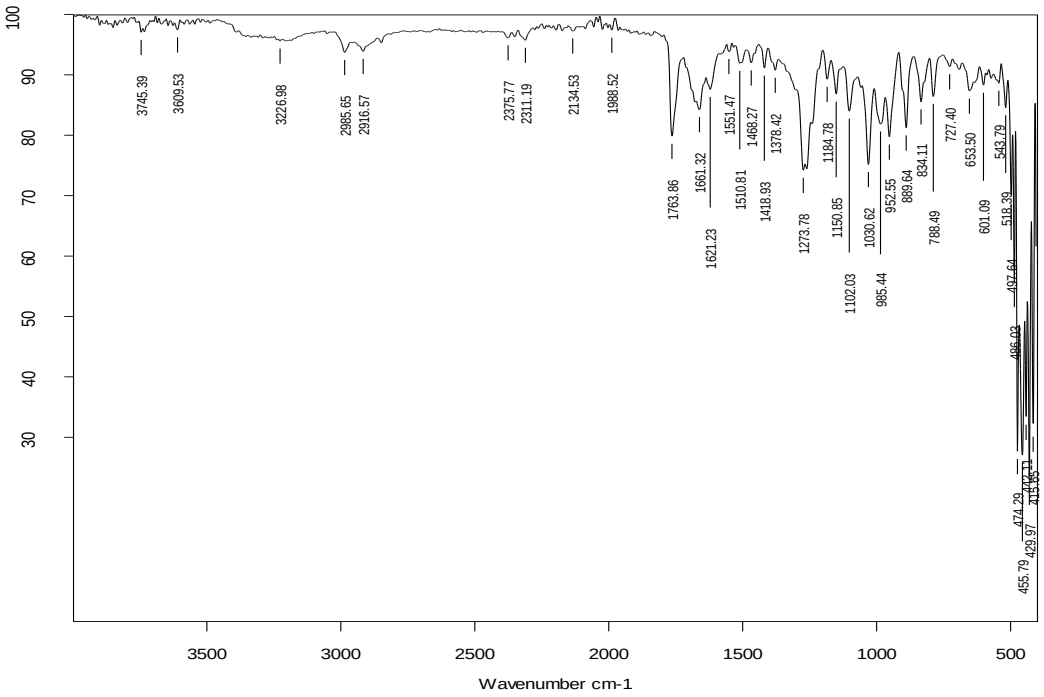
Encapsulation efficiency:

Encapsulation efficiencies were calculated and results are as following, 59±1.7% for CNPs, 53±1.4% for TNPs and 61±1.6% & 64±1.2% for curcumin and TFV respectively from TCNPs. This suggests that maximum amount of drugs has been encapsulated in protein.

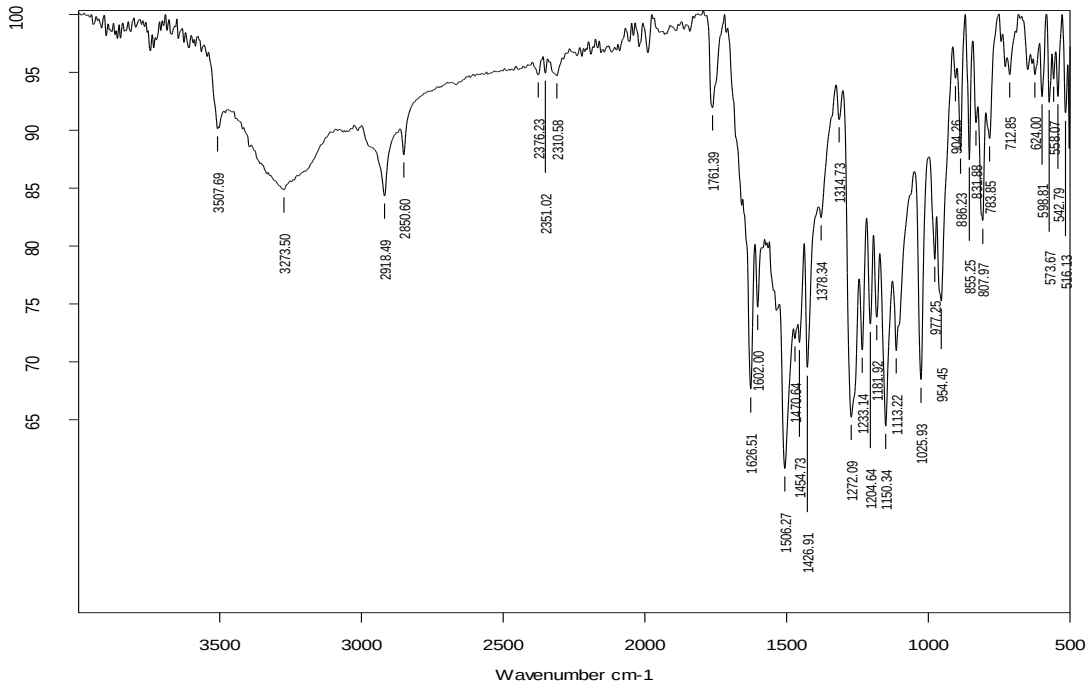
FT-IR analysis:

Fourier transform infrared spectroscopy (FT-IR) was used to assess potential interactions between protein and drug in nanoformulation. Spectra were recorded for Lf, curcumin and TFV in both (soluble and nano) and single & combination forms. Comparative spectral analysis showed following observations: important peaks of soluble Lf; amide I bond is 1632.7 cm^{-1}, amide II bond is 1536.74 $cm^{-1}$and in nano form of Lf peaks were slightly shifted to 1641 cm^{-1} and 1538.32 cm^{-1} respectively. For soluble curcumin, C=O and C=C peak is 1626.20 cm^{-1}, aromatic ring peak is 1600.74cm^{-1} and C-O –C peak is 1024.62 cm^{-1}, where as in nano curcumin these peaks are slightly shifted to 1626.50 cm^{-1}, 1601.62 cm^{-1}, 1025.13 cm^{-1} respectively. For soluble TFV, aromatic C–H stretch peak is 2986.86 cm^{-1}, P═O stretch peak is 1,661.32 cm^{-1} and a medium stretch of C–N deformation 1,273.78 cm^{-1} where as in TNPs slightly peaks were shifted to 2923.62 cm^{-1}, 1625.43 $cm^{-1}$and 1272.82 cm^{-1} respectively. Combination of soluble curcumin and TFV of peaks are as follows; aromatic C–H stretch peak is 2986.06 cm^{-1}, medium stretch of C–N deformation pea is 1,271.86 cm^{-1}, C=O and C=C peak is 1626.77 cm^{-1}, aromatic ring peak is 1601.50 cm^{-1} and C-O –C peak is 1026.93 cm^{-1} where as in nano form of TCNPs these peaks were slightly shifted to 2922.68 cm^{-1}, 1238.22 cm^{-1}, 1631.17 cm^{-1}, 1601.62 cm^{-1}, 1015.14 cm^{-1} respectively(Fig 4.5). This data suggests that majority of the peaks were remained same , only slight shift in some peaks were observed that may be due to dipole moment of a bond as the interaction between drug and protein may be electrostatic.

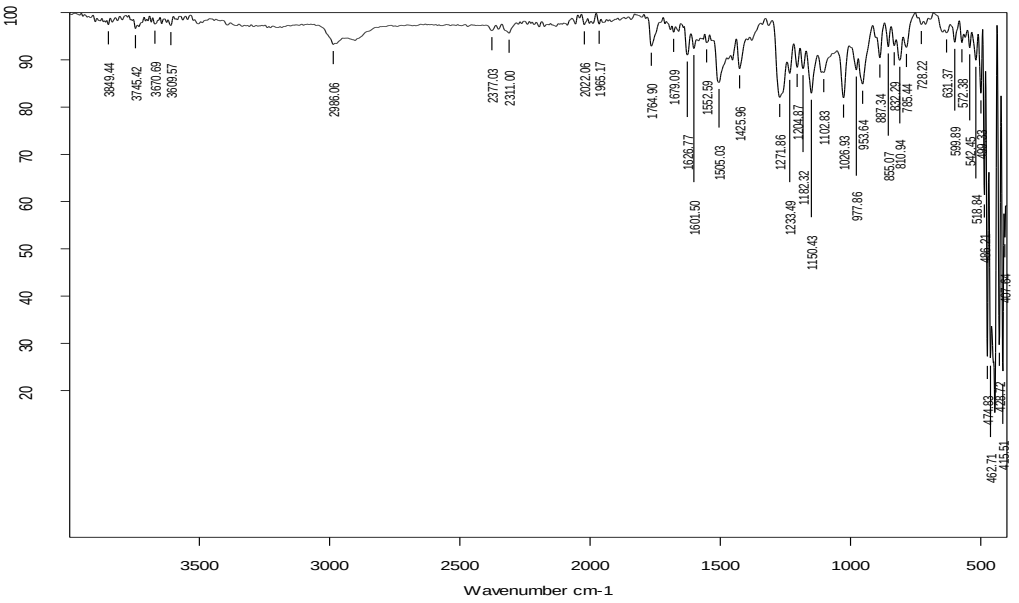
A. Soluble TFV:



B. TNPs:



C. Soluble (Curcumin+TFV):



D. TCNPs:

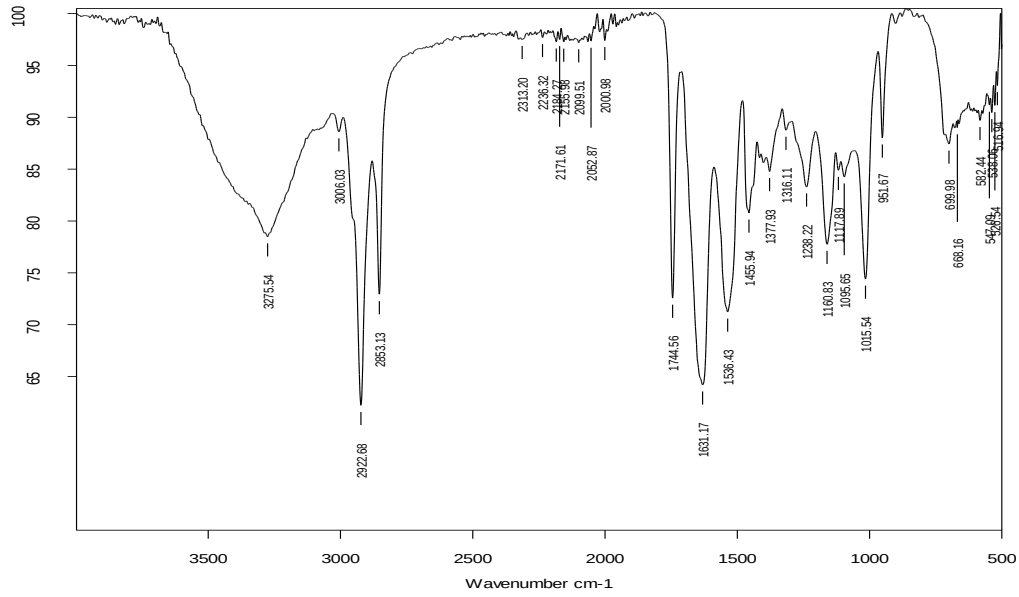


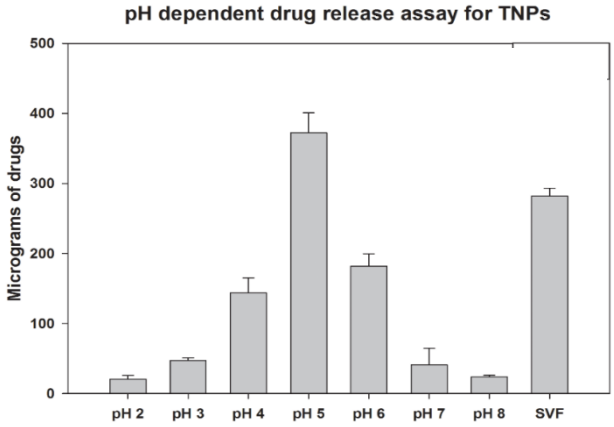
Fig 4.5: FT IR analysis:

NPs were lyophilized prior to scanning. The lyophilized NPs and soluble drug in powder form, both were scanned and data was collected using OPUS software. IR spectra run for samples: TFV and combination of Curcumin and TFV. These results suggest that there is no change in the functional groups of drugs.

pH-dependent drug release studies:

Drug release studies at different pHs revealed that highest drug release was found to be at pH5 followed by pH4 & 6 and a good amount of drug release was observed in a simulated vaginal fluid, (Fig 4.6). The above results suggest that drug-loaded NPs were stable at their corresponding pH and vaginal pH is optimum for the drug release.

A. TNPs:



B. TCNPs:

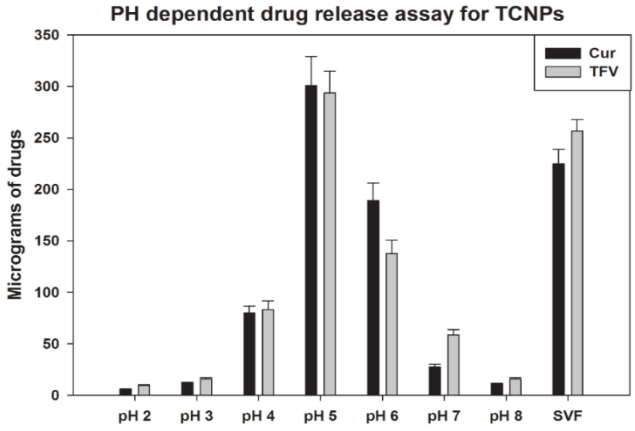


Fig 4.6: pH dependent release of NPs: pH dependent drug release assay for TNPs and TCNPs particles were incubated in the buffers of different pH, and simulated vaginal fluid. The release of TFV and curcumin was found to be maximum at pH-5 followed by pH-6 and pH-4. Optimum drug relese was observed at SVF, Each data points were repeated in triplicate (n = 3) and presented as Mean ± standard deviation (S.D).

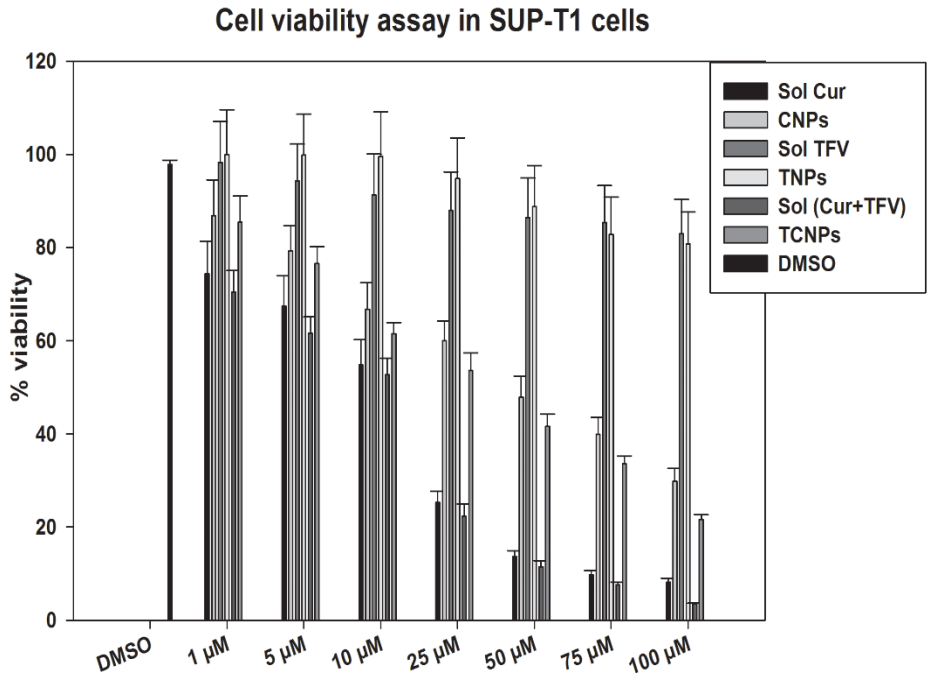
In-vitro studies:

Cell viability assay:

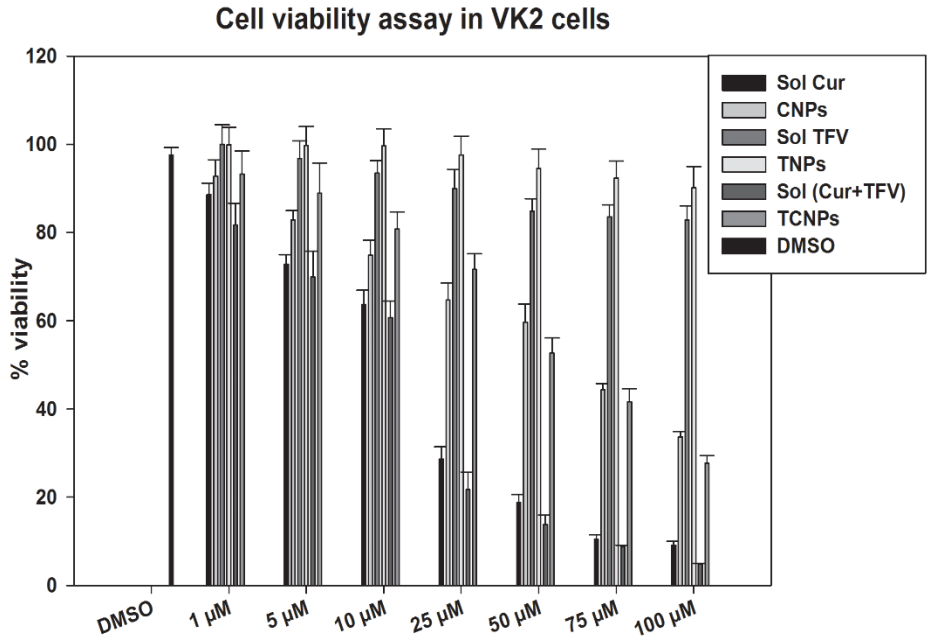
SUP-T1 cells, HL2/3 and VK2 cells (Fig 4.7) were incubated with a series of increasing concentrations (1, 5, 10, 25, 50 and 100 μM) of both soluble and nano formulations and cell viability was evaluated by MTT assay. Compared to soluble curcumin, CNPs were found to be less toxic at all concentrations. Soluble curcumin was highly cytotoxic even at higher concentration while CNPs at the same concentration was comparatively less cytotoxic.

The lower cytotoxic properties of CNPs highlights the perception that direct administration of soluble curcumin is lethal to cells at 10 μM concentrations and above. Both soluble TFV and TNPs formulations were found non-toxic at all concentrations. In combination drug treatment, soluble combination form showed more toxicity when compared to TCNPs.

A. SUPT1 cells:



B. VK2 cells:



C. HL2/3 cells:

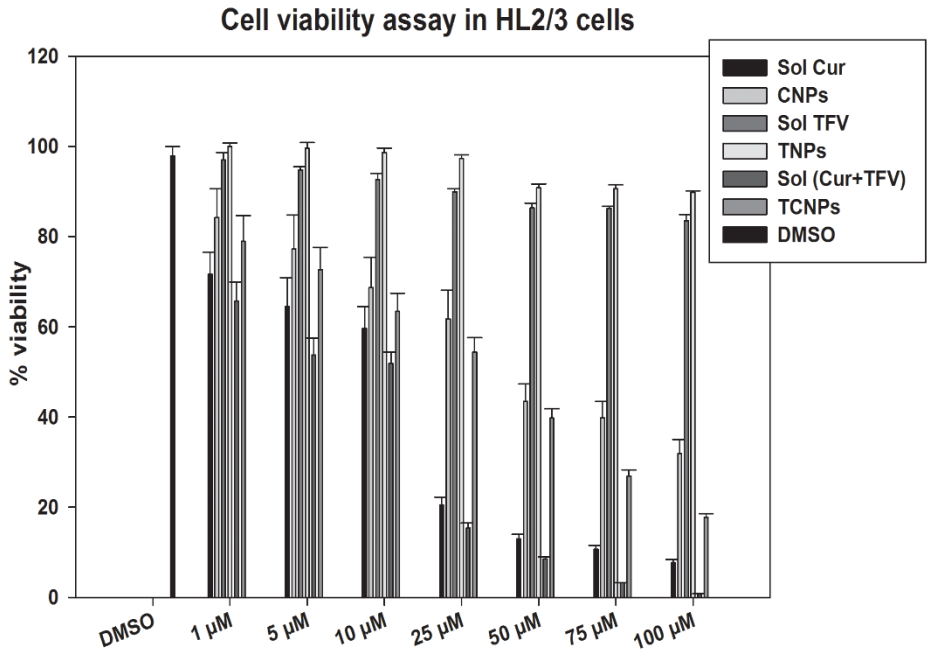


Fig 4.7: Cell viability assay: SUP-T1, VK2 and HL2/3cells were treated with a series of concentrations (1, 5, 10, 25, 50, 75 and 100 µM) of soluble curcumin, soluble TFV, soluble (Curcumin+TFV), CNPs, TNPs and TCNPs. Later the cells viability were tested a by MTT reagent and results were plotted.

Lactobacillus safety analysis by disc diffusion test:

Lactobacilli were incubated with increasing concentrations (1, 2 and 5 mg) of drugs both in soluble and nano forms of both curcumin, TFV individually and in combination. We did not find significant bacterial growth inhibition in all treated drugs either in soluble form or in nano form. These results suggested that soluble drugs and their nano formulation are safe to be a microbicide (Fig 4.8).

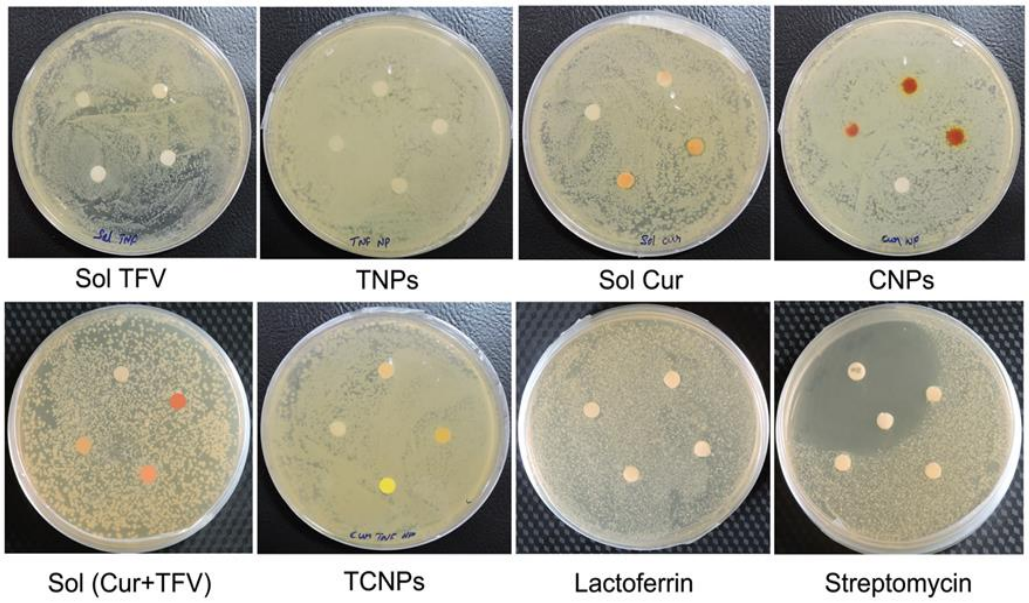


Fig 4.8: *Lactobacillus* viability assay: Soluble TFV, TNPs, Soluble curcumin, CNPs, Soluble (TFV + Curcumin), TCNPs and Lf were placed on disc with increased concentrations of 1 mg, 2 mg and 5 mg and one disc with negative control. In separate plate along with negative control, positive control Streptomycin 10 mcg was placed. Negligible bacterial growth inhibition was observed in both soluble and nano forms of drugs.

Sperms viability assay.

Microbicide having contraceptive property is an additional asset to any microbicidal formulation that to act as a multipurpose microbicide against HIV. Sperms (Fig 4.9) were incubated with increasing concentrations (10, 50, 100, 200, 300, 400, 500 and 600 μM) of the two formulations and cell viability was estimated by MTT assay. Both soluble curcumin and CNPs and combination formulations (TCNPs) were found to be non-toxic at very low concentrations. Greater than 200 μM concentration of curcumin was showed nearly 80% cytotoxic to sperms in both forms. This data suggests that these formulations can also act as a contraceptive due to its spermicidal activity of curcumin.

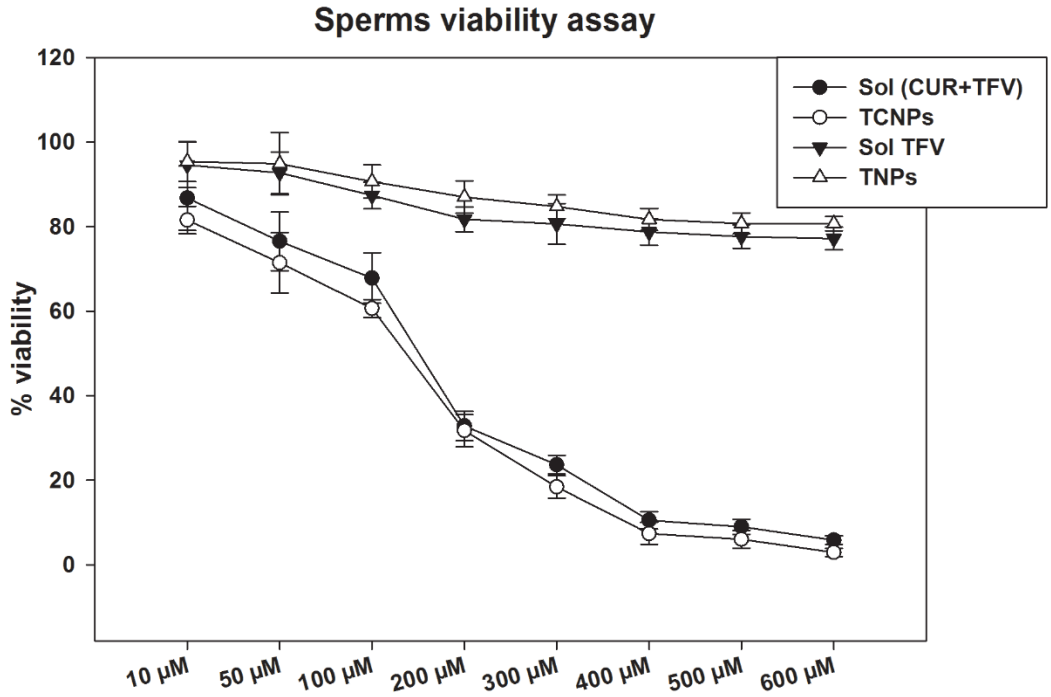


Fig 4.9: Sperms viability assay: Sperms were treated with increasing concentration (10, 50, 100, 200, 300, 400, 500 and 600 μM) of soluble TFV, soluble (Curcumin+TFV), CNPs, TNPs and TCNPs. Later the sperms viability were tested a by MTT reagent and results werd plotted.

Anti-HIV activity of NPs:

The in-vitro anti-HIV-1 activity of TCNPs was evaluated as individual and in combination for soluble and nano formulation. The individual IC_{50} for soluble curcumin (5.1 μM) and CNPs (1.75 μM), soluble TFV (4.78 μM) & TNPs (2.8 μM). Further, the anti-HIV activity of TCNPs have been found to be improved over its soluble formulation when used at 1:1, 0.5:0.5 and 0.25:0.25 while no significant advantage is observed at 0.1:0.1 and 0.05:0.05. The percent HIV-1 replication vs. drug combination ratio have been plotted (Fig 4.10). The Anti-HIV activity in a combination of curcumin + TFV are found to be improved or intact even after 50% reduction in dose. The antiviral activity was improved in combination nano form, so reducing the dose frequency and prevent unwanted side effects and toxicities.

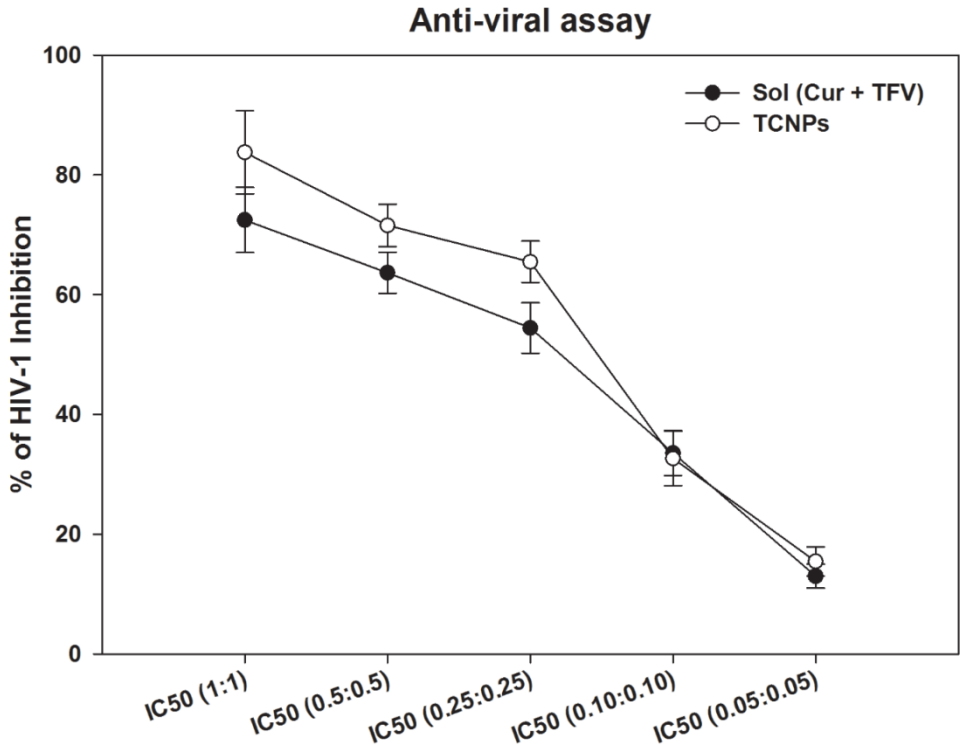


Fig 4.10: Anti-viral assay: Various different IC_{50} ratios of curcumin and TFV drugs were mixed in soluble and nano form. Later the antivral assay were performed and plotted.

***In-vivo* studies:**

Pharmacokinetic studies in vaginal lavage:

To evaluate the pharmacokinetic parameters, both soluble and nano combination forms of drugs were measured separately in vaginal lavage after administration of single dose. Drug levels were observed at nine different time points of post application (0.5, 1, 1.5, 2, 2.5, 4, 6, 12 & 24 hrs). PK profile was enhanced in nano treated group when compared to the soluble counterpart (Table 4.1). Three time's increase in AUMC and two times in AUC was observed from both drugs in nano form. Likewise greater than two-fold increase in T_{max} and $t_{1/2}$ in nano treated group. These results suggest that higher bioavailability of drugs at the site of application when TCNPs was given (Fig 4.11).

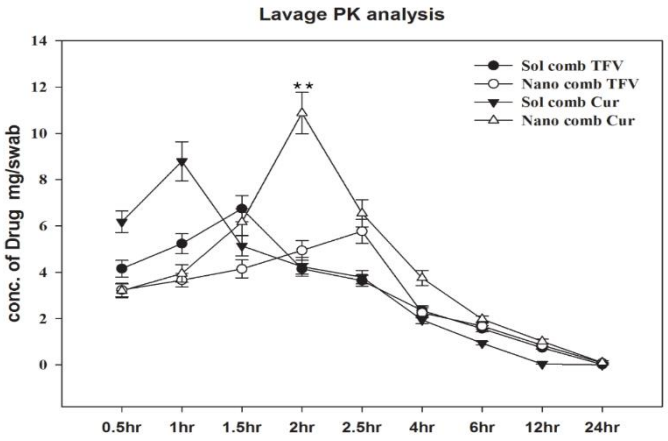


Fig 4.11: Pharmacokinetic study result in vaginal lavage: 20 mg of soluble curcumin plus 10 mg of soluble TFV and an equivalent amount of TCNPs were applied single dose in vagina for indicated time points. Lavages were collected after these time points and curcumin and TFV concentration were calculated separately. Abbreviation: Sol comb Cur and Sol comb TFV represents Curcumin and TFV drugs released from soluble (Curcumin+TFV). Nano comb Cur and Nano comb TFV represents Curcumin and TFV drugs released from TCNPs. Sample data were recorded as Mean ± S.D, n=3. The difference between two groups was calculated by using student's t-test and the value of significance expressed as ***P<0.0005, **P< 0.005, *P< 0.05.

Table 4.1: Pharmacokinetic profile in vaginal lavage

Parameters	Units	TFV		Curcumin	
		Nano	Soluble	Nano	Soluble
AUC	(h)*(mg/ml)	30.7092	28.236	40.642	21.6729
AUMC	(h)^2*(mg/ml)	192.867	141.632	239.94	57.2804
C_{max}	mg/ml	5.7671	6.7435	10.8735	8.7888
T_{max}	hr	2.5	1.5	2	1
$t_{1/2}$	hr	4.22595	2.8367	3.95154	1.78426

Values in the parenthesis designate the concentration of drugs in milligrams per swab.

Pharmacokinetic parameters.

AUC: The integral of the concentration-time curve (after a single dose or in steady state).

AUMC: Partial area under the moment curve between t start and t end.

C_{max}: The peak plasma concentration of a drug after vaginal administration.

T_{max}: Time to reach C_{max}.

$t_{1/2}$: The time required for the concentration of the drug to reach half of its original value.

In-vivo time course experiment:

To evaluate the extent of availability of drugs over a period of long time, time course study was performed in i*n-vivo*. The time-dependence was studied individually for the two drugs curcumin and TFV delivered in TCNPs. Results were plotted (Fig 4.12), it represents the concentration of curcumin and TFV in vaginal lavage at various time points, with a lag period of 1 hr (for soluble Curcumin), 1.5 hr (for soluble TFV), 2 hr (nano form of Curcumin) and 2.5 hr (nano form of TFV) hours of lag phase of after TCNPs administration. The results suggest that high concentration of drugs in the initial stage for both the soluble and nano form, which got reduced later exponentially. The nano formulations of drugs even after 2 hr and 2.5 hr of lag phase showed sustained and significant release of the drug up to 12 hr whereas both the drugs in the soluble form, showed a fast reduction (at 4 hr) in the concentration. We have indeed detected the presence TFV and curcumin up to 8–12 hr when drugs were given in nano-formulation but in case of the soluble form, the drugs were eliminated in 4–6 hrs.

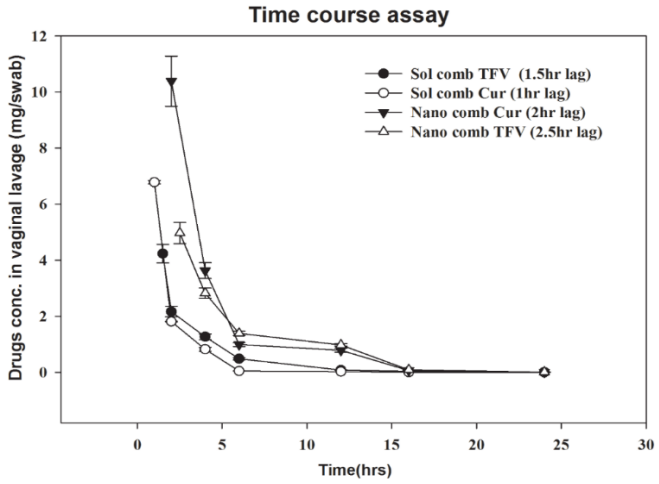


Fig 4.12: Drug release kinetic assay: 20 mg of soluble curcumin plus 10 mg of soluble TFV and an equivalent amount of TCNPs were applied topically to the rat vagina. Lavages were collected after above mentioned time points and Curcumin and TFV concentration were calculated separately. Abbreviation: Sol comb Cur and Sol comb TFV represents Curcumin and TFV drugs released from soluble (Curcumin+TFV). Nano comb Cur and Nano comb TFV represents Curcumin and TFV drugs released from TCNPs.

Dose-dependent toxicity and Bioavailability studies:

In Vaginal lavage:

After intravaginal application with fixed doses as mentioned in methods, vaginal lavage was collected after respective time points. Drugs (Curcumin & TFV) from both the formulations such as soluble and nano forms were measured and plotted (Fig 4.13). Results suggest that drugs from nano formulation both in a single form and in combination form showed 1.4 to 2.7 fold more availability of drugs at the applied site in a dose-dependent manner compared to soluble counterpart.

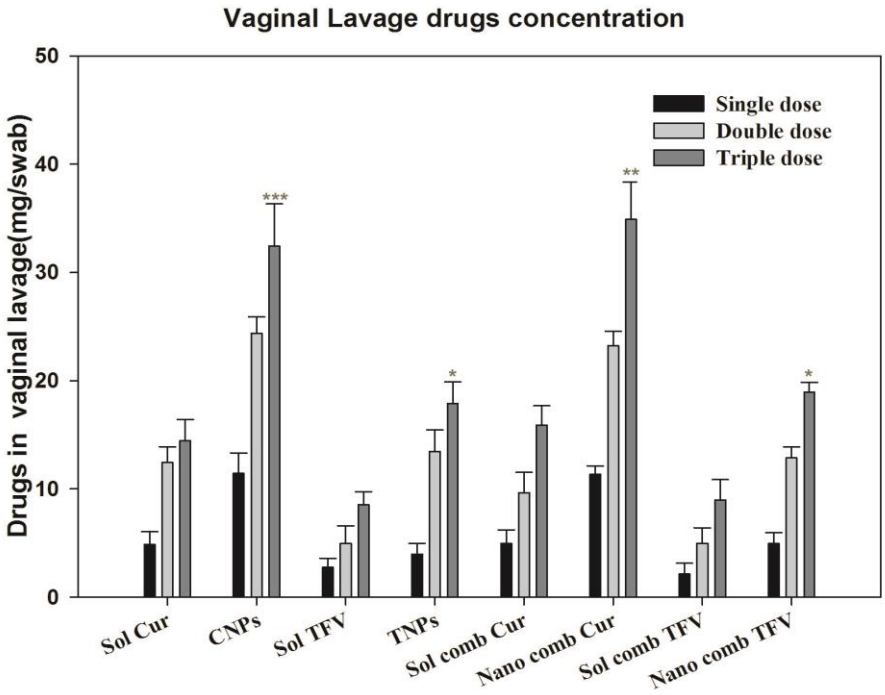
In Vaginal tissue:

Drugs from both the formulations such as soluble and nano forms were extracted from the homogenized vaginal tissue. Extracted drugs were estimated and plotted (Fig 4.13). Here drug concentrations were found to be in micrograms that are thousand times less than the vaginal lavage. In the case of TCNPs we found there was 1 to 2.4 times lower drug concentrations as compared to soluble counterpart. Whereas individual NPs didn't show significant variation in drugs exposure as compared to soluble counterpart.

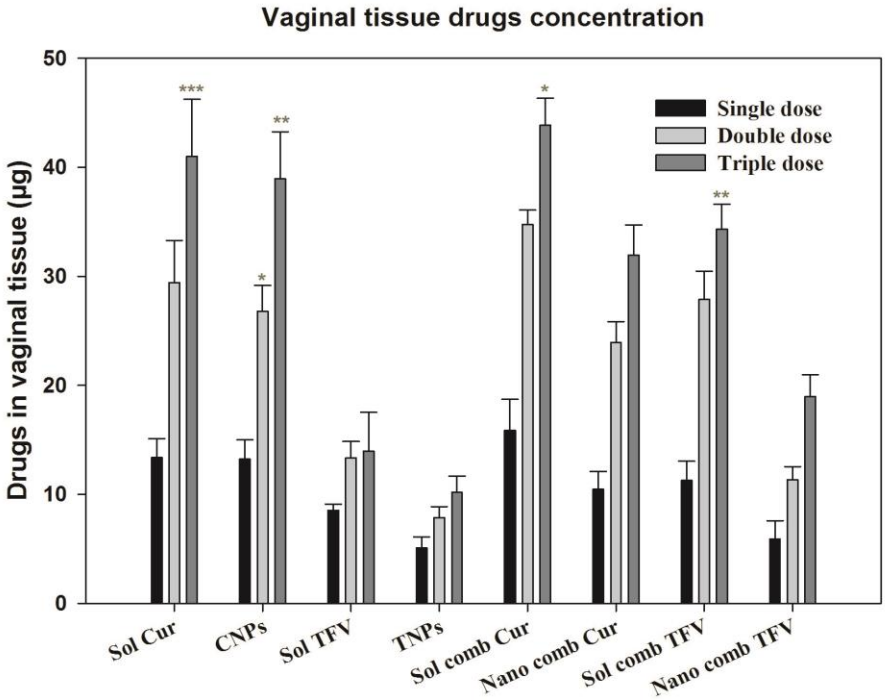
In systemic level:

Drugs from both the formulations such as soluble and nano forms were measured in the systemic circulation and plotted (Fig 4.13). Here results showed that 1.6 to 5.2 times high accumulation of drugs from soluble form when compared to the nano counterpart; it causes more organ-related toxicity in case of soluble formulation. But in TCNPs treated at the third dose, curcumin and TFV were found to be approx. 34 ng/ml & 21 ng/ml where as in soluble form 46 ng/ml and 40 ng/ml respectively it suggests that even at higher doses also the NPs reaching into the systemic circulation is less and it could be noticeable as safe.

A. Vaginal lavage:



B. Vaginal tissue:



C. Systemic Bioavailability:

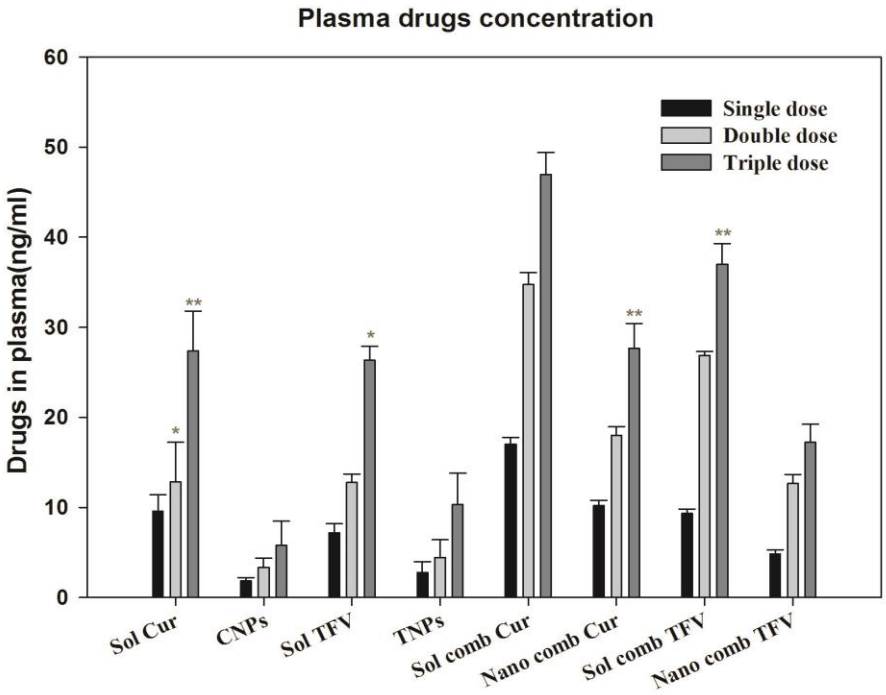


Fig 4.13: Bio availability studies in rats: Dose dependent toxicity study of drugs in single form or combination form either in soluble (TFV + Curcumin) and TCNPs were performed. The concentration of drugs in vaginal lavage, vaginal tissue and plasma were analyzed and plotted. Sample data were recorded as Mean ± S.D, n=3. The difference between groups was calculated using one-way analysis of variance and the value of significance expressed as ***P<0.0005, **P< 0.005, *P< 0.05.

Histopathological analysis:

H&E study of cervicovaginal epithelia showed that, the integrity of tissue was damaged in a dose dependent manner when the combination of soluble drugs was used. Where as in TCNPs treated vaginal epithelia showed less toxicity in a dose-dependent manner. This way TCNPs confirms that even at higher doses, this formulation could be marked as safe (Fig 4.14).

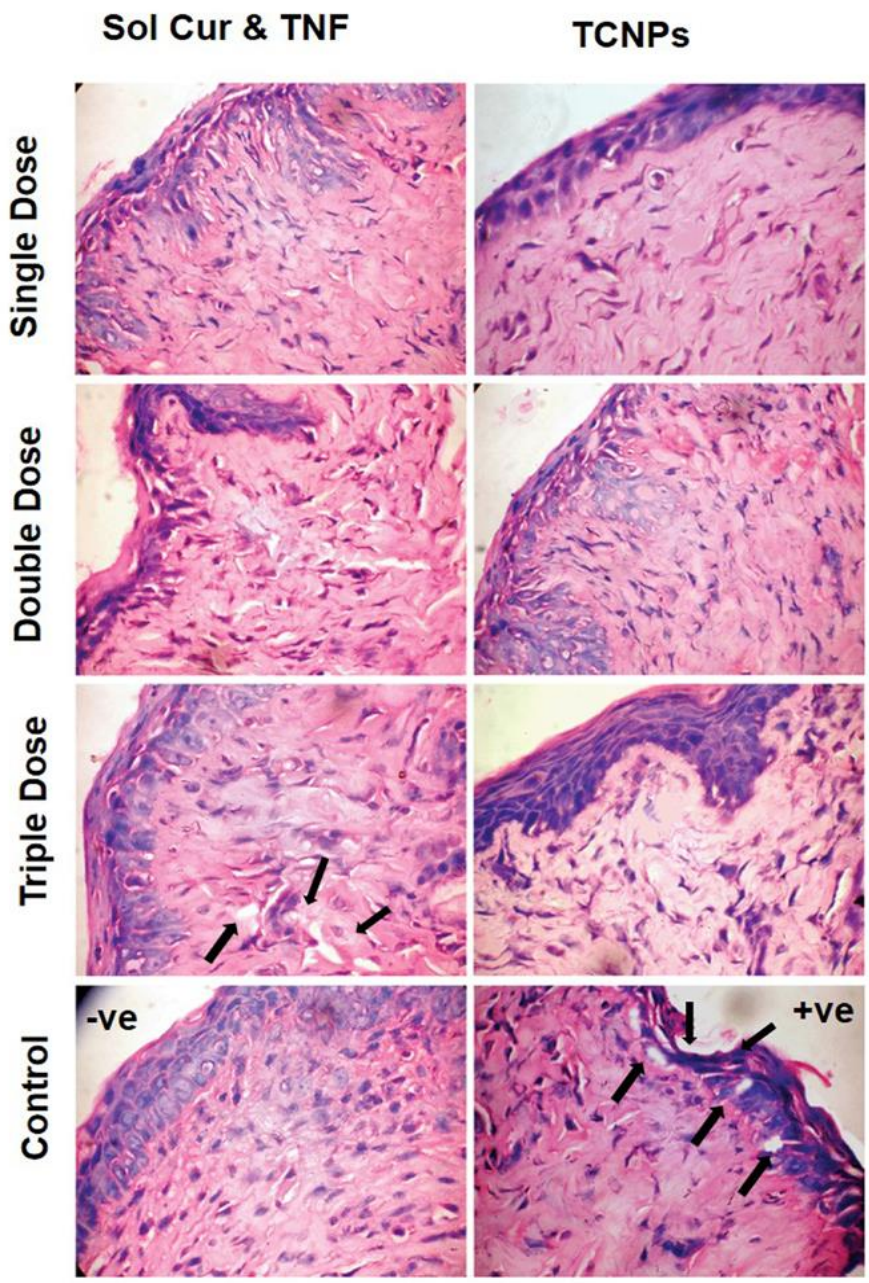
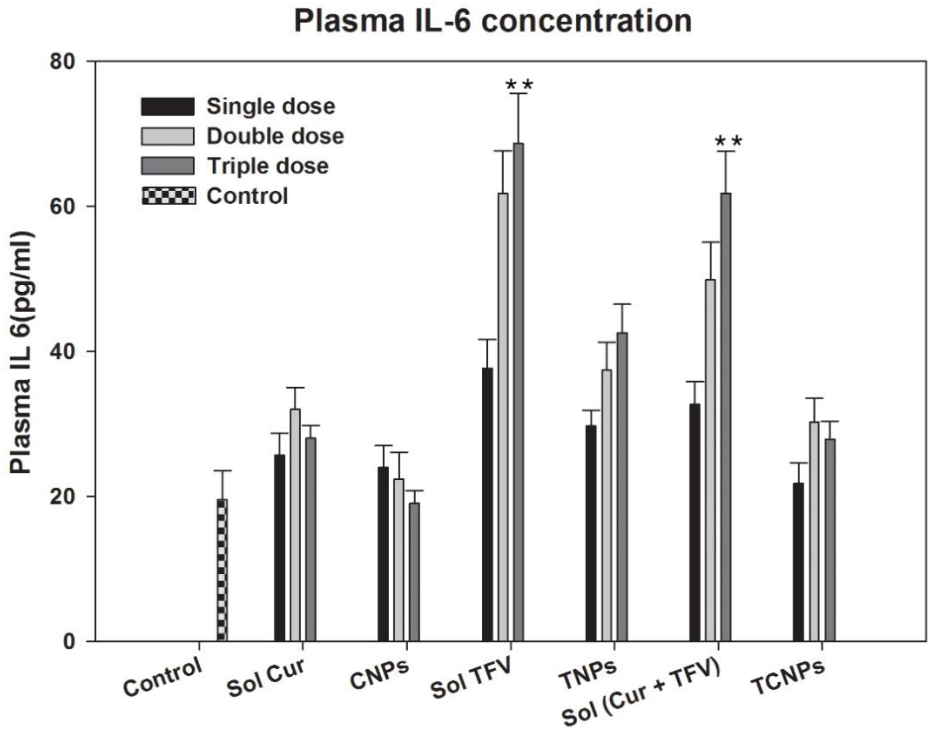


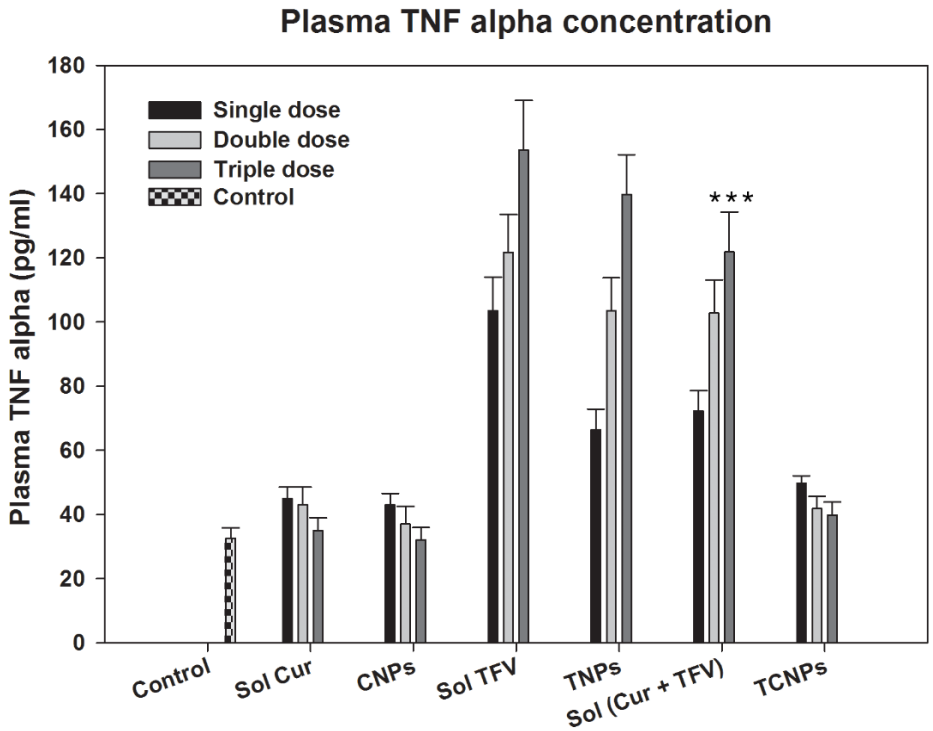
Fig 4.14: Histopathology: Right panel represent the vaginal epithelia treated with TCNPs for single, double and triple dose respectively and positive control. Left panel represent the vaginal tissue integrity when applied topically with soluble (Curcumin+ TFV) for single, double and triple dose respectively and negative control. Damaged site or lesions were shown through a printing arrow. Negative and positive controls were treated with PBS (pH-7.4) and nonoxynol-9 (10 mg/kg) respectively.

Systemic and vaginal Pro-inflammatory cytokine response:

The level of Inflammation has checked by estimating the Pro-inflammatory cytokine markers such as interlukine-6 (IL-6) and tumor necrosis factor alpha (TNF-alpha) from the isolated plasma and vaginal tissue. Soluble and nano form of TFV showed that IL-6 levels were increased by 3 fold. But in TCNPs treated, the IL-6 levels were measured as 2.6 times less when compared to soluble counterpart and 3 times less to a single form. At the third dose of TCNPs we couldn't detect any inflammation as compared to its soluble counterpart. But in soluble combination leads to more than three time's increase of IL-6 both in the vagina as well as in plasma. TNF-alpha is a cell signaling protein that involved in systemic inflammation. The rise in TNF-α level was significantly less, as similar to IL-6 in the case of TCNPs and CNPs at the third dose. (Fig 4.15).

A. Plasma:


Plasma IL-6 concentration
Plasma IL 6(pg/ml)
Single dose
Double dose
Triple dose
Control
**
**
0
20
40
60
80
Control
Sol Cur
CNPs
Sol TFV
TNPs
Sol (Cur + TFV)
TCNPs


Plasma TNF alpha concentration
Plasma TNF alpha (pg/ml)
Single dose
Double dose
Triple dose
Control
***
0
20
40
60
80
100
120
140
160
180
Control
Sol Cur
CNPs
Sol TFV
TNPs
Sol (Cur + TFV)
TCNPs

B. Vaginal tissue:

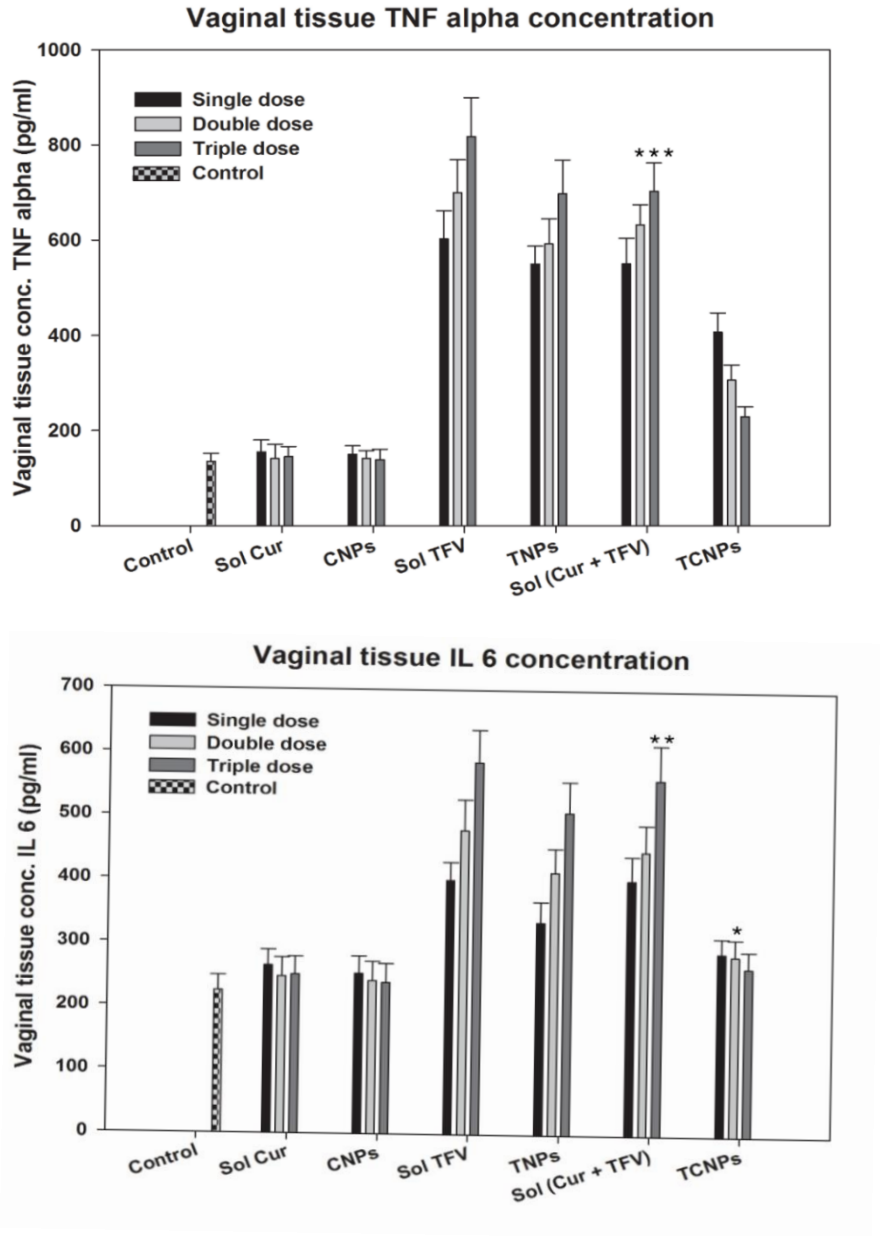


Fig 4.15: Proinflammatory cytokine response: Soluble TFV, Soluble Curcumin, Soluble (Curcumin+TFV), CNPs, TNPs and TCNPs were applied topically in vagina. IL-6 and TNF alpha levels in plasma and vaginal tissue were analyzed and plotted. Sample data were recorded as Mean ± S.D, n=3. The difference between groups was calculated using one-way analysis of variance and the value of significance compared with control and expressed as ***P<0.0005, **P< 0.005, *P< 0.05.

Discussion:

TFV is a proven microbicide when administered topically as a vaginal gel and as a NPs formulation. To improve its safety and efficacy, TFV was combined with the Curcumin in microbicidal formulations. The combination of two active molecules leading to a novel class of microbicides with improved biological activity profile. Curcumin has been shown to possess spermicidal, microbicidal & anti-inflammatory properties and TFV has reverse transcriptase inhibitor activity and also acts as a microbicide. Therefore introducing curcumin and TFV loaded Lf NPs in a single chemical entity could yield compounds with an anti-inflammatory, spermicidal and as well as anti-HIV activity. Apart from this, the use of Lf as a carrier provides protection to the vaginal flora that maintain low acidic environment supporting the growth of *Lactobacillus* species [163] and also has a wide variety of biological functions normally associated with the body's defense [164], such as immunomodulatory, antitumor, antioxidant and antimicrobial effects [101, 165]. Mixture design of experiments and theory was used to define a range of NPs compositions with varying rheological properties and to assess *in-vitro* drug release and tissue retention [166].

The present investigation was focused on exploring the potential of curcumin & TFV drug loaded Lf NPs for control release and safety. For successful vaginal delivery, the developed NPs should preferably have small particle size and ability to rapidly penetrate through vaginal mucus in order to deliver the ARV drug to the vaginal epithelium. We successfully developed 60-80 nm in dry form and with hydrodynamic radii of 110-120 nm of TFV loaded bovine Lf NPs, can show greater penetrability deep into vaginal epithelium as well as into the vaginal tissue. Drug loaded NPs released the maximum amount of their respective drug at pH 5 and PH 6. An impressive controlled drug release was observed with time in simulated vaginal fluid which mimics the conditions of the vaginal environment. Sperm viability assay proved that curcumin has effective spermicidal activity at the concentration of 200 μM onwards; hence this formulation can act as a contraceptive along with microbicide action. *Lactobacilli* predominantly present in the healthy woman vagina, which acts as a gatekeeper by playing an important role in protecting the woman

from urogenital infections (45-47). Here in this formulation drugs either in soluble form or in nano form showed negligible growth inhibition to *Lactobacillus*, it suggests that this formulation acts as a safe microbicide without altering natural vaginal micro biota. Anti HIV-1 data suggests that, the individual drug nano-formulation is found to be more effective than its soluble form as specified by the reduced IC_{50} values. Furthermore, 50% of IC_{50} concentration of the drug combination curcumin + TFV is adequate to confer anti-HIV activity of the corresponding soluble combination. This result suggests that even half amount of drug is sufficient to target HIV-1 leads to the reducing dose frequency and prevent unwanted side effects and toxicities. Based on all these properties, this formulation provides an effective anti HIV-1 environment in the vaginal area owing to its triple action. Even though the drug combination may be effective in soluble form, curcumin and TFV are not soluble in water. The NPs formulation was highly qualified to overcome these solubility limitations. These NPs shows triple action against HIV and can release active form of loaded drugs to improve the efficacy of the medicine. All the pharmacokinetic parameters were comparatively increased in NPs treated rats compared to soluble treated rats. A better improvement has been observed in AUMC, 4.2 fold high in case of nano form of curcumin and 1.4 fold in case of TFV as compared to its respective soluble form and AUC values also increased 1.9 fold of curcumin and 1.1 fold high in case of TFV as compared to its respective soluble form. It suggests that more exposure of drugs to the vagina and directly related to the bioavailability of a drug, hence it leads to a reduction in the dose frequency to the patient. T_{max} was 2 times in nano form of curcumin and for nano form of TFV was observed that 1.7 times more than that of respective soluble form. $t_{1/2}$ also increased 1.5 times in Nano form of TFV and 2.21 times in nano form of curcumin when compared to respective soluble form. Through this, we can say NPs could give protection for longtime due to its increased half-life and also show, slow and sustain release of drugs through NPs when compared to its soluble form. Time course analysis of curcumin and TFV in vaginal lavage was done to study the drug presence over a time period after administrating the drug topically in the vagina. The results found that up to 4 hrs, a significant amount of drug was observed in soluble treated rats whereas, in NPs

treated rats the concentration was found to be four times higher than soluble. The above formulation shows sustain and controlled drug delivery in the vaginal environment without causing any damage to the vaginal epithelium when compared to the soluble treated. In this present study, TCNPs has shown greater bioavailability than soluble forms when administered topically in rat vagina. Three different doses of soluble and nano form of curcumin (20 mg) and TFV (10 mg) was administered topically in three sets of Wistar rats and the drug presence in serum, vagina and vaginal lavage was analyzed by HPLC. Results showed that in the serum of nano drugs treated rats, both drugs were found in less concentration (nanograms) compared to soluble treated drugs. This suggested a less chance of systemic toxicity in nano formulation treated rats. In vaginal tissue, the amount of drug was observed in micrograms for both the forms. However, this amount of drug did not cause any tissue damage in nano formulation treated rats compared to soluble treated rats as shown by histopathological tissue sections. But in vaginal lavage amount of drug was found in milligrams, the comparatively high amount of drugs were observed in nano treated rats than soluble treated rats. The presence of higher concentrations of the drugs in vaginal lavage improves their availability for action. After treatment with NPs, serum and vaginal lavage were analyzed for any immunological response by measuring inflammatory markers IL-6 and TNF-alpha. We found a less inflammatory response in NPs treated group compared to soluble treated group. This might be due to the presence of having the anti-inflammatory nature of curcumin and Lf. Hence in NPs treated group the major inflammation was caused by TFV was neutralized by the presence of Lf together with curcumin. These NPs also provide better safety than the soluble forms while protecting against HIV-1.

Conclusion:

In this study, by using sol-oil method curcumin and TFV were successfully encapsulated and used as a controlled delivery system through vaginal administration as a microbicide by showing a synergistic effect. pH-dependent drug release studies showed that vaginal pH is favorable for drug release. In summary, curcumin and TFV loaded Lf NPs serves as a highly effective and efficacious microbicide formulation with a contraceptive asset.

Chapter 5

Development of Curcumin and Dapivirine (DPV) Loaded Lactoferrin Nanoparticles as a Microbicidal Formulation.

Introduction:

DPV is a non-nucleoside reverse transcriptase inhibitor; currently it is one of the active and potent microbicide against HIV-1 sexual transmission. DPV acts noncompetitively and binds hydrophobic binding pocket of HIV reverse transcriptase and blocks HIV activity [167]. Anti HIV activity of DPV was proved by using various cell lines in *in-vitro* and as well in a mouse model [168,169]. DPV has been formulated into a vaginal gel, vaginal ring, polymeric film, and nanoparticle delivery system [170]. As DPV in gel form is able to achieve remarkably higher levels in vaginal fluids and tissues upon vaginal administration, was proved by available animal and human data on pharmacokinetics [171-173]. Even it shows the higher levels, after administration of gel form of DPV, it is observed that a DPV concentration decreases rapidly. This leads to significant loss in efficiency, thus reduce protection which limits the development of coitally-independent microbicides. Later vaginal rings were developed to overcome and to answer the above problems [174,175]. Single-use microbicides and all usage patterns are most fonded over other products requiring continuous presence in the vagina because the above prolonged-release delivery methods may not fit to the patients [176]. Pharmacokinetics of microbicide drugs of any formulation has to deal with systemic exposure is another vital aspect, In order to avoid toxicity and evade potential viral resistance issues low systemic drug levels seem to be favored [177,178]. Now a day's nanotechnology based drug delivery systems are showing increasing interest as effective carriers for the vaginal delivery systems of microbicides. These nano systems may effectively help to overcome the solubility and stability issues of hydrophobic active drugs, Modulate drug release, increased mucosal drug distribution and retention, increase tissue penetration, and allow cell targeting [159, 179]. However, the true impact of nano mediated delivery in the fate of anti-HIV microbicide drugs is still poorly recognized.

Curcumin has broad spectrum of antiviral activity against different viruses along with other biological functions. It is an anti HIV compound, it can inhibit HIV virus by following ways, and those are Inhibition of HIV-1 LTR-directed gene expression

[180], Tat-mediated transactivation of HIV-1LTR [181], HIV-1 and HIV-2 proteases [182], HIV-1 integrase [183] and tat protein acetylation [184].

To prevent sexual transmission of HIV-1, ARVs in combination with Curcumin as a microbicides may lead to additive or synergistic effects that could enhance the ability of a vaginal or rectal product. These synergistic effects leads to an improved barrier to infection, overcoming of resistance issues, and lessening of the required dose of each drug which could also reduce the potential for toxicity.

Results:

Physicochemical Testing & characterization of NP:

Microscopic analysis:

FE-SEM: Freshly prepared nanoparticles (DNPs and DCNPs) were analyzed using FE-SEM (Fig 5.1). Particles were well dispersed with diameter of the drug loaded NPs were in the range of 55-7 0nm.

DNPs: **DCNPs:**

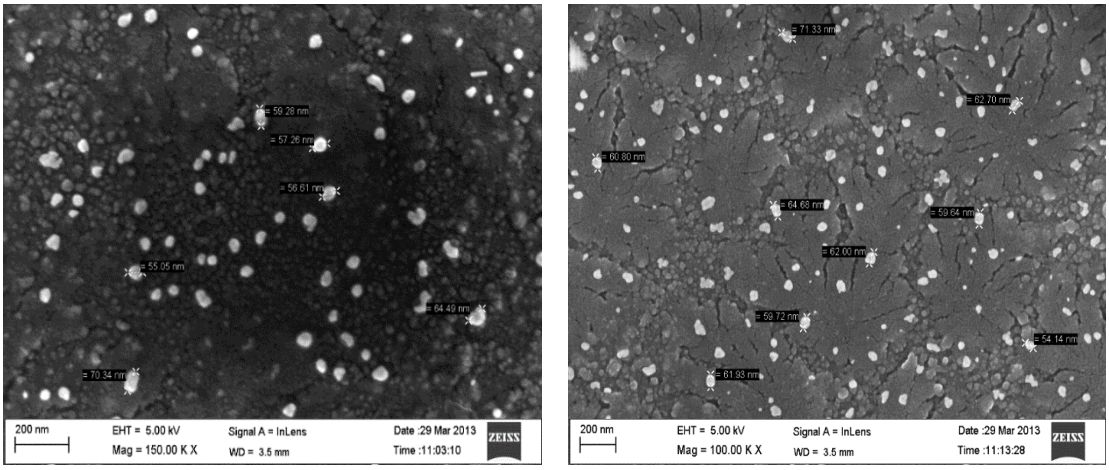


Fig 5.1: FE-SEM analysis of NPs: DNPs and DCNPs were analyzed by using the Field Emission Scanning Electron Microscope (FE-SEM). The range of diameter for DNPs and DCNPs were found to be in the range of 55-70 nm.

TEM analysis: The diameter of the drug loaded NPs were found to be approximately 60-80 nm (Fig 5.2).

DNPs: **DCNPs:**

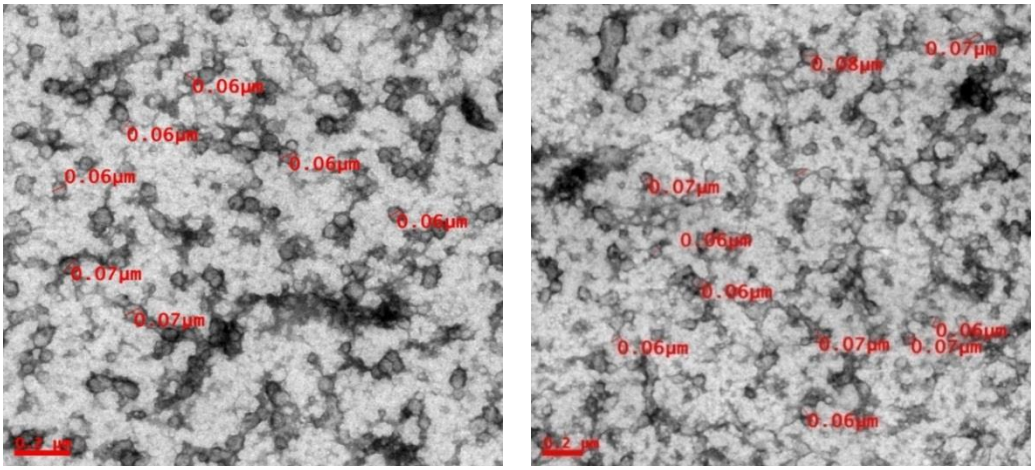


Fig 5.2: TEM analysis: DNPs and DCNPs were analyzed by using the Transmission Electron Microscope. The range of diameter for DNPs and DCNPs were found to be 60-80 nm.

AFM analysis: The size and surface morphology of NPs were analyzed using AFM (Fig 5.3). The diameter of the drug loaded NPs were found to be approximately 50-60 nm with a smooth surface.

DNPs: **DCNPs:**

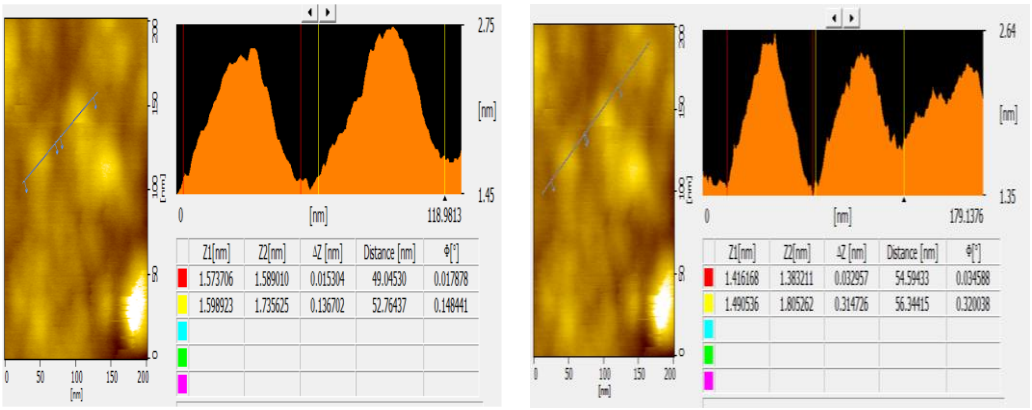


Fig 5.3: AFM analysis: DNPs and DCNPs were also analyzed by using the Atomic Force Microscope. The average diameter for DNPs and DCNPs were found to be 50-60 nm in range.

DLS analysis of NPs:

Hydrodynamic size of NPs was determined by DLS analysis. The size of DNPs and DCNPs are 102 nm and 116 nm respectively (Fig 5.4). These results showed that increased in particle size when compared to above microscopic analysis data. This may be due to the presence of hydrodynamic shell around the particle. Zeta potential and PDI values of prepared NPs were found in the range of -26 and -24 and 0.304 and 0.452 respectively.

DNPs: **DCNPs:**

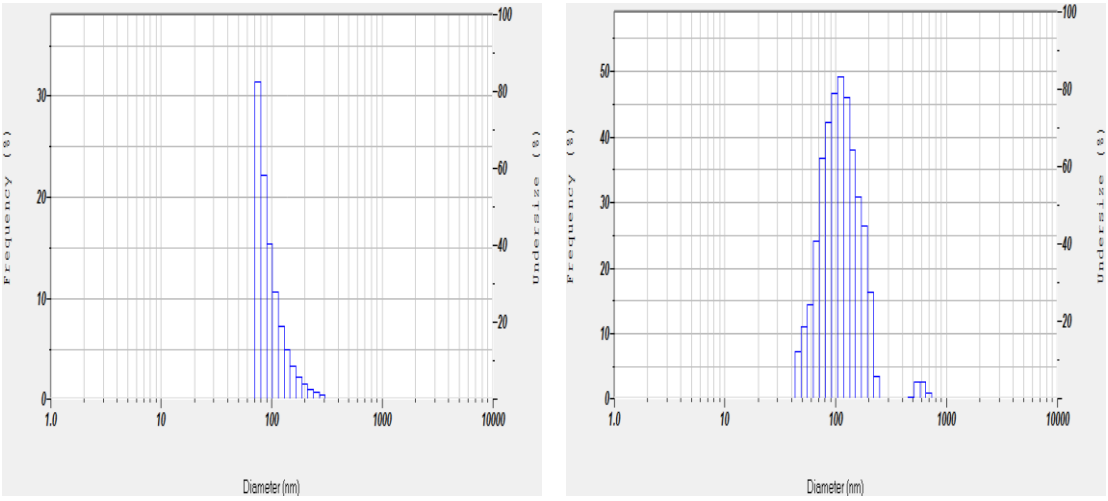


Fig 5.4: DLS analysis: DLS analysis was performed for the measurement of hydrodynamic radii. A mean size of DNPs and DCNPs 102 nm and 116 nm for drug loaded NPs were observed.

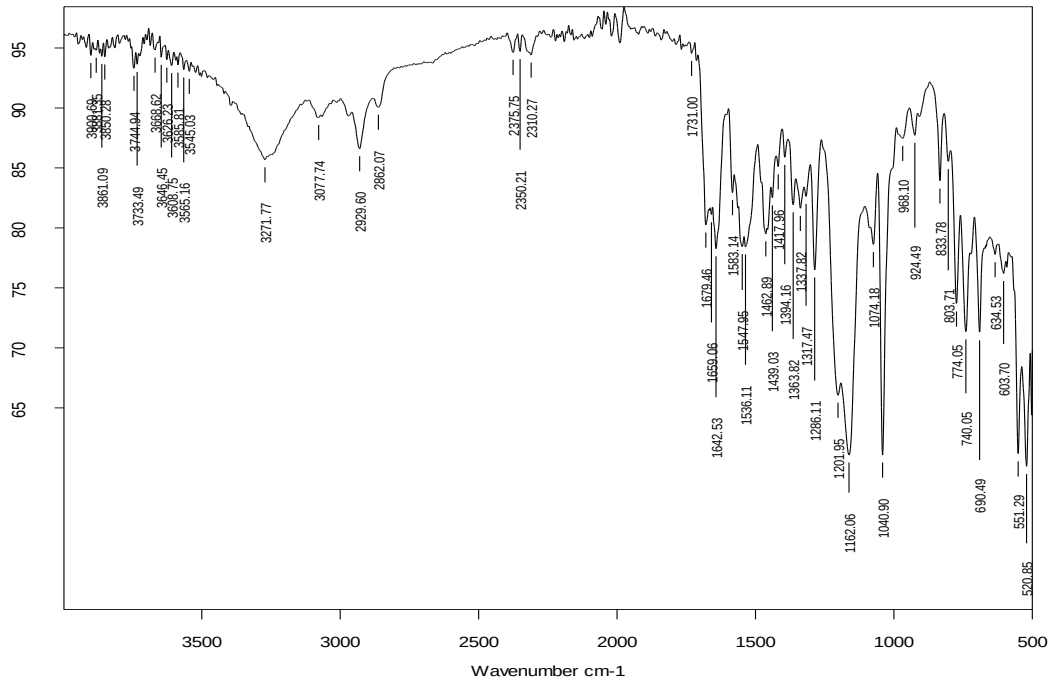
Encapsulation efficiency.

Encapsulation efficiencies were found to be, 61.6±1.6 %for DNPs and (Curcumin = 60.33±1.3%& DPV = 62.17±1.9%) respectively from DCNPs. This suggests that maximum amount of drugs has been encapsulated in protein.

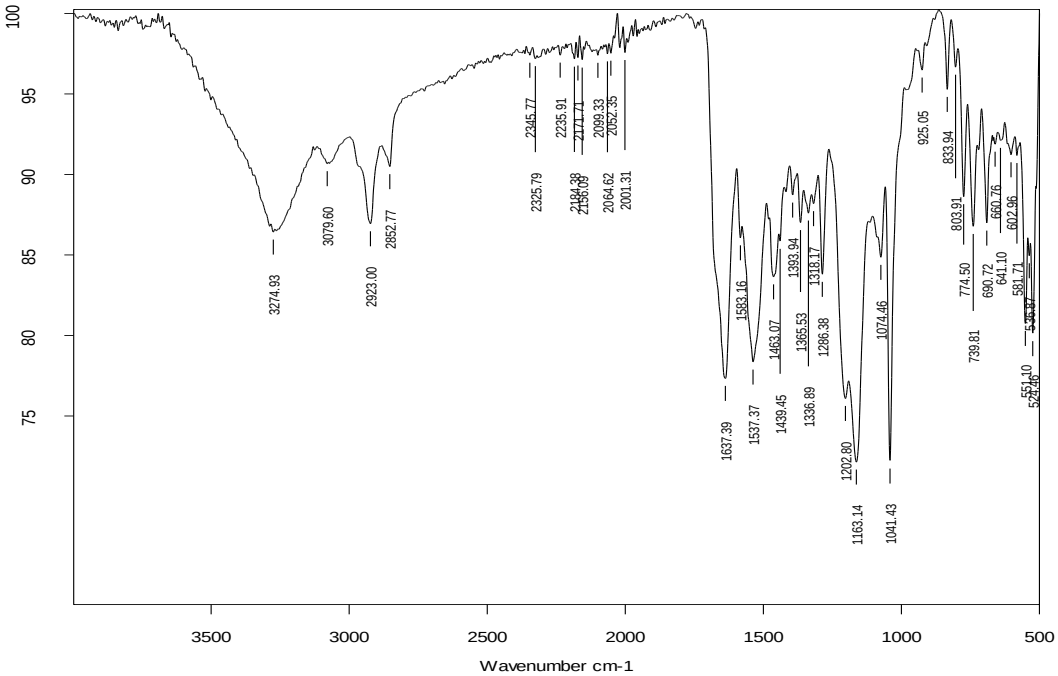
FT-IR analysis:

Fourier transform infrared spectroscopy (FT-IR) was used to assess potential interactions between protein and drug in nanoformulation. Spectra were run for Lf, Curcumin and DPV in both (soluble and nano) and single & combination forms. Comparative analysis was observed as follows, important peaks of soluble Lf; amide I bond is 1632.7 cm^{-1}, amide II bond is 1536.74 $cm^{-1}$and in nano form of Lf peaks were slightly shifted to 1641 cm^{-1} and 1538.32 cm^{-1} respectively. For soluble curcumin, C=O and C=C peak is 1626.20 cm^{-1}, aromatic ring peak is 1600.74 cm^{-1} and C-O –C peak is 1024.62 cm^{-1}, where as in nano curcumin these peaks are slightly shifted to 1626.50 cm^{-1}, 1601.62 cm^{-1}, 1025.13 cm^{-1} respectively. The IR spectrum of DPV was characterized by the absorption of N-H group at 3271.77 cm^{-1}(Soluble), 3274.93 cm^{-1} (nano) and C=N group at 2310.27 cm^{-1}(Soluble), 2325.79 cm^{-1} (nano). Combination of soluble curcumin and DPV of peaks are as follows; aromatic C–H stretch peak is 2920.71 cm^{-1}, medium stretch of C–N deformation pea is 1,282.84 cm^{-1}, C=O and C=C peak is 1631.18 cm^{-1}, aromatic ring peak is 1602.59 cm^{-1} and C-O –C peak is 1026.93 cm^{-1},N-H group at 3273.18 cm^{-1} and C=N group at 2309.93 $cm^{-1}$where as in nano form of DCNPs these peaks were slightly shifted to 2922.75 cm^{-1}, 1282.91cm^{-1}, 1631.08 cm^{-1}, 1602.87 cm^{-1}, 1041.10 $cm^{-1,}$ 3272.07 cm^{-1} and 2311.24cm^{-1}respectively (Fig 5.5). This data suggests that majority of the peaks were remained same , only slight shift in some peaks were observed that may be due to dipole moment of a bond as the interaction between drug and protein may be electrostatic.

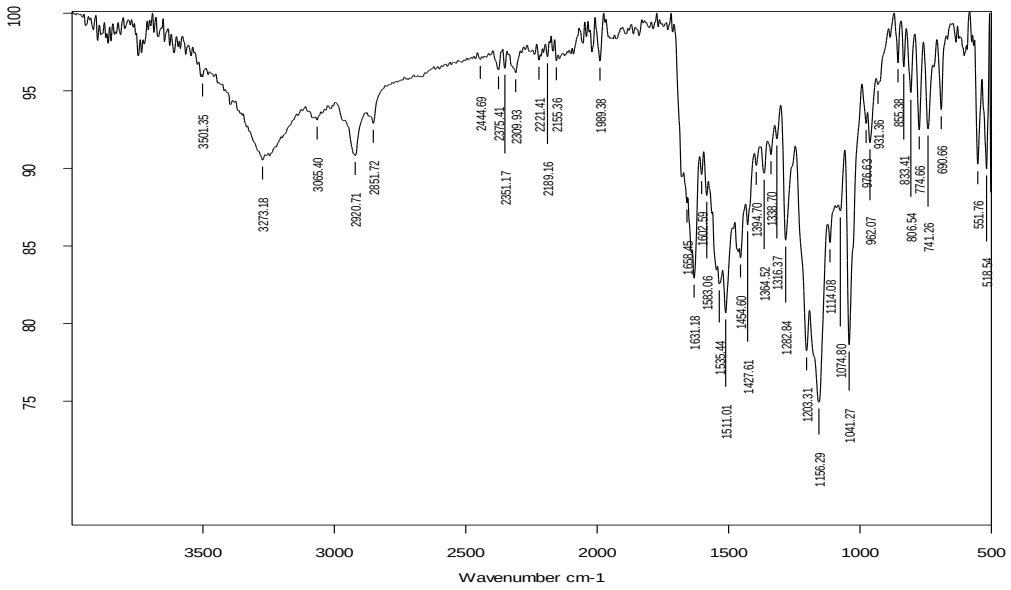
A. Soluble DPV:



B. DNPs:



C. Soluble (Curcumin+DPV):



D. DCNPs:

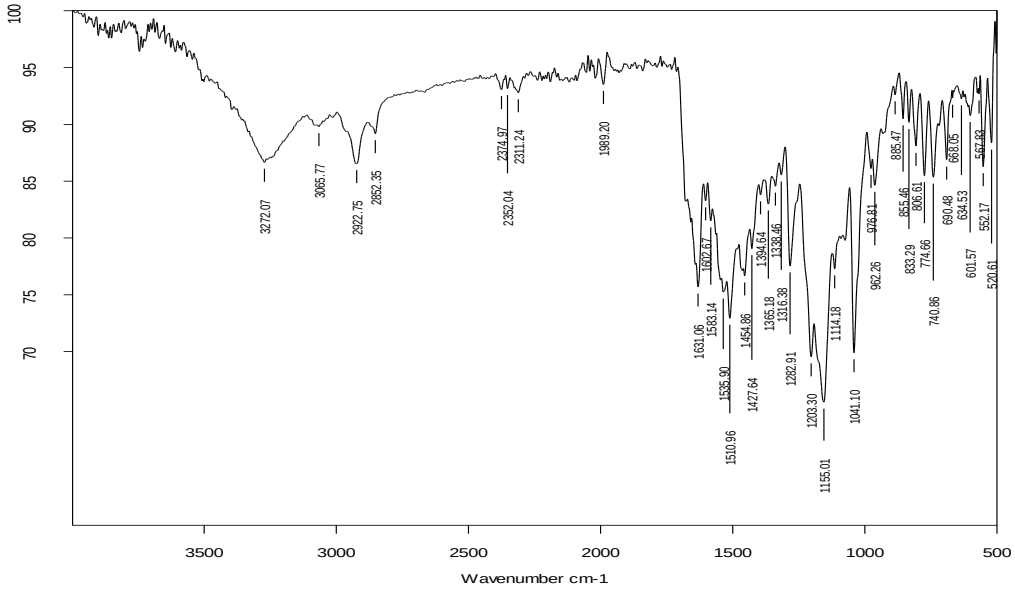
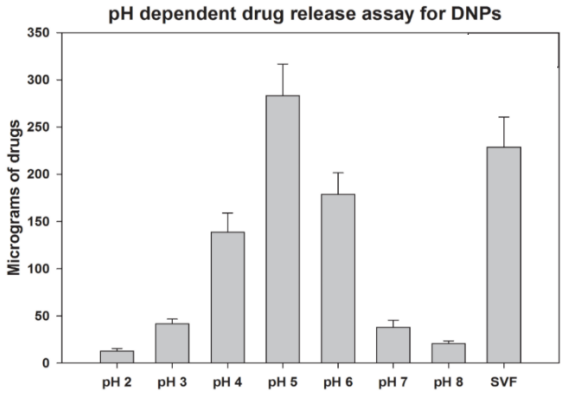


Fig 5.5: FT IR analysis: NPs were lyophilized prior to scanning. The lyophilized NPs and soluble drug in powder form, both were scanned and data was collected using OPUS software. IR spectra run for samples: DPV and combination of Curcumin and DPV. These results suggest that there is no change in the functional groups of the drugs.

pH-dependent drug release studies:

Drug release studies at different pHs revealed that highest drug release was found to be at pH-5 followed by pH4 & 6 and a significant amount of drug release was observed in a simulated vaginal fluid, (Fig 5.6). The above results suggest that DNPs and DCNPs were stable at their corresponding pH and vaginal pH provides optimum conditions for the drug release.

A. DNPs:



B. DCNPs:

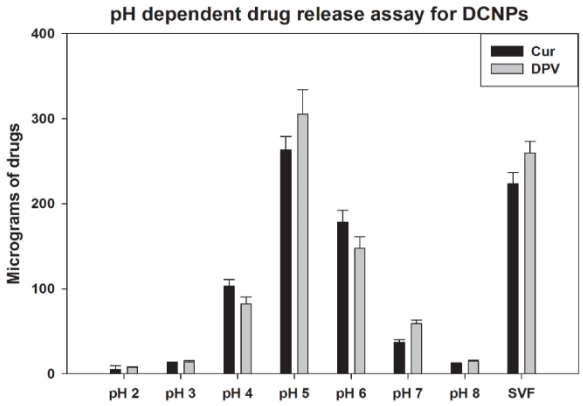


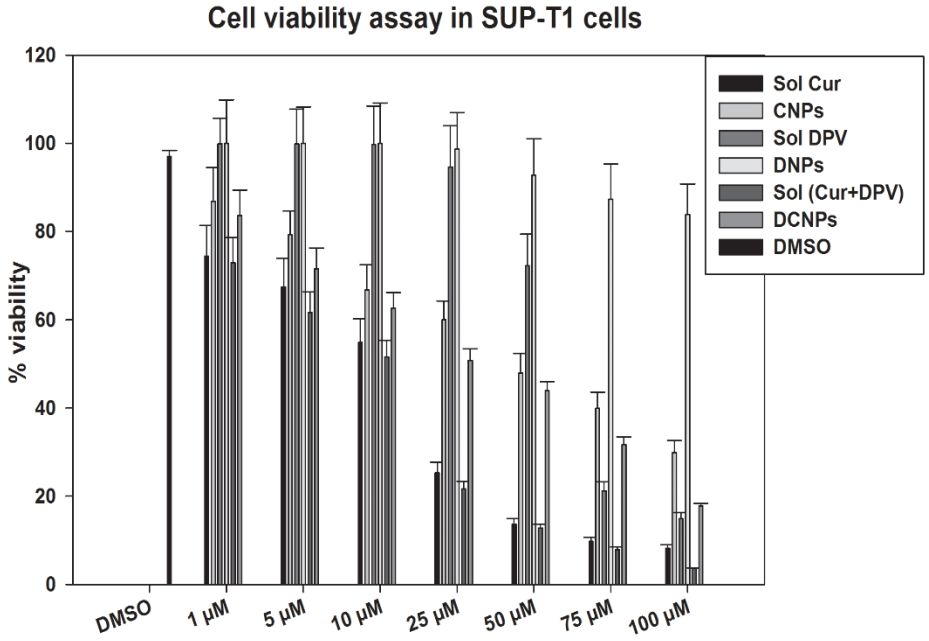
Fig 5.6: pH dependent release of NPs: pH dependent drug release assay for DNPs and DCNPs 300 µg of particles were incubated in the buffers of different pH, and simulated vaginal fluid. The release of DPV and curcumin was found to be maximum at pH-5 followed by pH-6 and pH-4. Optimum drug relese was observed at SVF, Each data points were repeated in triplicate (n = 3) and presented as Mean ± standard deviation (S.D).

In-vitro studies:

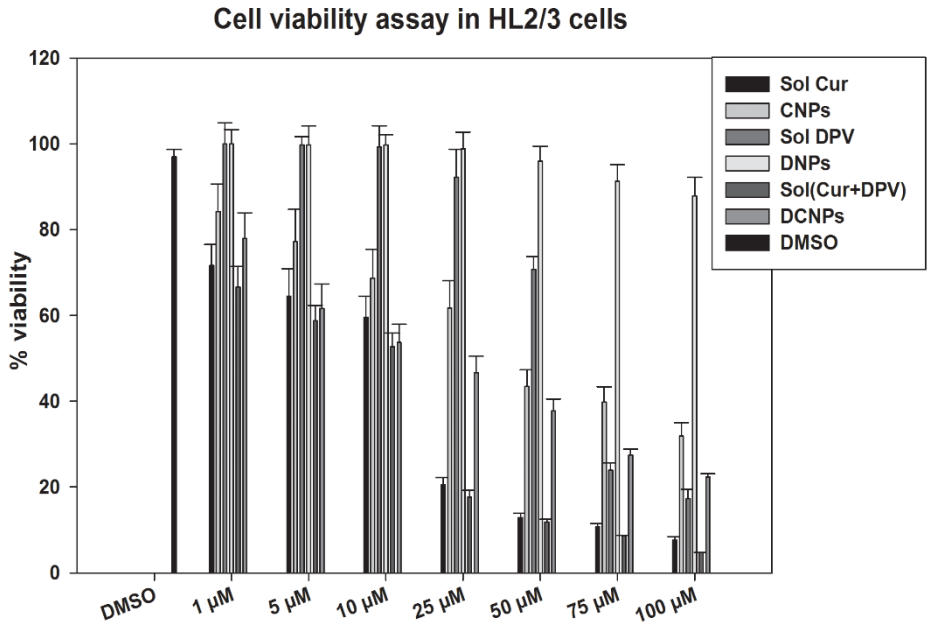
Cell viability assay: SUP-T1 cells, HL2/3 and VK2 cells (Fig 5.7) were incubated with a series of increasing concentrations (1, 5, 10, 25, 50 and 100 μM) of both soluble and nano formulations and cell viability was evaluated by MTT assay. Compared to soluble curcumin, CNPs were found to be less toxic at all concentrations. Soluble curcumin was highly cytotoxic even at higher concentration while CNPs at the same concentration was comparatively less cytotoxic.

The lower cytotoxic properties of CNPs highlights the perception that direct administration of soluble curcumin is lethal to cells at 10 μM concentrations and above. Both soluble DPV and DNPs formulations were found non-toxic at all concentrations. In combination drug treatment, soluble combination form showed higher toxicity when compared to DCNPs.

A. SUPT1 cells:



B. HL2/3 cells:



C. VK2 cells:

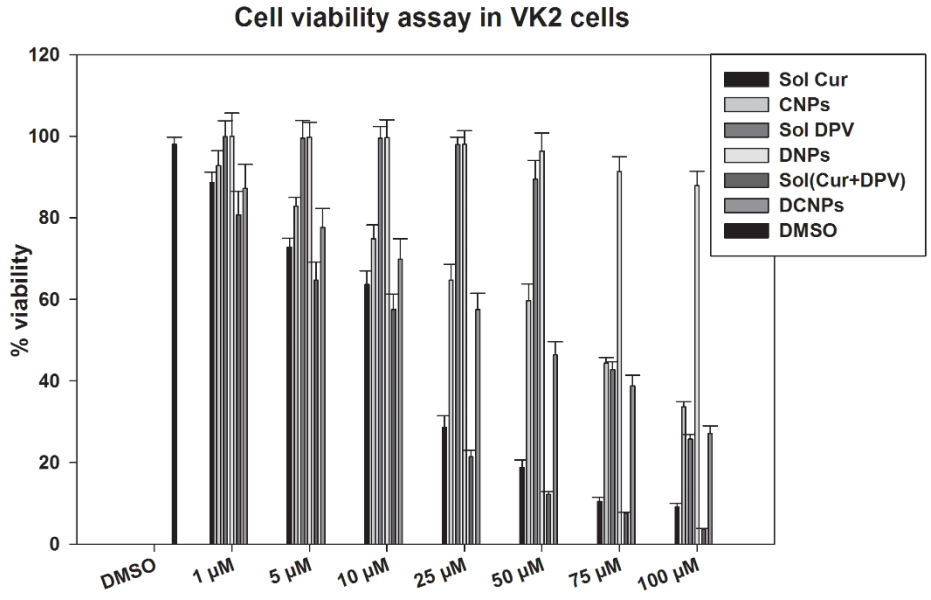


Fig 5.7: Cell viability assay: SUP-T1, HL2/3 and VK2 cells were treated with a series of concentrations (1, 5, 10, 25, 50, 75 and 100 µM) of soluble curcumin, soluble DPV, soluble (Curcumin+DPV), CNPs, DNPs and DCNPs. Later the cells viability were tested a by MTT reagent and results were plotted.

Lactobacillus safety analysis by disc diffusion test:

Lactobacilli were incubated with increasing concentrations (1, 2 and 5 mg) of drugs both in soluble and nano forms of both curcumin, DPV individually and in combination. We did not find significant bacterial growth inhibition in all treated drugs either in soluble form or in nano form. These results suggested that soluble drugs and their nano formulation are safe for microbicide application (Fig-5.8).

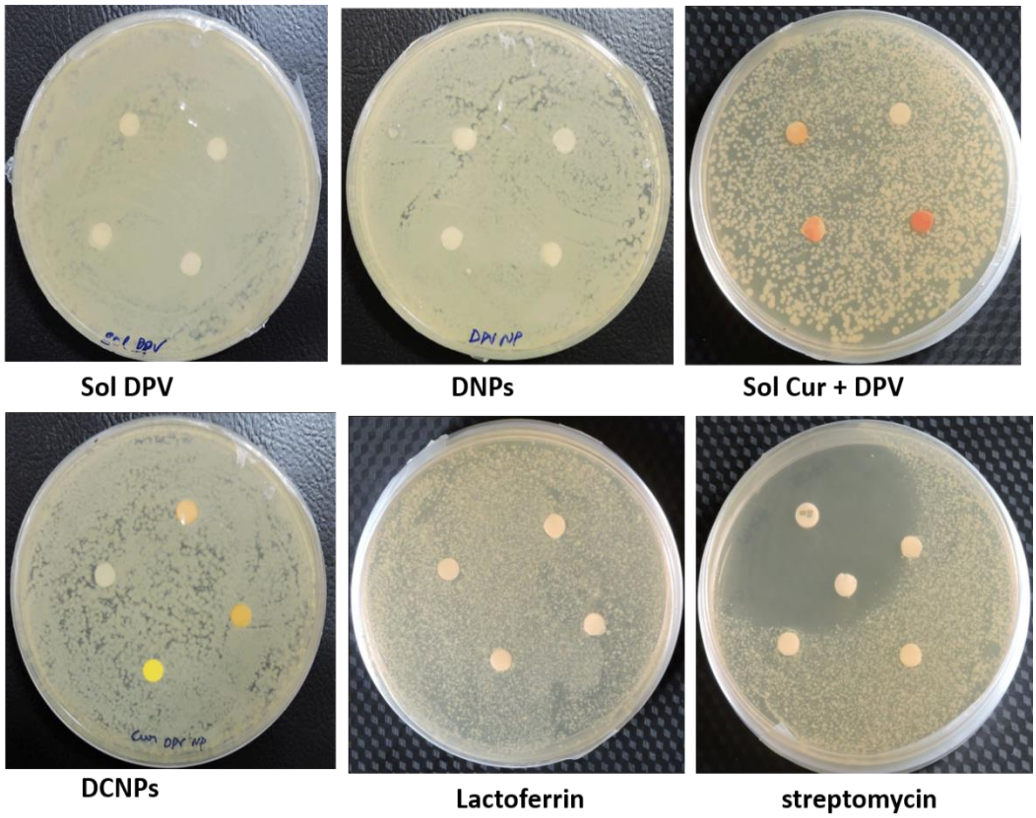


Fig 5.8: *Lactobacillus* viability assay: Soluble DPV, DNPs, Soluble curcumin, CNPs, Soluble (Curcumin+DPV), DCNPs and Lf were placed on disc with increased concentrations of 1 mg, 2 mg and 5 mg and one disc with negative control. In separate plate along with negative control, positive control Streptomycin 10 mcg was placed. Negligible bacterial growth inhibition was observed in both soluble and nano forms of drugs.

Sperms viability assay:

Microbicide having contraceptive property is an extra ability to any microbicidal formulation that to act as a versatile microbicide against HIV. Sperms (Fig 5.9) were incubated with increasing concentrations (10, 50, 100, 200, 300, 400, 500 and 600 μM) of the two formulations and cell survival was estimated by MTT assay. Both soluble curcumin, CNPs and combination formulations (DCNPs) were found to be non-toxic at very low concentrations. Greater than 200 μM concentration of curcumin was showed nearly 80% cytotoxicity to sperms in both forms. This data suggests that these formulations can also act as a contraceptive due to its spermicidal activity of curcumin.

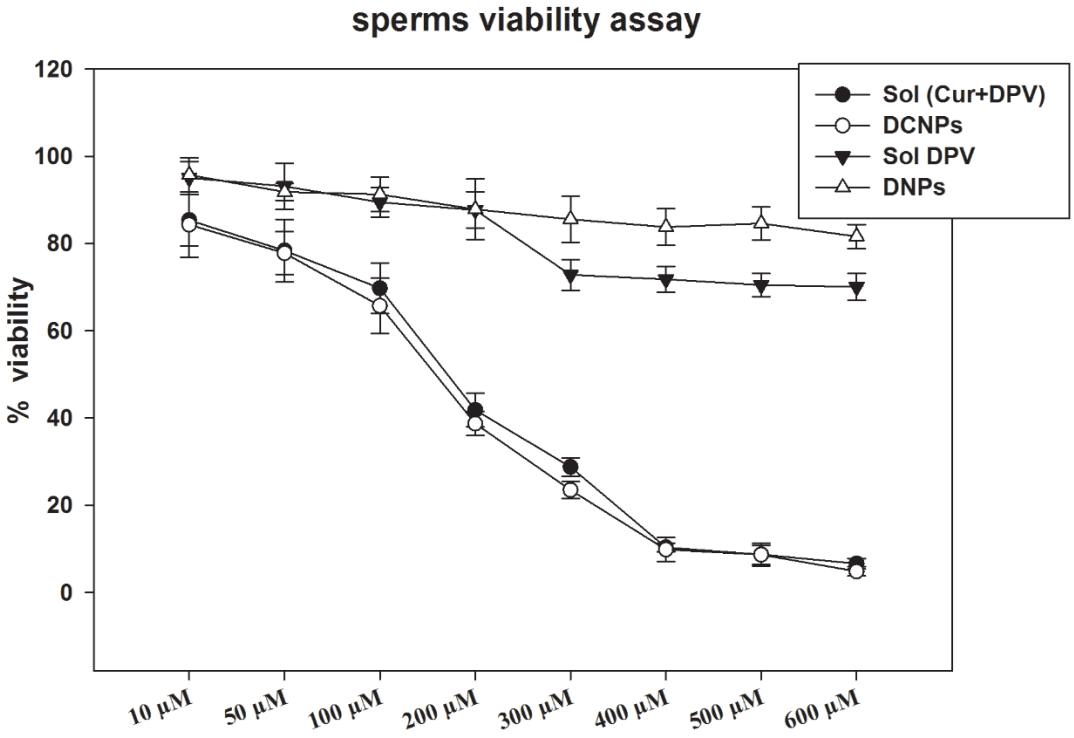


Fig 5.9: Sperm viability assay: Sperms were treated with increasing concentration (10, 50, 100, 200, 300, 400, 500 and 600 μM) of soluble curcumin, soluble DPV, soluble (Curcumin+DPV), CNPs, DNPs and DCNPs. Later the sperms viability were tested a by MTT reagent and results werd plotted.

Anti-HIV activity of NPs:

The in-vitro anti-HIV-1 activity of DCNPs was evaluated as single and combination drugs in both soluble and nano formulation. The IC_{50} for soluble curcumin (5.1 μM) and CNPs (1.75 μM), soluble DPV (28 nM) & DNPs (17 nM). Further, the anti-HIV activity of DCNPs have been found to be improved over its soluble formulation when used at 1:1, 0.5:0.5 and 0.25:0.25 while no significant advantage is observed at 0.1:0.1 and 0.05:0.05. The percent HIV-1 replication vs. drug combination ratio have been plotted (Fig 5.10). The Anti-HIV activities in a combination of curcumin + DPV are found to be improved or intact even after 50% reduction in dose. Since antiviral activity was enhanced by two-fold in combination nano form, it would help in reducing the dose frequency, prevent unwanted side effects and toxicities.

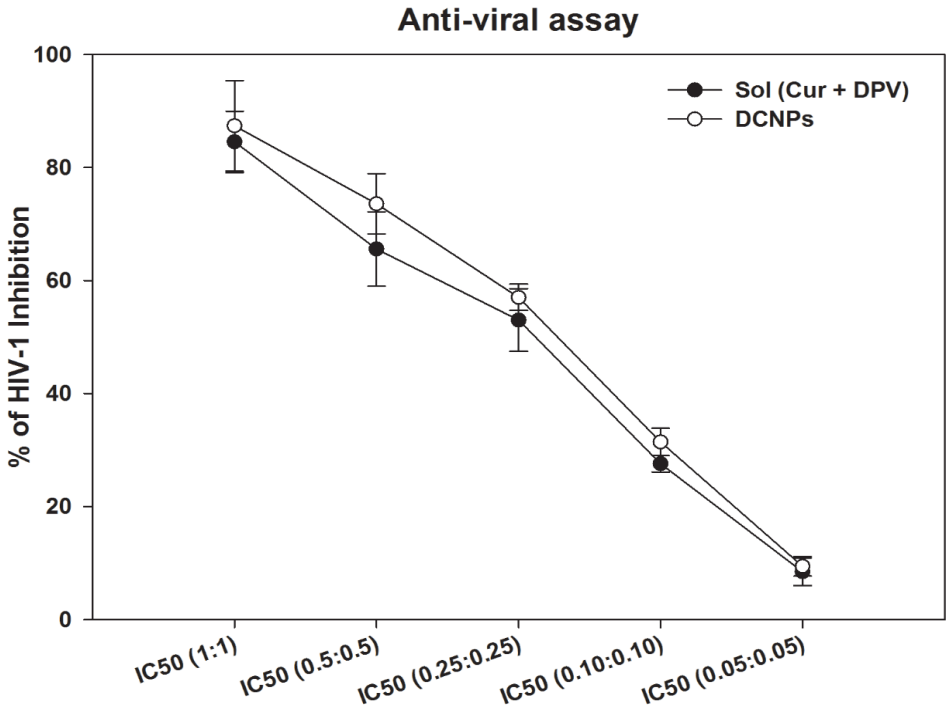


Fig 5.10: Anti-viral assay: Various different IC_{50} ratios of curcumin and DPV drugs were mixed in soluble and nano form. Later the antivral assay were performed and plotted.

***In-vivo* studies:**

Pharmacokinetic studies in vaginal lavage:

To evaluate the pharmacokinetic parameters, both soluble and nano combination forms of drugs were measured separately in vaginal lavage after administration of single dose. Drug levels were observed for nine different time points (0.5, 1, 1.5, 2, 2.5, 4, 6, 12 & 24 hrs). PK profile was enhanced in nano treated group when compared to the soluble counterpart (Table 5.1). Greater than three time's increase in AUMC and two times in AUC was observed from both drugs in nano form. Likewise greater than two-fold increase in $T_{max,}$ two-fold increase in $t_{1/2}$ and C_{max} increased to greater than one-fold in nano treated group when compared to soluble treated group. These results suggest that higher bioavailability of drugs at the site of application when DCNPs were given (Fig 5.11).

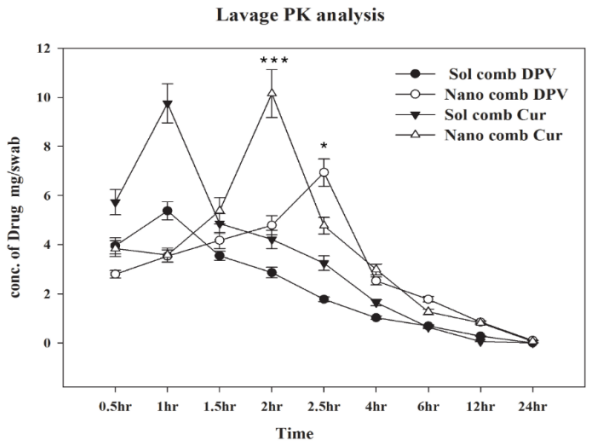


Fig 5.11: Pharmacokinetics: 20 mg of soluble curcumin plus 10 mg of soluble DPV and an equivalent amount of DCNPs were applied as single dose in vagina for indicated time points. Lavages were collected after these time points and curcumin and DPV concentration were calculated separately. Abbreviation: sol comb Cur and sol comb DPV represents curcumin and DPV drugs released from soluble (Curcumin+DPV). Nano comb Cur and Nano comb DPV represents curcumin and DPV drugs released from DCNPs. Sample data were recorded as Mean ± S.D, n=3. The difference between two groups was calculated by using student's t-test and the value of significance expressed as ***P<0.0005, **P< 0.005, *P< 0.05.

Table 4.1: Pharmacokinetic profile in vaginal lavage

Parameters	Units	DPV		Curcumin	
		Nano	Soluble	Nano	Soluble
AUC	(h)*(mg/ml)	32.4757	15.3342	32.2141	20.1822
AUMC	(h)^2*(mg/ml)	206.007	57.5105	176.238	51.5535
C_{max}	mg/ml	6.9347	5.3746	10.1473	9.7498
T_{max}	hr	2.5	1	2	1
$t_{1/2}$	hr	4.28923	2.10652	3.64984	1.88261

Values in the parenthesis designate the concentration of drug in milligrams per swab.

Pharmacokinetic parameters.

AUC: The integral of the concentration-time curve (after a single dose or in steady state).

AUMC: Partial area under the moment curve between t start and t end.

C_{max}: The peak plasma concentration of a drug after vaginal administration.

T_{max}: Time to reach C_{max}.

$t_{1/2}$: The time required for the concentration of the drug to reach half of its original value.

In-vivo Drug kinetic release (time course) experiment:

Time course study was performed in i*n-vivo,* to evaluate the extent of availability of drugs over a period of long time. The time-dependence was studied individually for the two drugs curcumin and DPV delivered in DCNPs. Results were plotted (Fig 5.12), results represents the concentration of curcumin and DPV in vaginal lavage at various time points, with a lag period of 1 hr (for soluble curcumin), 1.5 hr (for soluble DPV), 2 hr (nano form of curcumin) and 2.5 hr (nano form of DPV) hours after DCNPs were applied topically in vagina. The results suggest that high concentration of drugs in the initial stage for both the soluble and nano form, which undergo reduction exponentially. The nano formulations of drugs even after 2 hr and 2.5 hr of lag phase showed sustained and significant release of the drug up to 12 hr whereas both the drugs in the soluble form, showed a fast reduction (at 4hr) in the concentration. We have indeed detected the presence DPV and curcumin up to 8–12 hr when drugs were given in nano-formulation but in case of the soluble form, the drugs were eliminated in 4–6 hrs.

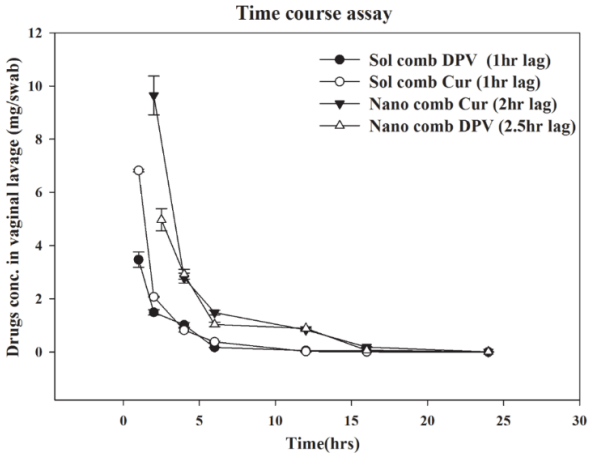


Fig 5.12: Drug release kinetic assay: 20 mg of soluble curcumin plus 10 mg of soluble DPV and an equivalent amount of DCNPs were applied topically to the rat vagina. Lavages were collected after above mentioned time points and curcumin and DPV concentration were calculated separately. Abbreviation: Sol comb Cur and Sol comb DPV represents curcumin and DPV drugs released from soluble (Curcumin+DPV). Nano comb Cur and Nano comb DPV represents curcumin and DPV drugs released from DCNPs.

Dose-dependent toxicity and Bioavailability studies:

In Vaginal lavage:

After intra-vaginal application with fixed doses as mentioned in methods, vaginal lavage was collected after respective time points. Drugs (Curcumin & DPV) from both the formulations such as soluble and nano forms were measured and plotted (Fig 5.13). Results suggest that drugs from nano formulation both in a single and in combination nano forms showed 1.4 to 2.5 fold more availability of drugs at the applied site in a dose-dependent manner compared to soluble counterpart.

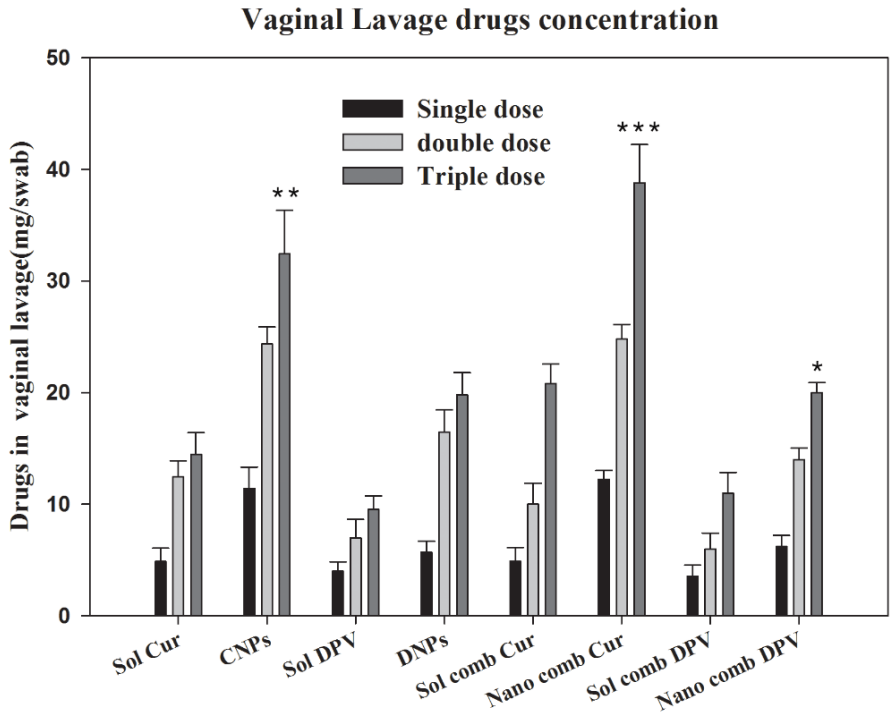
In Vaginal tissue:

Drugs from both the formulations such as soluble and nano forms were extracted from the homogenized vaginal tissue. Extracted drugs were estimated and plotted (Fig 5.13). Here drug concentrations were found to be in micrograms that are thousand times less than the vaginal lavage. In the case of DCNPs we found there was 1 to 2.3 times lower drug concentrations as compared to soluble counterpart. Whereas individual NPs didn't show any much variation in drugs exposure as compared to soluble counterpart.

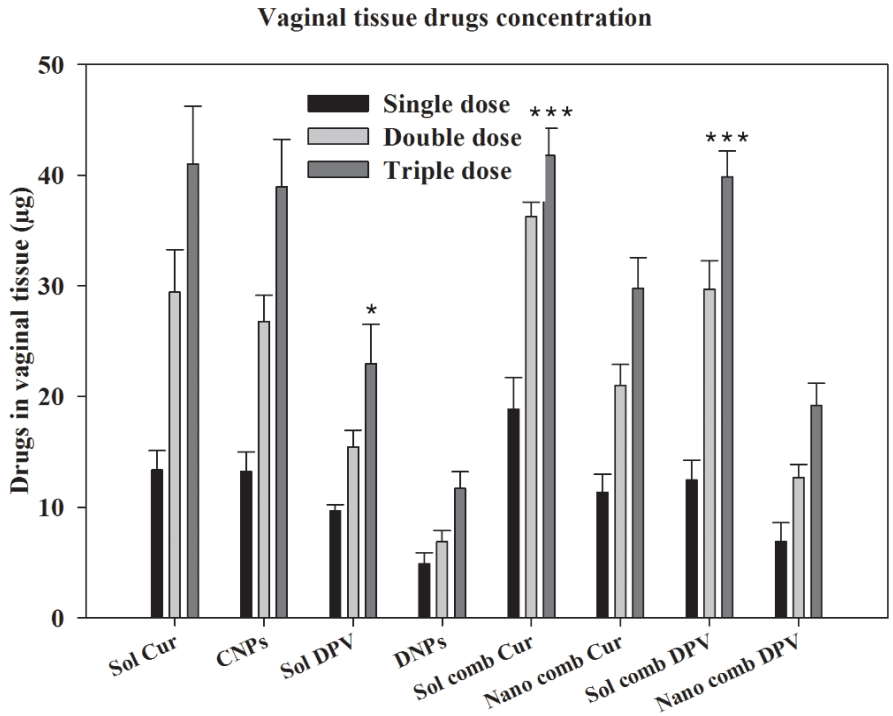
In systemic level:

Drugs from both the formulations such as soluble and nano forms were measured in the systemic circulation and plotted (Fig 5.13). Here results showed that 2 to 5.25 times high accumulation of drugs from soluble form when compared to the nano counterpart; thus suggesting higher absorption of soluble form leads to enhanced risk due to drug-related systemic toxicity. But in DCNPs treated at the third dose, curcumin and DPV were found in less concentration than soluble, it suggests that even at higher doses also the NPs exhibit low systemic absorption due to maintenance of optimal dose of drug from controlled release of drug from NPs.

A. Vaginal lavage:



B. Vaginal tissue:



C. Plasma:

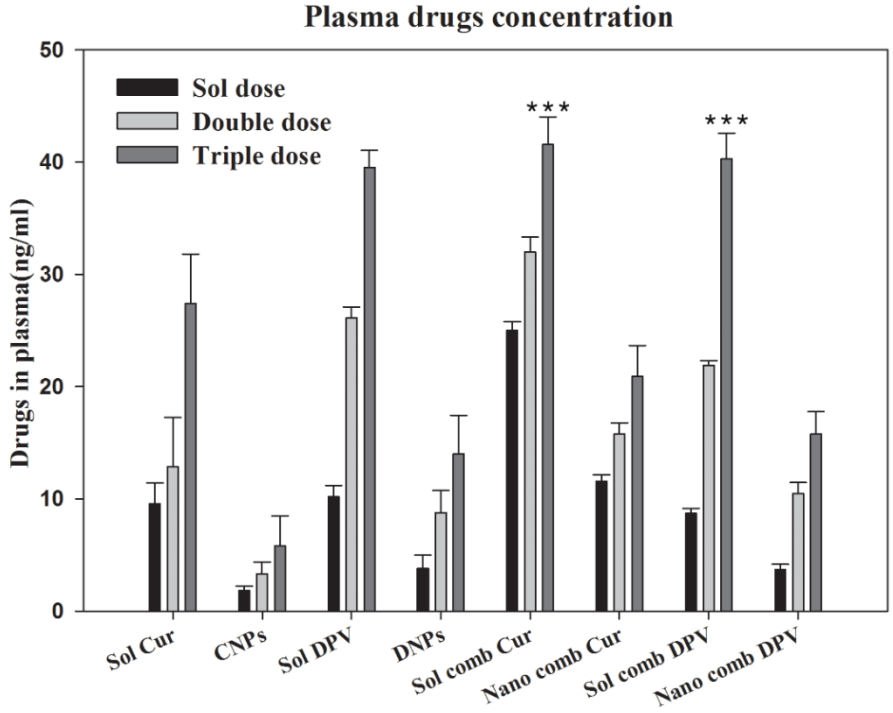


Fig 5.13: Bio availability studies in rats: Dose dependent toxicity study of drugs in single form or combination form either in soluble (DPV + Curcumin) and DCNPs were performed. The concentration of drugs in vaginal lavage, vaginal tissue and plasma were analyzed and plotted. Sample data were recorded as Mean ± S.D, n=3. The difference between groups was calculated using one-way analysis of variance and the value of significance expressed as ***P<0.0005, **P< 0.005, *P< 0.05.

Histopathological analysis:

H&E study of cervicovaginal epithelia showed that, the integrity of tissue was damaged in a dose dependent manner when the combination of soluble drugs was used. Where as in DCNPs treated vaginal epithelia showed less toxicity in a dose-dependent manner. These results confirm the safety advantage of DCNPs compared to soluble counterparts (Fig 5.14).

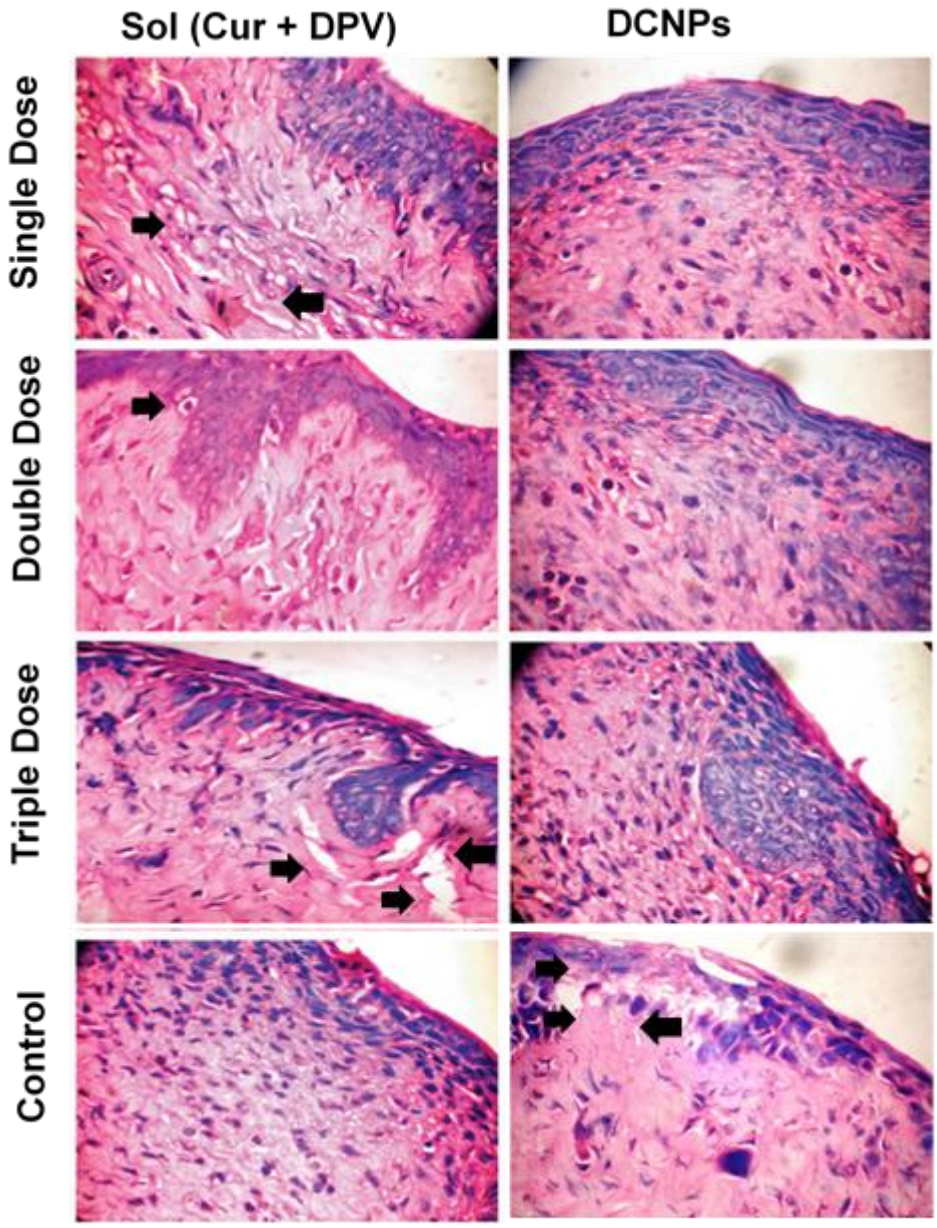
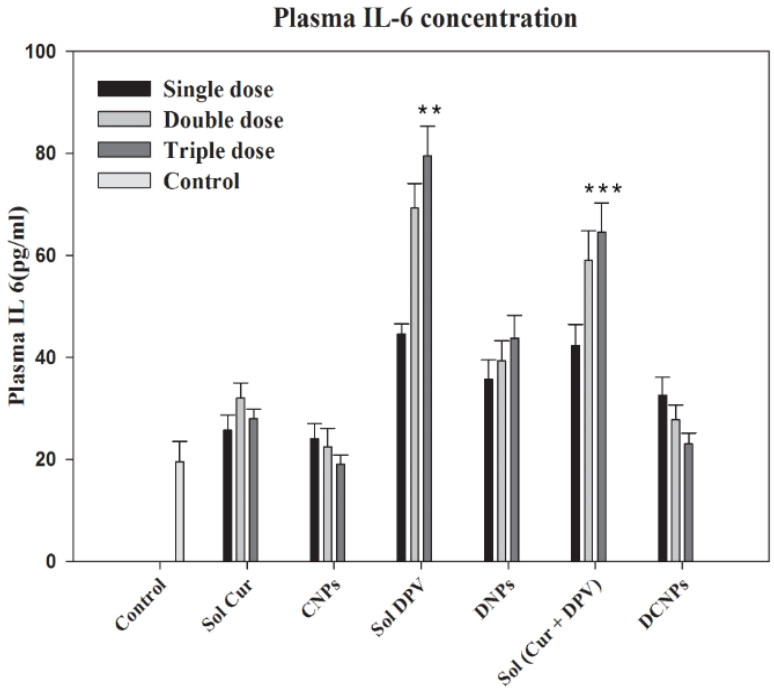


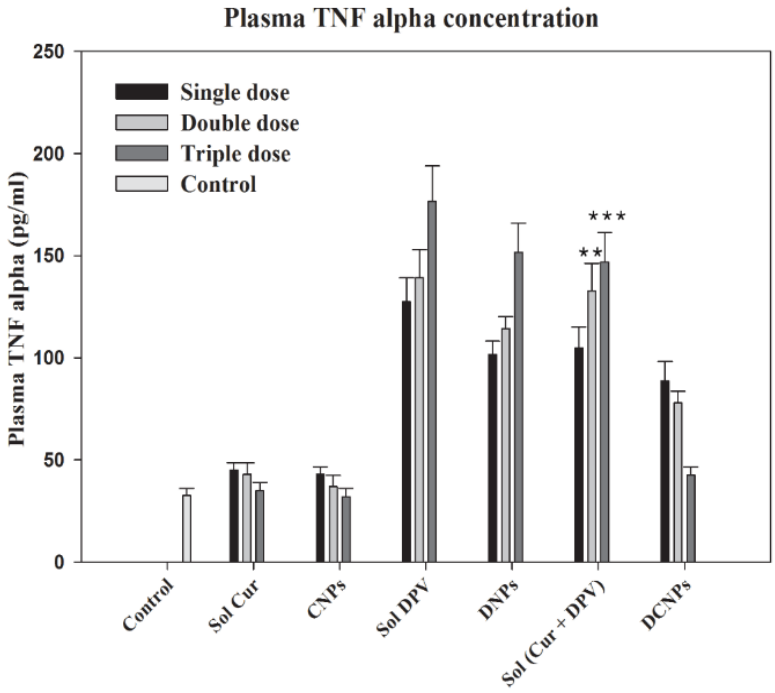
Fig 5.14: Histopathology: Right panel represent the vaginal epithelia treated with DCNPs for single, double and triple dose respectively and positive control. Left panel represent the vaginal tissue integrity when applied topically with soluble (Curcumin+ DPV) for single, double and triple dose respectively and negative control. Damaged site or lesions were shown through a printing arrow. Negative and positive controls were treated with PBS (pH-7.4) and nonoxynol-9 (10 mg/kg) respectively.

Systemic and vaginal Pro-inflammatory cytokine response:

The level of Inflammation has checked by estimating the Pro-inflammatory cytokine markers such as interlukine-6 (IL-6) and tumor necrosis factor alpha (TNF-alpha) from the isolated plasma and vaginal tissue. In DCNPs treated condition, the IL-6 levels showed 2.8 times lower than soluble counterparts in plasma. Even at the third dose of DCNPs we couldn't detect significant inflammation. In contrast, treatment with soluble combination leads to nearly 2.5 times increase of IL-6 in the vaginal tissue. TNF-alpha is a cell signaling protein that involved in systemic inflammation. Furthermore, the TNF-alpha levels were significantly low, in the treatment with DCNPs and CNPs at the third dose. (Fig 5.15).

A. Plasma:


Plasma IL-6 concentration
Single dose
Double dose
Triple dose
Control
Plasma IL 6(pg/ml)
100
80
60
40
20
0
**
***
Control
Sol Cur
CNPs
Sol DPV
DNPs
Sol (Cur + DPV)
DCNPs


Plasma TNF alpha concentration
Single dose
Double dose
Triple dose
Control
Plasma TNF alpha (pg/ml)
250
200
150
100
50
0
**
***
Control
Sol Cur
CNPs
Sol DPV
DNPs
Sol (Cur + DPV)
DCNPs

B. Vaginal tissue:

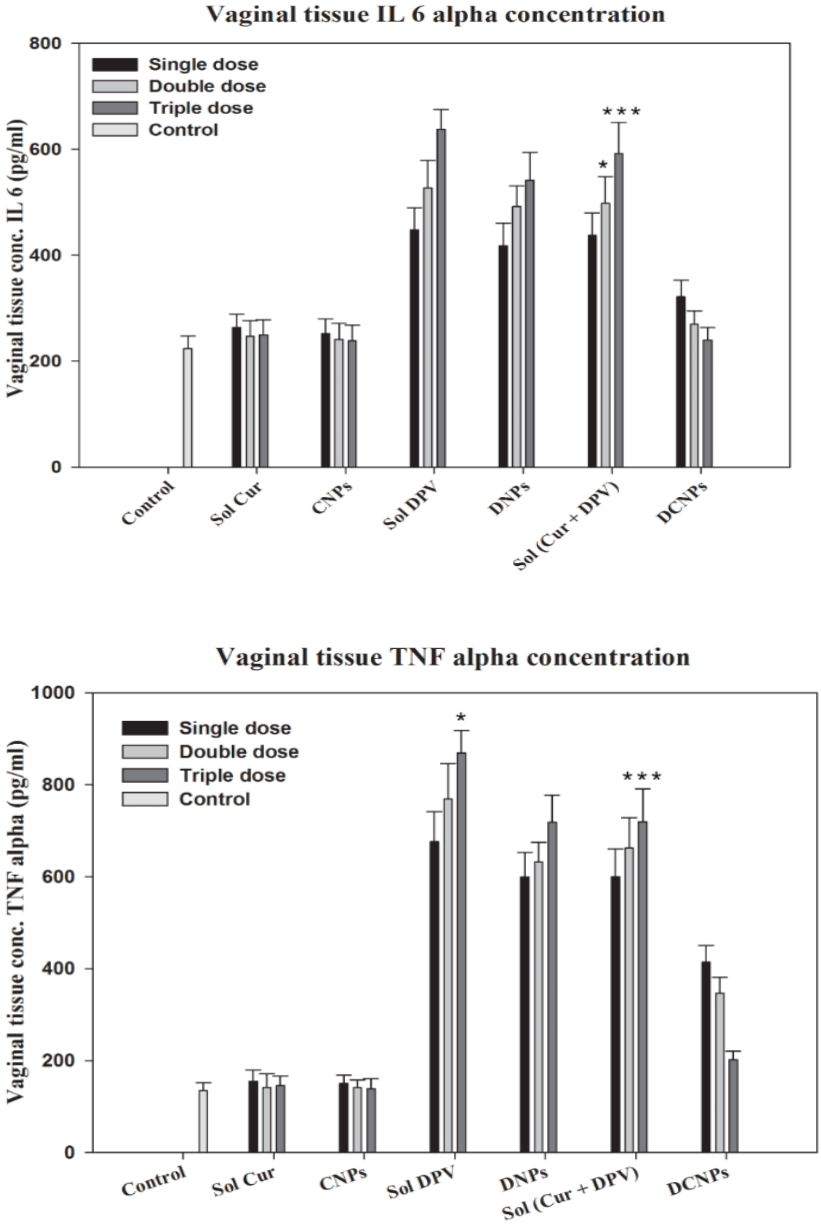


Fig 5.15: Proinflammatory cytokine response: Soluble DPV, Soluble Curcumin, Soluble (Curcumin+DPV), CNPs, DNPs and DCNPs were applied topically in vagina. IL-6 and TNF alpha levels in plasma and vaginal tissue were analyzed and plotted. Sample data were recorded as Mean ± S.D, n=3. The difference between groups was calculated using one-way analysis of variance and the value of significance expressed as ***P<0.0005, **P< 0.005, *P< 0.05.

Discussion:

The standard method for microbicide development has been a single entity product designed for vaginal use. Combination of drugs like the use of highly active antiretroviral therapy (HAART) for treatment of HIV could help in further reducing the likelihood of becoming infected with HIV [185]. Ideally, drugs that target different steps in the viral replication cycle would be advantageous especially with virus that may anchorage drug resistance mutations to one of the drugs in the formulation [186, 187]. Here we evaluated DPV a NNRTI and Curcumin alone and combination Lf NPs loaded form for better bioavailability, efficacy and safety.

Curcumin and DPV loaded Lf NPs were prepared by sol-oil chemistry method which is simple and cost effective method without effecting a chemical nature of the drug/vehicle. This procedure offers a chance to produce various materials with novel predefined properties in a very simple procedure and relatively at low cost [110]. The main advantage of this method is to produce particles with very high purity and uniform nanostructure as assessed through FE-SEM, TEM and AFM analysis; Particles have an average size between 45-80 nm, the NPs were spherical in shape and having smooth surface. In both types of nanoformulation, either blank or drug/s loaded, the DLS sizes were found to be 102-116 nm greater than their SEM or AFM sizes like as earlier formulations with EFV and TFV. The reason for this is reasonably normal because the size was measured using light scattering method that could be exaggerated by interaction of nanoparticle with the water shell and charge present on the surface that may lead to overall upsurge in size. DCNPs with a polydispersity index (PDI) of 0.452 are found to as homogeneous mixture.

The measured zeta potential was around −24.0 mV. This shows relatively negative charge on the surface of NPs. Toxicity and stability of NPs was depends on size and zeta potential of the NP, as well as tissue uptake and interstitial trafficking [188]. It was shown that topical administration of Lf NPs with a strong negative charge (around −20 mV) and size around 102-116 nm, can display greater permeability deep into vaginal epithelium as well as into the ectocervical tissue. DPV has higher EE% because it is highly hydrophobic (Log p=5.3), and it also has a higher molecular

weight (329.4 g/mol).Human vaginal vicinity pH is around 4.5 maintained by the *Lactobacilli*, is the most colonizing bacteria and it maintains low acidic pH by secreting lactic acid. To know the pH sensitivity of the protein NPs for the drug release, pH dependent drug release was performed; drug loaded NPs (DNPs & DCNPs) release maximum amount of drug at pH 5 and PH 6. Later drug release was tested in simulated vaginal fluid which mimics the condition of vaginal environment, this experiment showed significant amount of drug release at that particular pH. It suggests that these drug loaded nano-formulation can deliver drug in the vaginal environment without any failure.

Fourier transform infrared spectroscopy (FTIR) was used to assess potential interactions between protein and drug in nano-formulation. Even though minor shift was observed in functional positions in nano formulation when compared to soluble form, this change considered should be negligible. These results suggests that drugs are chemically stable in nano-formulation as like in soluble drug formulation, here we can say that protein and drug involved in physical interactions. In our earlier studies also we proved that this method of nanoparticle preparation is excellent to retain and improve the activity of drug. Most of the anti HIV drugs having solubility problem, hence drug bioavailability is very poor in the site of action. Current formulation may overcomes this problem and at the same time HIV undergoes resistance when single drug is employed, a combination of ARV and curcumin with Lf, may reduce frequency of acquiring resistance due to negligible systemic and tissue exposure of drug along with the maintenance of optimum concentration of drug. Due to this combination together, by showing synergistic effects of all three compounds against HIV we can say that, this formulation is more efficacious than other formulations. Normally the *Lactobacillus* species found predominantly in vaginal flora, for any microbicide formulation it is critical that to not disturb this normal vaginal flora. This flora maintenance is very important to maintain low pH environment and the secretion of hydrogen peroxide, which provides natural barrier against HIV transmission [189]. Our data suggested that Lf NPs formulation do not effect growth of the normal vaginal flora. Together with other safety data, Lf NPs has the potential to be a safe matrix for vaginal delivery of microbicide. Anti HIV-1

data suggests that, the individual drug nano-formulation is found to be more effective than its soluble form as quantified by the reduced IC_{50} values. Furthermore, 50% of IC_{50} concentration of the drug combination curcumin + DPV is adequate to confer anti-HIV activity of the corresponding soluble combination. This result suggests that even half amount of drug is sufficient to target HIV-1 leads to the reducing dose frequency and prevent unwanted side effects and toxicities. Curcumin and DPV combination with curcumin in both soluble and nano forms showed effective spermicidal activity at >250 μM concentration onwards, this was proving the contraceptive ability of the nano formulation. This contraceptive nature is an additive asset to this formulation along with anti-HIV and anti-inflammatory properties.

Coming to *in-vivo* analysis, to see the pharmacokinetic parameters response in the vaginal lavage with two formulations, local pharmacokinetic study was performed and results showed that, pharmacokinetic parameters such as $t_{1/2}$, T_{max}, C_{max} are almost double in NPs treated group when compared to soluble treated group, compared to earlier drugs EFV & TFV with same formulation DPV showed better PK parameters. This confirmed that NPs could give efficient protection against HIV in women for long time, it shows improved bioavailability of the drug in nano treated groups when compared to soluble drug treated groups. Drug sustainability and longevity of the drug was measured by time course analysis experiment; after administration of drug topically in to the rat vagina, it showed that significant amount of drug was observed up to 16 hrs of time in NPs treated group whereas soluble treated group was only showed up to 4 hrs, this experiment proved that drug residence is significantly longer time in NPs treated group comparatively soluble treated.

Dose dependent bioavailability (Triple dosage) study with 2 hrs time gap showed the advantage of NPs compared to soluble treated by means of low drug levels in serum, significant amount of drug levels in Vaginal tissue and vaginal lavage when compared to soluble treated groups as similar to our earlier work with EFV and TFV. It suggests that maximum amount of drug retained in the vaginal lavage which can help in preventing HIV transmission during infection. In vaginal tissue, the amount of

drug was observed in micrograms for both the forms. However, this amount of drug did not cause any tissue damage in nano formulation treated rats compared to soluble treated rats as shown by histopathological tissue sections. Due to the detection of drug levels in serum (nanograms) and vagina (micrograms); here we checked safety analysis in the systemic level and vagina by measuring inflammatory markers such as, IL-6 and TNF alpha. This was showed that low levels of IL-6 and TNF alpha in nano treated group compared to soluble treated group. The possible reason for the low levels of inflammation in NPs treated rats; even though the presence of significant amount of drug, is due to anti-inflammatory nature of curcumin and Lf. while in the soluble form high concentration of ARV and curcumin is exposed at the same time. Since inflammatory response is concentration dependent higher, ARV and curcumin induces high levels of toxicity thus synergistic effect lost, therefore loose its benefit.

Conclusion:

In this study, curcumin and DPV were successfully encapsulated using sol-oil method and used as a microbicide by showing a synergistic effect with controlled delivery system through vaginal administration. Drug release studies at different pHs showed that vaginal pH is favorable for drug release. These microbicide as nano formulations showed negligible toxicity to vaginal microflora *Lactobacillus*, vaginal epithelial tissue and with improved PK parameters. Thus DCNPs serves as a highly effective microbicide formulation with a contraceptive ability.

Combination nanoparticles such as, ECNPs, TCNPs and DCNPs can act as an effective microbicides when compared to its soluble forms. Safety assessment suggests that, all the three formulations are found to be safe as compared to their soluble counterpart. From Bioavailability point of view DCNPs are the most bioavailable formulation among the three. Because of ARV combination with curcumin loaded Lf NP combination, these microbicidal formulation can act as MPTs (Multiple Prevention Technologies) such as microbicidal, anti-inflammatory and spermicidal activity when compared to its soluble formulation.

Conclusions

Conclusions:

- Three different types NPs (single drug loaded Lf NPs and curcumin combination ARV loaded Lf NPs) have been prepared using sol-oil chemistry. Size of NPs 20-26 nm (lacto) and 40-80 nm (ARV lacto) were observed by FE-SEM, AFM and TEM.
- The hydrodynamic radii and zeta potential have been measured using DLS and found to be an average of 40 nm (Lf) 102 to 120 nm (drug loaded) and -19 to -27 mV respectively.
- The physical stability of drug/s loaded NPs have been estimated through FT-IR study, and found that the key functional groups related to respective drugs are intact in the nanoformulation.
- The encapsulation efficiency percentage (EE %) was calculated for each type of NPs and found as follows, curcumin from CNPs = 59.6±1.34%, EFV from ENPs =58.4 ± 1.79%, TFV from TNPs = 53.5±1.3%, DPV from DNPs = 61.6±1.6%, ECNPs (Curcumin = 63 ± 1.9%, EFV = 61.5% ± 1.6%), TCNPs (Curcumin = 61.75±1.6%& TFV= 64.52±1.8%) and DCNPs (Curcumin = 60.33±1.3%& DPV = 62.17±1.9%).
- pH dependent drug release assay of NPs shows that maximum drug release (40-60%) was observed at pH-5 in pH dependent drug release. Optimum drug release showed in vaginal simulated fluid (pH 4.5)
- Antiviral properties of all ARV drugs and curcumin have been found to be improved with significant decrease in IC_{50} (Curcumin; soluble-5.1 μM & nano-1.75 μM, EFV; soluble-2.56 nM & nano-1.1 nM, TFV; soluble-4.78 μM & nano-2.8 μM and DPV; soluble-28 nM & 17 nM) of nano form as compared to its soluble counterpart.
- The combination forms (ECNPs, TCNPs and DCNPs) are found to be more efficacious in their nano form as compared to its soluble form. As the percent HIV inhibition is found to be equal or improved even after treated with 50% of their soluble form combination.

- Main observation is that antiviral activity was enhanced in combination nano form, this gives the advantage of using drug combination in the nano form there by reducing the dosage leading to the prevention of unwanted side effects and toxicities.

- Due to negligible toxicity to *Lactobacillus* exhibited by all forms of drugs; there may not to be any threat to vaginal micro flora, considered as a safety Microbicide.

- The comparative in-vivo pharmacokinetics (PK) studies were performed on Wistar female rats. Results showed a significant improvement of PK profile of drug when nanoformulation is administered topically (C_{max}, $t_{1/2}$, AUC and AUMC), suggesting higher bioavailability of drugs as compared to its soluble equivalents.

- In time course assay drugs were present up to 8-12 hrs in nano form treated but in case of soluble treated drugs were eliminated in 4-6 hrs, this proved that drug retention is high in nano treated groups. When compared to soluble treated.

- Bioavailability studies shown that in nano form (ECNPs, TCNPs and DCNPs) treated rats, maximum amount of drugs present in the lavage, 10^3 and 10^6fold less concentrations of drugs were observed in the vaginal tissue and in the systemic circulation when compared to the lavage respectively.

- The integrity of epithelia was found heavily damaged when the combination of soluble drugs were used. In case of ECNPs, TCNPs and DCNPs the integrity of tissue was found to be same as that of control or very lesser extent. Further IL-6 and TNF- α levels were estimated in vaginal tissue and in systemic level, they found 1.5 to 3 fold less in NPs treated when compared to soluble form treated.

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Publications

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Triple combination MPT vaginal microbicide using curcumin and efavirenz loaded lactoferrin nanoparticles

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We report that a combination of anti-HIV-1 drug efavirenz (EFV), anti-microbial-spermicidal curcumin (Cur) and lactoferrin nanoparticles (ECNPs) act as MPT formulation. These nanoparticles are of well dispersed spherical shape with 40–70 nm size, with encapsulation efficiency of 63 ± 1.9% of Cur & 61.5% ± 1.6 of EFV, significantly higher than that of single drug nanoparticles (Cur, 59 ± 1.34%; EFV: 58.4 ± 1.79). ECNPs were found to be sensitive at pH 5 and 6 and have not effected viability of vaginal micro-flora, *Lactobacillus*. Studies in rats showed that ECNPs delivers 88–124% more drugs in vaginal lavage as compared to its soluble form, either as single or combination of EFV and Cur. The ECNPs also shows 1.39–4.73 fold lower concentration of absorption in vaginal tissue and plasma compared to soluble EFV + Cur. Furthermore, ECNPs show significant reduction in inflammatory responses by 1.6–3.0 fold in terms of IL-6 and TNF-α in vaginal tissue and plasma compared to soluble EFV + Cur. ECNPs showed improved pharmacokinetics profiles in vaginal lavage with more than 50% of enhancement in AUC, AUMC, C_{max} and $t_{1/2}$ suggesting longer exposure of Cur and EFV in vaginal lavage compared to soluble EFV + Cur. Histopathological analysis of vaginal tissue shows remarkably lower toxicity of ECNPs compared to soluble EFV + Cur. In conclusion, ECNPs are significantly safe and exhibit higher bioavailability thus constitute an effective MPT against HIV.

Across the globe, a major number of women, especially in developing countries, needs protection from various sexual transmitted infections (STIs) like HIV/AIDS as well as sexual and reproductive health (SRH) risk that includes unintended pregnancy. Further due to STIs, the women in low and middle income countries like sub-Saharan region experience about 41% of unintended pregnancies that leads to approximately 70,000 deaths[1,2]. Hence, there is an urgent need to develop technologies which can target these conditions at a time. The Multiple prevention technologies (MPT) are currently the most promising and intricate class of product under development which can simultaneously prevent transmission from the STIs like HIV/AIDS and unwanted pregnancy[3]. Currently three types of MPT are available; which include male and female condoms, female Diaphragms and microbicide (chemical barrier)[4]. Our current study focus on the vaginal microbicide based MPT.

Several research groups have already reported various combinations of microbicides[5,6]. Some of the formulations have undergone different stages of clinical trials, and a large numbers of formulations have failed due to safety, toxicity and efficacy issues[7–9]. In addition to this other microbicide such as PRO 2000, Carraguard (Phase III) and BufferGel were also not found to be successful at different phases of clinical trials[10,11]. The major requirement of a microbicide is, to release optimum concentrations of active drug in the vagina along with higher bioavailability for longer period without causing discomfort and adverse effects to the biological barriers. Polyanions and surfactants based vaginal microbicides against HIV were also not successful; while nonoxynol-9 has increased the risk of HIV acquirement[12,13]. In contrast, Anti-retroviral (ARV) drugs based microbicides have shown improved neutralization and thus are clinically more significant[14]. The main property of microbicide either as semi-solid gels, vaginal films, tablets, or ring is to provide a sustained release of drug over a longertime[15]. Further, Tenofovir (TDF) based intravaginal rings (IVR) provide a controlled release of TDF in to the vagina at

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a rate of $76.4 \pm 54.8\,\mu g g^{-1}$ and remained constant for seven days after the removal of IVR[15]. Duration of drug release is 8 hr for vaginal tablets[16], 6 hr for vaginal gels[17] and 72 hr for vaginal rings[18].

Curcumin, an active principle of unique herbal compound turmeric has been used as the main spice ingredient in most parts of Asian subcontinent since centuries. It exhibits pleiotropic effect like anti-HIV[19], anti-inflammatory[20], anti-oxidant[21], vaginal contraceptive[22] and many more. Curcumin shows concentration-dependent inhibition of sperm motility and complete block at concentration of $\geq 250\,\mu M$[23]. But its hydrophobic nature and low bio-availability apparently put limitations on its use in conventional therapeutic application[24]. Many approaches were attempted to overcome these limitations; and nanoparticle mediated drug formulation is one of approaches investigated. Nanoparticles (NPs) size ranges from 1–100 nm, which themselves act as a whole unit that is capable of transport as well as release of drug[25]. NPs also provide several additional benefits, e.g. protection of the drugs against degradation, facilitates targeted action, and delivery of varieties of biological bits, like peptide, protein and nucleotides. They have the ability to overcome many classical drug delivery challenges such as controlled drug delivery, greater cellular acceptance of poorly permeable drugs, problems of physicochemical stability and reduction of immunogenic response. The therapeutic efficacy and the degree of safety of drugs can be considerably improved by targeted delivery using nanocarriers[26,27]. Many protein based nanoparticles have been already developed for therapeutic uses to treat cancer, AIDS and Parkinson's[28,29].

The main benefit of protein nanoparticles is that, these can be prepare in relatively mild condition without use of any toxic chemicals[29–31]. Protein could be an ideal vehicle for drug transportation in nano-form because its amphiphilic nature helps its cooperation with drugs as well as solvent[32]. Curcumin loaded-apotransferrin nanoparticles are shown to inhibit HIV-1 through inhibition of virus infection and down-regulation of host inflammatory responses[33].

Natural protein such as lactoferrin; is water soluble, metabolizable, biodegradable; Surface modification of such natural protein could be done very easily to facilitate required interaction of drugs and ligands[34]. Lactoferrin protein has several pleiotropic functions like immune modulation, anti-viral, and anti-cancer, and thus can act as first line of defense to inhibit inflammation and infection[35–39].

The objective of our study is to develop a triple-combination topical formulation that can simultaneously act on HIV, HIV-mediated inflammation, other viral and bacterial infections with contraceptive action, based on the principle of multipurpose prevention technologies (MPT). This is a triple combination of broad spectrum lactoferrin (as vehicle) and curcumin as preventive and protective agent and EFV as therapeutic agent against HIV. The principle steps in realizing the objectives of this studies are (1) preparation and characterization of lactoferrin nanoparticles loaded with curcumin and EFV; (2) Studying bioavailability and pharmacokinetic profile of curcumin and EFV in vaginal lavage upon topical application of nanoformulation, and (3) Evaluation of safety of nanoformulation in terms of inflammation.

Results

Preparation and characterization of efavirenz and curcumin loaded lactoferrin nanoparticle.

Curcumin loaded Lactoferrin NPs (Lacto-Cur-nano), Efavirenz loaded Lactoferrin NPs (Lacto-EFV-nano) and Efavirenz plus curcumin loaded lactoferrin nanoparticles (ECNPs) were prepared using sol-oil chemistry as described in materials and methods section. The nanoparticles prepared were characterized through FE-SEM (Field Emission Scanning Electron Microscope), AFM (Atomic-force microscopy), TEM (Transmission electron microscopy) and DLS (Dynamic light scattering). Results presented in Fig. 1 shows that nanoparticles were uniformly dispersed spherical particles with size in the range of 40–70 nm. AFM images provide a three-dimensional surface profile reveal a particular type of projection which may help in binding with the receptor. DLS analysis of blank or drug/s loaded NPs has showed the hydrodynamic size in a range of 40 nm and 91–125 nm respectively. Increased apparent size in DLS is due to the surface water shell that contribute in DLS measurements. The zeta potential of freshly prepared blank or drug/s loaded NP were found to be in a range of -21 to -25 mv respectively (Supplementary Table 1) indicating their stability.

FT-IR spectral analysis. FT-IR spectral data showed the stability of lactoferrin, efavirenz, curcumin and efavirenz plus curcumin combination which remained conserved in their nanoformulation (Fig. 2). All relevant FT-IR peaks related to the soluble and nanoformulation were indicated with black arrows and found to be almost identical with minor deviations in their intensities (Supplementary Table 2).

Assessment of loading efficiency. Loading efficiency of Lacto-Cur-nano, Lacto-EFV-nano and ECNPs were assessed. ECNPs were prepared at four different concentrations of efavirenz by keeping the concentration of lactoferrin and curcumin constant (Table 1). Maximum loading was observed in formulation ratio II for ECNPs ($63\% \pm 1.9$ of Cur. and 61.5 ± 1.6 of EFV), IIA for Lacto-Cur-nano ($59\% \pm 1.34$) and IIIB for Lacto-EFV-nano ($58.4\% \pm 1.79$). This suggests that maximum amount of drugs has been entrapped in protein. It has also been observed that combination of EFV and Cur are synergistic in loading of one drug to the other.

pH dependent release of drugs from NPs. ECNPs were incubated at different pH conditions to mimic the *in vivo* environment of rat vagina; $300\,\mu g$ of drug loaded nanoparticles were incubated with different pH values (1–9) of PBS and simulated vaginal fluid (SVF). Results showed that ECNPs are more sensitive at pH 5 and 6 with maximum drug release observed at pH 5 (Fig. 3). All three types of nanoparticles either in single or combination form showed more than 80% of drug release at pH 5. At pH below 4 and above 6, only 10% of drug release was found. Thus suggests that nanoparticles slowly release drugs in the vaginal lavage in the pH range of 4 to 4.5. Furthermore, higher concentrations of Cur and EFV will be released at $\geq$pH 4.5, a condition where higher virus infectivity was detected in vaginal lavage[40]. In addition, bacterial vaginosis related-pH increase was seen which

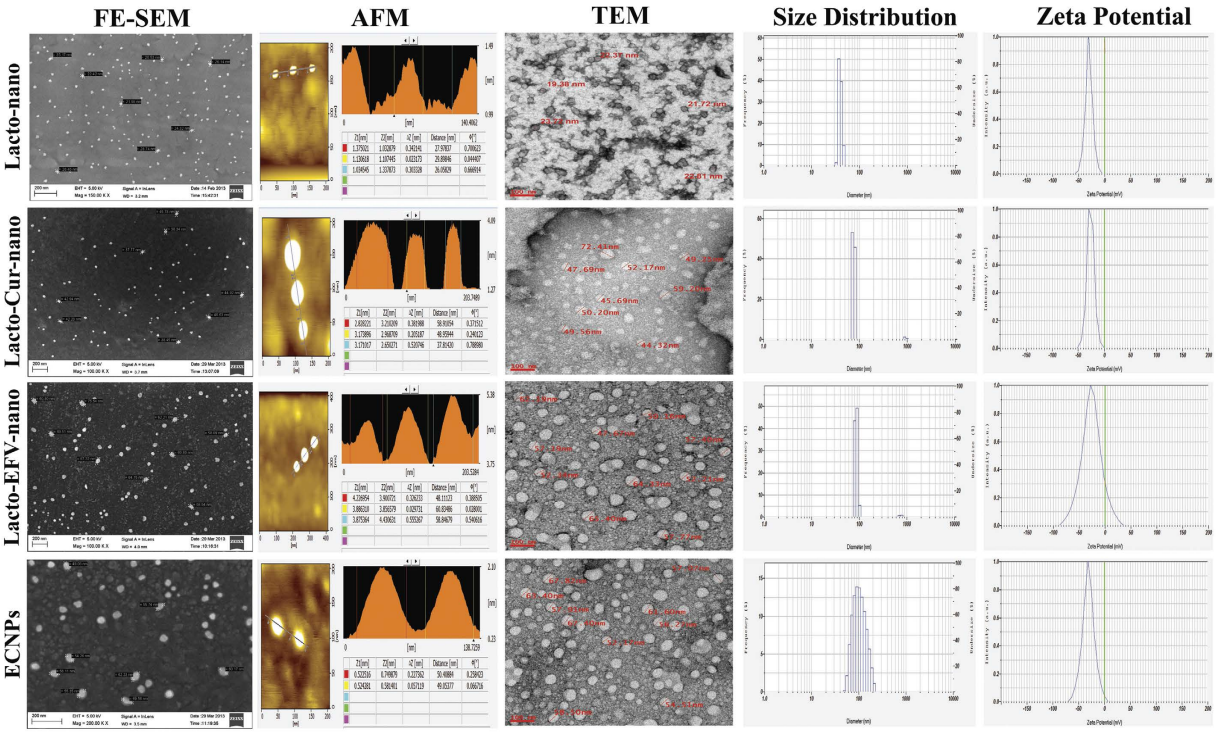

Figure 1. Microscopic and DLS analysis of nanoparticle. Left to right panel indicates the FE-SEM, AFM, TEM, size distribution and zeta potential. Top to bottom; Blank lactoferrin nanoparticle (Lacto-nano), Curcumin loaded lactoferrin nanoparticle (Lacto-Cur-nano or CNP), Efavirenz loaded lactoferrin nanoparticles (Lacto-EFV-nano or ENP), Cur+EFV loaded Lactoferrin nanoparticles (ECNPs).

allows virus shedding[41] and under these pH conditions ECNPs release higher concentrations of cur and EFV thus promoting higher viral neutralizing environment.

Stability studies of nanoparticles-*in vitro*. The stability of ECNPs in PBS (phosphate buffer saline, pH7.4) suspension form was analyzed for at least 20 days at 4 °C and 25 °C. Data presented in Supplementary Table 1 shows that all the four parameters were found to be quite steady at both temperatures. An average negative charge of −25 mV and a PDI of 0.4 indicates the high stability and homogenous colloidal solution property of nanoparticles. The loading efficiency and size distribution of ECNPs were found to be reasonably constant.

Anti-HIV activity of nanoparticles. Virus inhibition in the presence of nano forms either single or in combination remains sustained for a period up to 24 h of prior-exposure to cells while soluble forms show a slight decrease in activity upon this pre-exposure (Supplementary Fig. S1).

Pharmacokinetic (PK) study result in vaginal lavage. Curcumin and efavirenz levels were measured separately in vaginal lavage after administration of single dose of combination drugs either in soluble or nano-form. Drug levels were observed at nine different time points up to 24 h (Fig. 4a). We found enhanced PK profile (Table 2) when drugs were delivered via nanoparticles. The lavage PK shows more than 3-fold increase in AUMC both in the case of efavirenz as well as curcumin. Similarly there was more than 2-fold increase in T_{max} and $t_{1/2}$ (Table 2). AUC values were also seen to be increased by 50% when nanoparticles were used as delivery system. This PK profile suggest the higher bioavailability of drugs when given through lactoferrin nanoparticles.

Evaluation of drug release kinetics of ECNPs. To investigate the extent of availability of drugs over a period of long time, the time-dependent release study (study 2) was performed. The time-dependence was studied individually for the two drugs curcumin and efavirenz delivered in ECNPs; the results are shown in in Fig. 4b. The figure represents that the concentration of curcumin and efavirenz in vaginal lavage at various time point, after one (sol drugs combination) and two (ECNPs) hours of lag phase of drug administration. The results suggest high concentration in the initial stage for both the sol and nano form, which got decreased later exponentially. The nano formulations of drugs even after 2 hr of lag phase showed sustained and significant release of drug up to 12 hr whereas both the drugs in the sol form, showed fast reduction (at 4hr) in the concentration. We have indeed detected the presence efavirenz and curcumin up to 8–12 h, when drugs were given in nanoformulation but in case of the soluble form, the drugs were eliminated in 4–6 hrs.

Viability assay profile of *Lactobacillus*. *Lactobacillus* is the most colonizing bacteria in the vagina, and is generally considered as a gatekeeper of the vaginal ecosystem. Any microbicide could be considered as safe until it is non-toxic to the growth of *Lactobacillus*. *Lactobacillus* naturally produces hydrogen peroxide which provide a

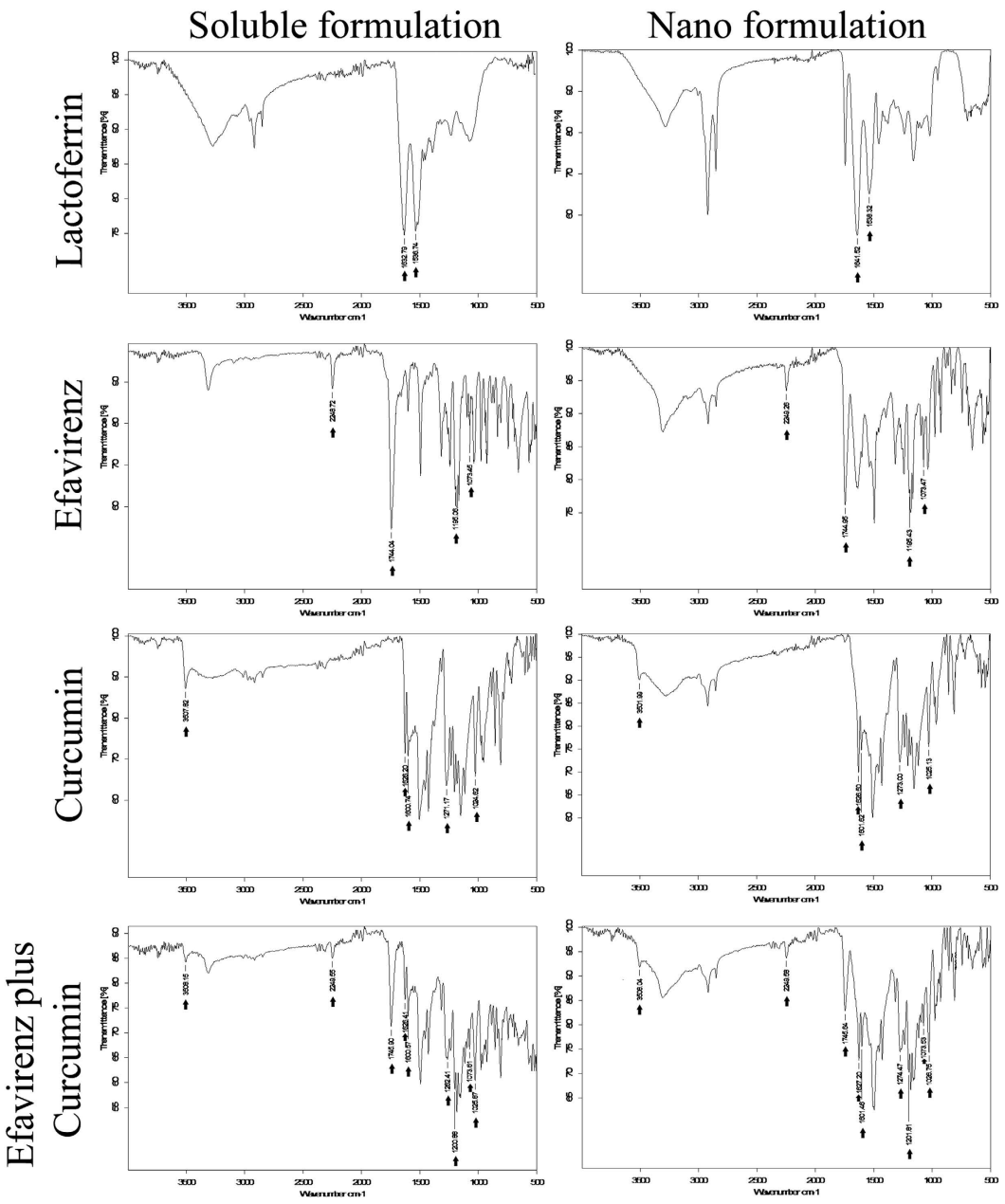

Figure 2. Fourier transform infrared spectroscopy (FTIR) spectral analysis of nanoparticle. Left and right panel indicate the spectra of soluble and nanoformulation respectively. Top to bottom: - Lactoferrin, Efavirenz, curcumin and combination of efavirenz and curcumin. All samples were lyophilized prior to scanning and data were collected using OPUS software.

natural barrier for HIV transmission. From Fig. 4c it is clear that there is no difference in the viability of bacteria between the media control and ECNPs, which confirms that ECNPs could be a safe microbicide.

Dose dependent toxicity and bioavailability studies.

Assessment of efavirenz and curcumin in vaginal lavage. The fixed doses mentioned in dose schedule (study 1) were administered intravaginally and the drugs estimated in lavage are plotted (Fig. 4d). Results suggest that the nano formulation of drugs either in single form or combination showed 1.8 (1st dose) to 2.2 (3rd dose) fold more availability of drugs at the topical site in a dose-dependent manner.

Efavirenz and curcumin concentration in vaginal tissue. Absorption of drugs in local cervical-vaginal tissue has been estimated and represented in Fig. 4e. Efavirenz and curcumin were found in very low concentrations in the range of micrograms, 1000-fold less than vaginal lavage. Cur NPs and EFV NPs didn't show appreciable difference

Formulations	Lactoferrin concentration (mg)	Curcumin concentration (mg)	Efavirenz concentration (mg)	Loading Efficiency of curcumin	Loading Efficiency of efavirenz
LE of ECNPs					
I	40	20	5	47% ± 2.5	49% ± 2.4
II	40	20	10	63% ± 1.9	61.5% ± 1.6
III	40	20	15	57.4% ± 3.2	51.7% ± 2.7
IV	40	20	20	48% ± 2.8	53% ± 3.7
LE of Lacto-Cur-nano					
IA	40	5	0	38% ± 1.45	NA
IIA	40	10	0	59% ± 1.34	NA
IIIA	40	15	0	52% ± 2.2	NA
IVA	40	20	0	49.5% ± 2.5	NA
LE of Lacto-EFV-nano					
IB	40	0	5	NA	41% ± 1.73
IIB	40	0	10	NA	47.6% ± 2.8
IIIB	40	0	15	NA	58.4% ± 1.79
IVB	40	0	20	NA	57.83% ± 2

Table 1. Loading efficiency (LE) of drug(s) loaded lactoferrin nanoparticle. NA: Not applicable.

in exposure of drugs as compared to sol counterpart. But in the case of ECNPs, we found there was 1.2 (1st dose) to 1.8 (3rd dose) fold lower drug concentrations as compared to sol drugs in combination.

Systemic bioavailability of efavirenz and curcumin. Figure 4f represents the nature of accumulation of drugs in the systemic circulation. We found that the sol form of drugs either in single or combination showed 1.5 (1st dose) to 1.7 (3rd dose) fold more accumulation in plasma as compared to its nano form; leading to higher organ-associated toxicity in the case of sol formulation. In the comparison study between the ECNPs and its soluble combination form at the third dose, curcumin and efavirenz were found to be approx. 30 ng/ml & 20 ng/ml and 44 ng/ml & 35 ng/ml respectively, suggest that at higher doses also the NPs could me marked as safe.

Systemic and vaginal Proinflammatory cytokine response. The proinflammatory cytokine markers; interlukine-6 (IL-6) and tumor necrosis factor alpha (TNF-α) levels were estimated in isolated plasma and vaginal tissue. Figure 5a,c represent the IL-6 level in vaginal tissue and plasma respectively. Sol EFV and Nano EFV showed at least three-fold increase in the level of IL-6. When EFV was encapsulated into lactoferrin together with curcumin, the IL-6 levels significantly decreased by 2.5 fold as compared to its sol formulation. At the third dose of ECNPs, we couldn't detect any inflammation, in contrast to Sol (Cur + EFV) which leads to more than three-fold increase of IL-6, both in the vagina as well as in blood. Figure 5b,d represents the TNF-α level in vaginal tissue and plasma respectively. TNF-α is a major cytokine which stimulates the inflammatory response and leads to many autoimmune diseases and other clinical problems. In the case of ECNPs and Lacto-Cur-nano at the third dose, the TNF-α levels was found significantly decreased. These results suggest that nanoformulation provides an effective anti-inflammatory environment along with anti-HIV-1 drug EFV in vagina for protection from infection.

Histopathological analysis. Histopathological studies of cervicovaginal epithelia showed that ECNPs causes a reduced amount of toxicity in a dose dependent manner. The integrity of epithelia was found heavily damaged when the combination of sol drugs were used. In the case of ECNPs, the integrity of tissue was found to be same as that of control or very lesser extent. Tissue section pictures revealed that even at higher dose, ECNPs caused very less amount of tissue damage and at the same time soluble drug combination shows higher tissue damage. Negative (Fig. 6g) and positive controls (Fig. 6h) were treated with saline and nonoxynol-9 respectively. Left panel (Fig. 6a,c,e) represents the vaginal epithelia treated with ECNPs at first, second and third dose respectively. Right panel (Fig. 6b,d,f) represent the epithelia lining treated with Sol (Cur + EFV) at first, second and third dose respectively.

Discussion

While several of the existing MPT have their own limitations[42–45], currently the microbicide based MPT would be one of the finest products which could be adopted against HIV. Our study shows that, Protein nanoparticles formulation comprising of a combination of drugs would form one of the multipurpose prevention technologies (MPT) against HIV transmission. Curcumin and efavirenz loaded lactoferrin were developed using the sol-oil method, with a simple and fast approach without causing any chemical modifications to the drugs or vehicle protein[46]. In this method, a liquid-solid interface is formed from small molecules under macromolecular assemblies. Protein aggregation at this solid-liquid interface was prevented by sonication followed by exposure at low temperature. Particles could be easily precipitated and washed, and at this stage, particles can be stored over very long period even at room temperature. At room temperature this nanoparticle suspension appears as a collection of uniformly dispersed particles (Ex. Colloid). This methodology offers a possibility to produce various materials with novel, predefined properties in a very simple process and relatively at low cost. The main advantage of this

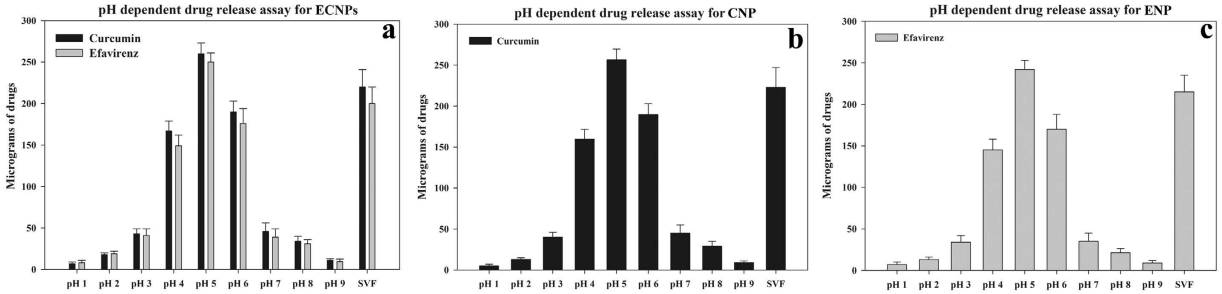

Figure 3. pH and simulated vaginal fluid dependent release profile of curcumin and efavirenz from ECNPs (a), curcumin from CNP (Lacto-nano-curcumin) (b) and efavirenz from ENP (Lacto-nano-efavirenz) (c). SVF represent simulated vaginal fluid. Each data points were repeated in triplicate (n = 3) and presented as Mean ± standard deviation (S.D).

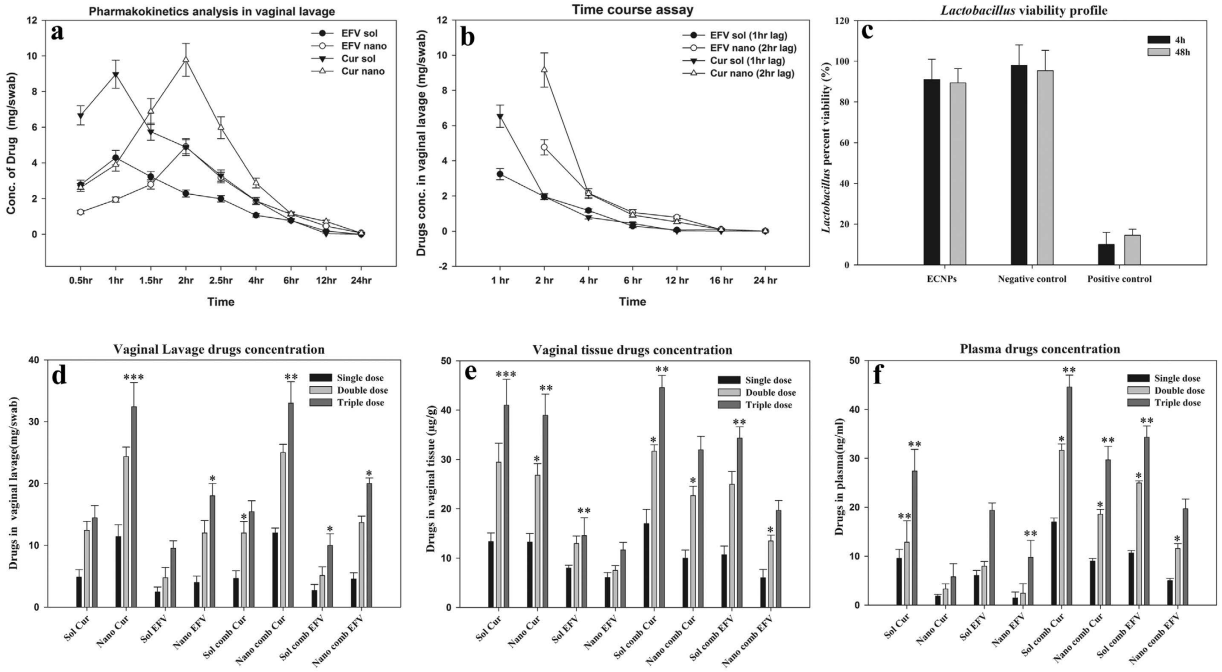

Figure 4. (**a**) Pharmacokinetics study: 20 mg of sol curcumin plus 10 mg of sol EFV and an equivalent amount of ECNPs were applied as single dose in vagina for indicated time points. (**b**) Time course experiment: same drug doses have been topically applied at a lag period of 1 h (for sol EFV + Cur) and 2 hr (for ECNPs). Lavages were collected after these time points and curcumin and efavirenz concentration were calculated separately. Abbreviation: EFV sol and Cur sol – concentration of EFV and Cur when delivered via Sol (EFV + Cur). EFV nano and Cur nano – concentration of EFV and Cur when delivered via ECNPs. (**c**) Viability percentage of *Lactobacillus cripatus* when treated with ECNP, at 4 h and 48 h, media without ECNPs and 1% triton X served as negative and positive control respectively. (**d–f**) Dose-dependent study of drugs in single form or combination form either in soluble EFV + Cur or ECNPs. d, e and f represents the concentration of drugs in vaginal lavage, vaginal tissue and plasma respectively. Sample data were recorded as Mean ± SD, n = 3 and value of significance expressed as ***P < 0.0005, **P < 0.005, *P < 0.05. Abbreviation: EFV-comb-sol & Cur-comb-sol: - efavirenz and curcumin concentration delivered as soluble combination. EFV-comb-nano & Cur-comb-nano: - efavirenz and curcumin concentration delivered via ECNPs. EFV-sol: Soluble EFV. Cur-sol: Soluble Cur. EFV-nano: EFV released form ENP. Cur-nano: - Cur release form CNP.

method is to produce particles with very high purity and uniform nanostructure as assessed through FE-SEM and AFM analysis. In both types of nanoformulation, either blank or drug/s loaded, the DLS sizes were found to be greater than their SEM or AFM sizes (Fig. 1). The reason for this is quite rational because here the size was measured using light scattering method that could be affected by interaction of nanoparticle with the water shell and charge present on the surface that may lead to overall increase in size. ECNPs with a polydispersity index (PDI) of 0.435 are found to as homogeneous mixture. ECNPs was found to be more stable up to 20 days, as the parameters

Parameters	Units	EFV*		Curcumin#	
		ECNPs	Sol (EFV + Cur)	ECNPs	Sol (EFV + Cur)
AUC	(h)*(mg/ml)	20.4164	13.7581	32.4251	21.9066
AUMC	(h)^2*(mg/ml)	132.482	52.448	172.922	56.496
$\mathbf{C_{max}}$	mg/mL	4.9126	4.2897	9.7794	8.9723
$\mathbf{T_{max}}$	hr	2	1	2	1
$\mathbf{t_{1/2}}$	hr	4.63222	2.14916	3.97347	1.8513

Table 2. Pharmacokinetic profile in vaginal lavage. Pharmacokinetic parameters. AUC: The integral of the concentration-time curve (after a single dose or in steady state). AUMC: Partial area under the moment curve between t start and t end. C_{max}: The peak plasma concentration of a drug after oral administration. T_{max}: Time to reach C_{max}. $t_{1/2}$: The time required for the concentration of the drug to reach half of its original value. *EFV credentials measured individually when delivered via ECNPs and Sol (EFV+Cur) respectively. #Cur credentials measured individually when delivered via ECNPs and Sol (EFV+Cur) respectively.

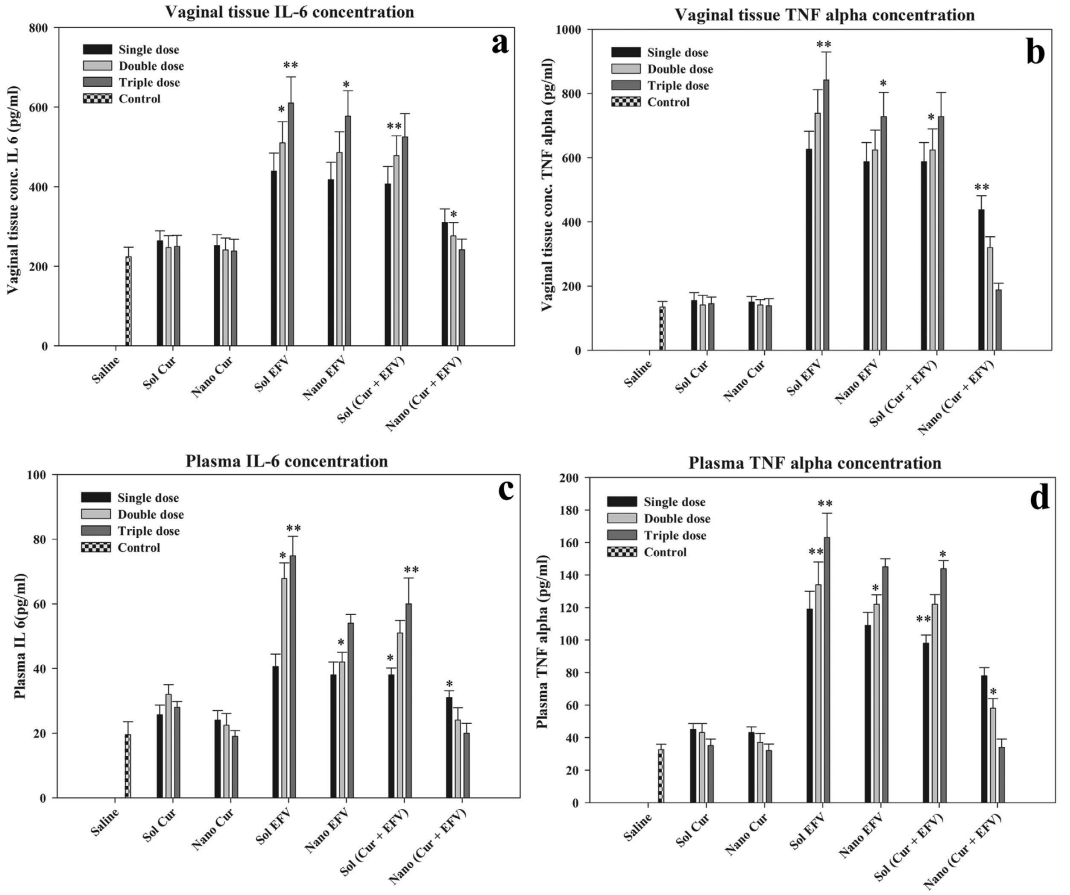

Figure 5. Dose dependent cytokine response. (**a**,**c**) Represent the IL-6 concentration in vaginal tissue and plasma respectively. (**b**,**d**) Represent the TNF-α concentration in vaginal tissue and plasma respectively. Sol Cur, Sol EFV, CNP, ENP, Sol (Cur + EFV) and ECNPs were applied topically in vagina and IL-6 and TNF-α were estimated. Value of significance ***$P < 0.0005$, **$P < 0.005$, *$P < 0.05$.

related to stability are found to be quite constant. This shows the versatile character of lactoferrin nanoparticles (Supplementary Table 1).

FT-IR data suggest the stability of efavirenz and curcumin in their nano form, as the majority of characteristics bands are still conserved (Fig. 2). In case on ECNPs all the major functional groups related to curcumin, efavirenz and lactoferrin were remains constant or little shift in the peaks may be observed. This shift is caused by the dipole moment present in the molecules, as the bond characteristics is electrostatic in nature. Results of this study showed that more than one drug can be encapsulated into the protein. Furthermore the study show a co-operativity in loading by synergistic action and molar equivalences. Since HIV-1 can acquires resistance to different combination of drugs and such a resistance in some cases is patient-dependent and this necessitates the design and development of formulation that include multiple drug regimens with multi-targeting capabilities.

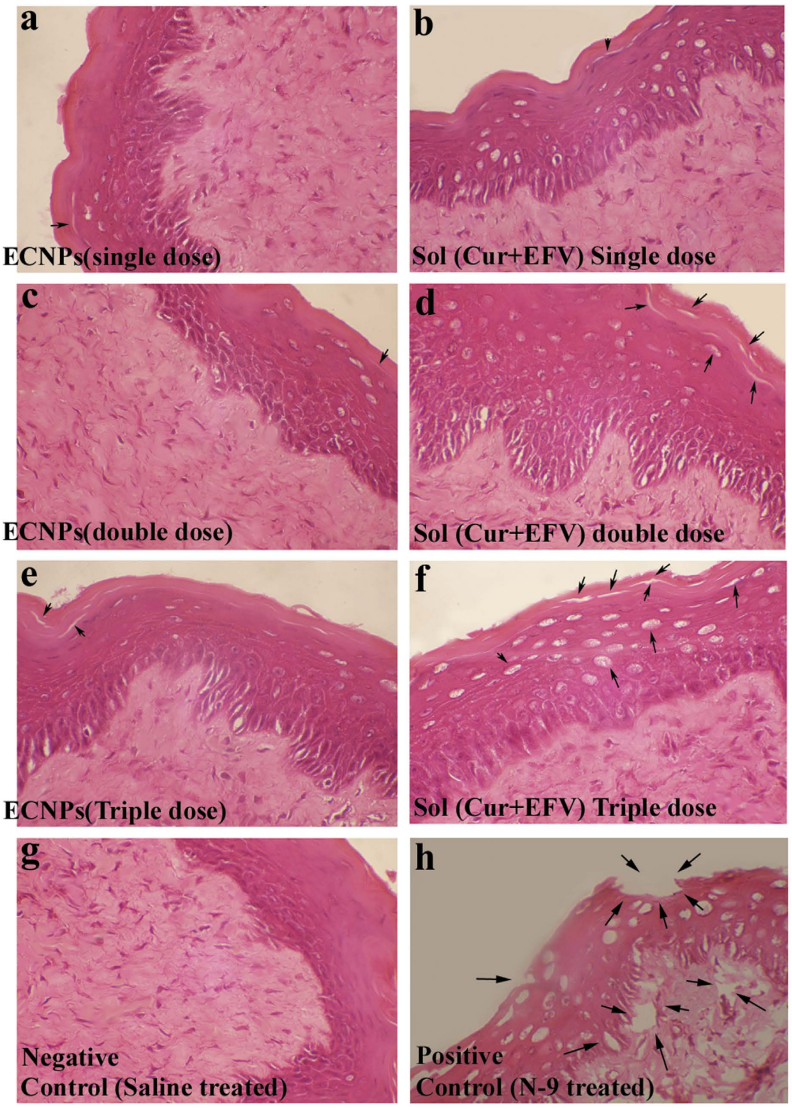

Figure 6. Histopathology vaginal tissue Hematoxylin and Eosin staining images. Left panel (**a**,**c**,**e**) represent the vaginal epithelia treated with ECNPs at first, second and third dose respectively. Right panel (**b**,**d**,**f**) represent the vaginal tissue integrity when applied topically with Sol (Cur+EFV) at first, second and third dose respectively. Damaged site or lesion were shown through a printing arrow. Negative (**g**) and positive (**h**) Controls were treated with saline and nonoxynol-9 (10 mg/kg) respectively.

As curcumin and efavirenz exhibits very poor aqueous solubility, encapsulating these two drugs onto lactoferrin could solve the major issue of solubility limitation and the ECNPs designed, thus targets with its triple broad spectrum action comprising of curcumin, efavirenz and lactoferrin. Besides its anti-inflammatory properties, curcumin activity helps in inhibition of HIV-1 replication by binding to integrase and protease[47] thus providing additional specificity to formulation. While efavirenz targets the Reverse transcriptase enzyme and the lactoferrin blocks virus entry[48]. In addition, lactoferrin acts as an immune modulator[38] and exhibit broad spectrum anti-microbial activities[36]. Thus, this formulation would serve as a typical broad spectrum HIV microbicide. The main observation is the antiviral activity was enhanced for ECNPs when compared to their soluble and nanoforms individually (Supplementary Fig. S1). Here the maximum activity was obtained that is up to 98% even when the individual concentration of Curcumin, Efavirenz and Lactoferrin was reduced to half. This gives the advantage of using the drugs in combination with the nanoform there by reducing the dosage leading to the prevention of unwanted side effects and toxicities. The emergence of multi-drug resistance has led to an increase in failure of HIV treatment[49]. The vaginal lavage bioavailability studies showed an enhanced PK profile of drugs. The efavirenz and curcumin AUC (area under the curve) were found to increase by more than 50% when delivered via nanoparticles, thus pointing out that drug in nanoformulation gets exposed over long time to the vagina as compared to soluble formulation thus facilitating a giving long lasting protection against HIV. Similarly the other PK parameters such as T_{max} and $t_{1/2}$ show a 2-fold increase, suggesting an enhanced stability of encapsulated drugs in nanoparticles (Table 2). Further, in the time course study ECNPs showed minimum burst effect suggesting that

the drugs were released slowly and steadily Results of two hr. time lag indicated better performance (for nano drugs) as compared to one hour (for sol drugs) (Fig. 4b). ECNPs were found to release drugs up to 8–12 h while in soluble combination 70% of drug were eliminated from application site rapidly. The reason behind this could be that the nanoparticles are getting adsorbed on to the surface of vaginal epithelium and then releasing the drugs steadily into lavage[50].

Lactoferrin is also found in its natural form in human vaginal secretion with premenstrual concentration of 3.8–11.4 μg/mg which after menopause increases up to 62.9–218 μg/mg[51]. Owing to such abundance in vaginal secretions, lactoferrin in ECNPs, may escape its recognition by system, thus is not the source of inflammatory response. Furthermore, curcumin down regulates inflammatory responses induced by EFV, which may be one of the reasons of low inflammatory response as compared to soluble combination in single dose as well as in multiple dose application (Fig. 5). Thus during ECNPs topical application, the major inflammation caused by EFV is not further enhanced and indeed is neutralized by the presence of lactoferrin together with curcumin. Hence, EFV will get a hassle free environment and it will exhibit maximum antiviral properties in lavage[52]. Lower concentrations of drugs were present in systemic circulation viz. nanograms of drugs concentrations, which was 10^{-6} fold lower than that observed in lavage and 10^{-3} fold lower than that in vaginal tissue and at these concentrations EFV may not show any significant toxicity. In contrast when soluble drugs are employed significant amount of drug was found to be present in the plasma as well as in tissue indicating the risk of using soluble EFV for vaginal application. Due to low toxicity to the *Lactobacillus* exhibited by ECNPs, there may not be any threat to vaginal microflora if ECNPs are used, which is a key marker for the safety of any microbicide (Fig. 4c). Furthermore, ECNPs delivered more quantity of drugs as compared to its soluble counterpart at all the dose levels. The important feature of microbicide based drug usage is to ensure sustained and controlled drug delivery in the vaginal environment without altering the vaginal epithelium, a critical requirement that could be achieved by ECNPs. Previous studies show that intravaginal application of microbicide causes the toxicity of local cervicovaginal epithelial tissue which damages it and finally leads to loss of its integrity[53]. Damaged tissue causes the more exposure to virus and leads to more viral invasion and infection.

In conclusion, lactoferrin loaded curcumin and EFV nanoparticles serves as control release formulation with low toxicity and higher bioavailability.

Conclusion

In this study, curcumin and efavirenz were successfully encapsulated through sol-oil method and used as a controlled delivery system through vaginal route as a microbicide. The EE% and drugs release assay showed that vaginal pH is favorable for drug release. ECNPs showed less toxicity to *Lactobacillus cripatus* and vaginal epithelial tissue and release active drug entities in the vaginal lavage. In summary, lactoferrin loaded curcumin and EFV nanoparticles serves as a highly effective and efficient multi-protective and microbicide formulation.

Materials and Methods

Reagents. Curcumin and efavirenz were of 94% and 98% purity respectively. Lactoferrin was purchased from Symbiotics (Irvine, CA 92614 USA). Extra virgin olive oil was from Leonardo. All reagents and chemicals used were of analytical grade. IL-6 and TNF-α measurement kit were purchased from BD bioscience with cat no. 550319 and 558535 respectively

Animals. In this study we have used healthy female albino Wistar rats. Experiments were carried out in accordance with the approved guidelines. All experimental protocol were approved by Institute Animal Ethics Committee, University of Hyderabad. Except rats used for tissue isolation, all other rats were reused after a wash period of 48 hours.

Protein nanoparticle preparation. Lactoferrin nanoparticles (NPs) were prepared with the combination of curcumin and efavirenz through sol oil chemistry (patent filed)[26]. 40 mg of lactoferrin was dissolved in 500 μl ice cold PBS pH7.4. In a separate tube, 20 mg of curcumin was dissolved in 100 μl of DMSO and varying concentration (5, 10, 15, 20 mg) of efavirenz was dissolved in 100 μl of DMSO. Drug mixture was added to lactoferrin and incubated on ice for 1 hr. The sample was gently added to 25 ml of ice cold olive oil under continuous stirring. The mixture was sonicated on ice for 15 minutes using ultrasonic homogenizer with a pulse period of 30 sec and an amplitude of 5 μm at an interval of one min between sequential pulses. This mixture was frozen in liquid nitrogen for 15 min followed by 4 hr incubation on ice. The mixture so formed was centrifuged at 6000 g for 15 min; supernatant (containing olive oil) was discarded and the pellet was extensively washed with ice-cold diethyl ether to remove traces of oil. The pellet obtained was resuspended in PBS. The same protocol was followed for the preparation of individual nanoparticles of curcumin or efavirenz in different ratios mentioned in Table 1. Four different types of NPs were prepared (Table 1) for the present study; 1. Lactonano (without any drug), 2. Lacto-nano-EFV + Cur (ECNPs), 3. Lacto-cur-nano (CNP) and 4. Lacto-EFV-nano (ENP).

Characterization of nanoparticles. Nanoparticles assembled were morphologically characterized using Field Emission Scanning electron microscope (FE-SEM) (Philips XL-30), Atomic Force Microscopy (AFM), Transmission electron microscopy (TEM). For SEM, particles were coated with gold. For TEM, samples were fixed on 200 mesh type-B copper grid coated with carbon (TED PELLA, INC.) and staining was done using 2% Uranyl acetate. Particles characterized through AFM (SPM400) were spin coated on a cover slip. Hydrodynamic radaii were measured using nanopartica nanoparticle analyzer, SZ-100 (Horiba Scientific).

Fourier transform infrared spectroscopy (FTIR) of nanoparticles. FT-IR studies have been performed using ALPHA's Platinum ATR single reflection diamond ATR module (Bruker Corporation). Briefly one

to two milligrams of lyophilized samples were directly kept on the sample holder and scanned from 500 cm^{-1} to 4000 cm^{-1}. The spectra were visualized using OPUS software. Base line correction has been done according to manufacturer instruction.

Calculation of loading efficiency. Nanoparticles were incubated with 1 ml of 1X PBS (pH5) and kept under gentle rocking for 30 minutes at room temperature. 100 μl of 30% silver nitrate was added to resulting mixture to precipitate the protein. Then 1 ml of HPLC grade methanol was added to the mixture and this was centrifuged at 12000 rpm for 15 min and concentration of drugs in supernatant were estimated. Supernatant was analyzed in triplicate. Standard curve was developed using the different concentrations of curcumin and efavirenz through HPLC respectively. Loading efficiency was calculated as per equation (1).

$$\text{Loading Efficiency}(\%) = \frac{X_{\text{loaded}}}{X_{\text{total}}} \times 100 \tag{1}$$

$$X_{\text{total}} - X_{\text{lost}} = X_{\text{loaded}} \tag{2}$$

where X_{loaded} = amount of drug loaded, X_{total} = amount of total drug used and X_{lost} = amount of drug lost during preparation.

In vitro experiments.

Analysis of sensitivity of NPs under different conditions of pH and simulated fluid. Nanoparticle pellet were resuspended in 1 ml of 1X PBS (of different pH values in the range 1–9) and simulated vaginal fluid; These were then kept for incubation on rocker at room temperature for 4 h. Then 300 μl of 30% silver nitrate was added and drugs were extracted by adding 1 ml of methanol. These were centrifuged at 12000 rpm for 15 min and the supernatant was filtered using 0.2 micron syringe filter and analyzed by using HPLC. Every experiment was conducted in triplicate.

In vitro stability profile of nanoparticles. The stability profile of nanoparticles was performed for ECNPs in suspension form and represented in Supplementary Table 1. The stability was measured in terms of the loading efficiency, size distribution, zeta (ζ) potential and polydispersity index (PDI). Suspension ECNPs were incubated for indicated time points such as 0, 1, 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 days at two different temperature (4 °C and 25 °C). Loading efficiency was calculated according to equation (1). Size distribution, PDI and zeta potential were measured using dynamic light scattering methods.

Anti-HIV assay. Anti-HIV-1 activity of Lacto-Cur-nano, Lacto-EFV-nano and ECNPs along with their soluble counterparts was analyzed using HIV-1 NL4-3 virus clone. The concentrations of Curcumin, Efavirenz and Lactoferrin were taken as 10 μM, 2 nM and 1 μM respectively. But the concentrations of all the drugs and Lactoferrin were reduced to half in the preparation of ECNPs The experiment is conducted by adding drug to Sup T-1 cells and incubating for 1, 3, 4 and 8 hours respectively. After incubation, cells were washed and challenged with HIV-1_{NL4-3} and incubated for 5 days and virus replicated was estimated at day 5 using HIV-1 p24 antigen capture assay (ABL). Based on the virus replicated in the control in absence of drug, drug efficacy was measured in-terms of percent inhibition of HIV-1 replication.

In vivo experiments.

Animal experimental design. Animals (0.160–0.240 kg, 6months) were housed for seven days prior to experiment at the animal housing facility, University of Hyderabad for 12 h in a light/dark condition. Animals were euthanized using sodium pentobarbital (50 mg/kg, IP) and final sacrifice was done by cervical dislocation. Blood was collected through heart puncture during terminal anesthesia. All the animal experiments were done in triplicate. All the experiments were performed on healthy female rats. 54 rats were used for pharmacokinetics (PK) study (study1) and these were reused for study 3 after a wash period of 48 h. 6 rats were used for time course bioavailability studies (study 2), and among these, 3 rats were randomly selected and reused for study 3. Study3 (dose dependent toxicity and bioavailability studies) were performed on pooled rats from study 1 and 2. An outline of all the animal experiment is provided in Supplementary Fig. S2.

Dosage schedule. For studies 1 to 3, drugs used were in the combination of 20 mg of curcumin (sol curcumin) plus 10 mg of efavirenz (sol efavirenz) and an equivalent amount of combination nanoformulation per rat. For single drug, curcumin (20 mg) or EFV (10 mg) was used either in sol or nano form. The best formulation ratio such as II, IIA and IIIB as given in Table 1 were used for this study. Nano formulation of drugs used in this experiment was equivalent to its sol form. All the drugs were topically applied in vagina. Saline treated rats were considered as control.

Pharmacokinetic assessment in vaginal lavage. For study 1, Single dose of curcumin plus efavirenz either in sol combination or nano-combination as mentioned in the dosage schedule were topically applied in vagina to different groups of animals. After completion of various time points such as 0.5, 1, 1.5, 2, 2.5, 4, 6, 12 and 24 h, vaginal lavage was collected using sterile cotton swabs. Further, the swabs were mixed with methanol and the drug was extracted. Drugs were quantified using HPLC at the above mentioned time points; pharmacokinetics parameters were calculated using Kinetica v.5 software.

Time course experiment in vivo. Sustained release of drugs in vaginal lavage: For study 2, all the animals were administered with single dose of Sol (EFV+Cur) and nano (EFV+Cur) or ECNPs. On the basis of T_{max} obtained from PK study, the time course study was performed with a lag period of 1 hr (for sol drugs) and 2 hr (nano drugs). The vaginal lavages were manually collected at fixed time points viz. 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, 16 h and 24 h with sterile cotton swabs. Drugs present in swabs were extracted in methanol and estimated for efavirenz and curcumin separately using HPLC.

Lactobacillus viability assay. To evaluate the effect of ECNPs on the growth of *Lactobacillus crispatus*. Lactobacillus viability test was performed according to standard protocol[54]. The Bacterial density of 0.06 (OD at 670 nm) or 108 CFU/ml (100 μl) was seeded into a sterile 96 well plate and incubated with 100 μl of 1 mg/ml of ECNPs at 37 °C. Media without ECNPs was considered as negative control and triton X (1%) as a positive control. After 4 and 48 h of incubation, 20 μl of MTS reagent was added and absorbance was measured at 490 nm. The percentage viability was calculated using the formula; viability percentage = (absorbance of Test sample/absorbance of Control sample)/100. Where absorbance of Test and Control samples is represented by the quantity of formazan reduced by viable cells.

Dose dependent toxicity and bioavailability studies. For study 3, a dose dependent study was performed to examine the novelty of nanoparticles as a microbicide. The study was carried out to compare the effect of sol and nano form of drug either in single or combination form. In total six type of formulations, such as Sol Cur, Nano Cur, Sol EFV, Nano EFV, Sol (Cur+EFV) and ECNPs were used and the study was carried out up to three doses. Doses were applied according to the schedule described below at an interval of 2 h between each dose. After the completion of each dose, rats were sacrificed under proper anesthetic condition. Vaginal lavage, blood, and vaginal tissue were collected. Blood was collected through cardiac puncture. Plasma was isolated from blood and used for the estimation of drugs. Vaginal tissues were collected and used for the estimation of inflammatory markers. Cytokines (IL-6 and TNF-alpha) levels were estimated in plasma and in the vaginal tissue homogenate. A small part of vaginal tissue were saved in 10% neutral buffered formalin and further processed for histopathology through Hematoxylin and Eosin tissue staining. Major part of vaginal tissues were homogenized in PBS using homogenizer. After final processing of vaginal tissues, blood plasma, and vaginal lavage; concentrations of curcumin and efavirenz accumulation were determined using HPLC.

Drug extraction from vaginal tissue. The Vaginal tissue collected was homogenized in PBS using rotor stator homogenizer for 15 minutes. 300 μl of 30% silver nitrate per ml was added to the tissue extract to precipitate the protein. Drugs were extracted using 3 ml of HPLC grade methanol. Then curcumin and efavirenz were estimated through HPLC at 425 nm and 247 nm respectively.

Mobile phase chromatographic condition. A reverse phase C18 column (25 cm × 4.60 mm, particle size 5 μm) (Purospher® STAR RP-18 endcapped (5 μm) Hibar® RT 250-4.6, column No. 148837, Merck Millipore) was used for HPLC analysis. The mobile phase for efavirenz and curcumin were as follows. For efavirenz, mobile phase consists of 25% of 0.1% formic acid (Milli-Q water, pH 3.2) and 75% of acetonitrile, and the flow rate was set to 0.3 mL/min at ambient temperature[55]. For curcumin the mobile phase is composed of acetonitrile: 5% acetic acid in the ratio of (75:25, v/v)[56]. All solvents were filtered using 0.4 μm nylon syringe filter and degassed prior to use. 10 μl of sample were analyzed for detection of EFV and curcumin.

Histopathology study. The Histopathology study was done only for combination form of drugs either in soluble form or nanoformulation (ECNPs). Doses of soluble drugs in combination and ECNPs were given as described in dose schedule (n=3). Negative and positive controls animals were treated with saline and 10 mg/kg nonoxynol-9 (N-9) respectively. Multiple doses were repeated up to three doses at time gap of 2 h. After the completion of measurements at the time points, animals were sacrificed under standard protocol. Vaginal tissue was fixed in 10% Neutral Buffered Formalin, followed by sectioning using cryomicrotome and Hematoxylin& Eosin staining. All pictures included in the results section were taken at 100× zoom using Olympus BX51P polarizing microscope.

Statistical analysis. All studies were performed in triplicate. Data were presented as mean and standard deviation. The significance difference were calculated using one way ANOVA. The level of significance was used as $^{***}P < 0.0005$, $^{**}P < 0.005$, $^{*}P < 0.05$.

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Author Contributions

P.K. and A.K.K. have conceived and designed the experiments. Y.S.L. and P.K. performed the experiments. K.G. and Y.S.L. have analyzed the data. ELISA experiment were performed by C.B., A.K.K. and P.K. wrote the manuscript.

Additional Information

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RESEARCH ARTICLE

Improved Safety, Bioavailability and Pharmacokinetics of Zidovudine through Lactoferrin Nanoparticles during Oral Administration in Rats

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Abstract

Zidovudine (AZT) is one of the most referred antiretroviral drug. In spite of its higher bioavailability (50–75%) the most important reason of its cessation are bone marrow suppression, anemia, neutropenia and various organs related toxicities. This study aims at the improvement of oral delivery of AZT through its encapsulation in lactoferrin nanoparticles (AZT-lactonano). The nanoparticles (NPs) are of 50–60 nm in size and exhibit 67% encapsulation of the AZT. They are stable in simulated gastric and intestinal fluids. Anti-HIV-1 activity of AZT remains unaltered in nanoformulation in acute infection. The bioavailability and tissue distribution of AZT is higher in blood followed by liver and kidney. AZT-lactonano causes the improvement of pharmacokinetic profile as compared to soluble AZT; a more than 4 fold increase in AUC and AUMC in male and female rats. The serum C_{max} for AZT-lactonano was increased by 30%. Similarly there was nearly 2-fold increase in T_{max} and $t_{1/2}$. Our in vitro study confirms that, the endosomal pH is ideal for drug release from NPs and shows constant release from up to 96h. Bone marrow micronucleus assay show that nanoformulation exhibits approximately 2fold lower toxicity than soluble form. Histopathological and biochemical analysis further confirms that less or no significant organ toxicities when nanoparticles were used. AZT-lactonano has shown its higher efficacy, low organs related toxicities, improved pharmacokinetics parameter while keeping the antiviral activity intact. Thus, the nanoformulation are safe for the target specific drug delivery.

Introduction

Zidovudine is the first drug approved for the treatment of HIV infection and is still in the part of the first line regimen in Highly Active Antiretroviral Therapy (HAART) [1]. Despite of its efficacy, the factors that limit its clinical use are its toxicity, suboptimal bioavailability and

pharmacokinetics which includes bone marrow aplasia [2], inhibition of mitochondrial machinery [3], short plasma half-life [4] and high hepatic first-pass metabolism [5] etc. This will eventually lead to the increase in the frequency and dosage of the regimen resulting in the unwanted side effects that compromise in the adherence to the antiretroviral treatment. AZT (300 mg twice a day oral 1 mg per kg intravenous infusion over 1 hour every 4 hours), was the first drug approved by the Food and Drug Administration (FDA). Commercially, AZT is available in various forms such as capsules, tablets, syrup and intravenous injection. Further, FDA has approved the fixed tablet formulation of AZT for HIV naive patient in combination with other ART drugs, these includes *Combivir* (300mg AZT plus 150mg lamivudine) and *Trizivir* (300mg AZT plus 150mg lamivudine plus 300mg abacavir) [6]. This necessitates zero order and targeted delivery of AZT since excess plasma concentration occur immediately after its administration [7]. Hence, a successful treatment of HIV infection requires a uniform systemic level of the drug throughout the course of the therapy.

Apart from T-lymphocytes, reticuloendothelial cells namely monocytes and macrophages act as major reservoirs for HIV and are thought to be responsible for its distribution throughout the body and brain [8–11]. So a sterilizing cure for HIV is not possible without the access of drug regimens into macrophage system. In addition, AZT is reported to be a substrate of diverse drug efflux mechanisms present in cells of CNS, Immune system and Intestinal epithelium which is mainly mediated by ATP binding cassette (ABC) family of proteins viz., P-glycoprotein [12]. This consequently will lead to the evolution of drug resistant strains. This is one of the main reasons of the intra- and inter-patient variability and nonlinearity observed in the bioavailability of AZT [13,14]. To circumvent all of the above limitations associated with AZT delivery various methods were employed that encompasses delivery as prodrugs, encapsulation in polymeric and non-polymeric nanocarriers like nanoconjugates, micelles, surface engineered liposomes, SLNs etc. [15–18]. The various advantages in using nanoparticles as delivery vehicles are their size, surface charge, large surface area to volume ratio, stability, multifunctional and biomimetic properties [19–21].

In spite of having so much of beneficial record of AZT [22], the previous (pharmacokinetics) PK studies showed that, AZT is responsible for the bone marrow toxicity [23] which leads to bone marrow suppression [24] and finally results to various alteration related to hematopoiesis [25–27]. Further AZT has been proven as a genotoxic compound [28,29] which cause the induction of micronuclei in the mouse bone marrow cells [30].

Earlier research in our lab involved in the development of nanoformulation of doxorubicin, carboplatin and curcumin with transferrin family of proteins namely apotransferrin and lactoferrin and these were successfully applied for the treatment of hepatocellular carcinoma in rats and HIV-1 infection in cell line [31–35]. Since oral administration is the best modality available for the drug delivery, the present work involves the improvement in the delivery of AZT using the lactoferrin as nanocarrier system. So the advantage with the present nanoformulation of AZT with lactoferrin is bidirectional, where the carrier itself has antiviral activity along with AZT itself. The present work showed the improved safety, bioavailability and pharmacokinetics of AZT encapsulated in lactoferrin nanoparticles (AZT-lactonano).

Materials and Methods

Materials

Lactoferrin and olive oil used for preparation of nanoparticle were purchased from Symbiotics (USA) and Leonardo (Italy) respectively. AZT used was the pharmaceutical production of Sigma. All other reagents are of molecular biology grade. 24 well plates were from Corning (USA). For the administration of drugs 18 Standard Wire Gauge bend oral dosing/gavage

needle was used. Syringe filters were purchased from Pall, HPLC (Waters) was used for the estimation of drug. All Safety analysis experimental kits were purchased from Span diagnostics India and Cayman chemical USA. p24 ELISA kit was purchased from ABL (USA).

Animals

All the animals (Wistar rats) used in this study were approx. 6 months old and 0.160 kg to 0.250 kg weight, obtained from Sainath Agencies Hyderabad. Animals were housed in animal house facility of University of Hyderabad and all animal experiments were conducted as per the approval from Intuitional Animal Ethics Committee, University of Hyderabad.

Preparation of Lactoferrin Nanoparticles

AZT loaded lactoferrin nanoparticles were prepared through sol-oil chemistry [31]. 10 milligram of AZT was dissolved in 1000μl of Milli-Q water and was gently mixed with 40 mg of lactoferrin dissolved in 1ml of ice cold phosphate buffer saline (pH 7.4). Mixture was incubated in ice for an hr. Then it was slowly added to 25ml of olive oil with gentle vortexing. The sample was sonicated for 15 min at 4°C with the help of narrow stepped titanium probe of ultrasonic homogenizer (300V/T, Biologics Inc., Manassas, Virginia, USA). Resulting mixture was immediately transferred in liquid nitrogen for 10 min then thawed on ice for 4hr. Particles formed were centrifuged at 6000 rpm for 10 min at 4°C. Pellet formed was extensively washed twice with ice cold diethyl ether (to completely remove oil) and then dispersed in 1ml of PBS.

Nanoparticle Characterization

Nanoparticle morphology were examined through two different methods, field emission scanning electron microscope (FE-SEM, Philips FEI-XL 30 ESEM; FEI, Hillsboro, OR, USA) operated at 20 KV, and Atomic force microscope (AFM; SPM400). Gold coated Nanoparticles were used for FE-SEM and in AFM where samples were spin coated on glass slides. The characterization was done according to the protocol described as per manufacturer's instructions.

FT-IR Spectral Analysis (Drug-Nanoparticle Interaction Study)

Spectral analysis were done using KBr pellet method. All the nanoparticles were lyophilized before FT-IR analysis. 0.1 to 1.0% sample was well triturated into 200 to 250 mg of KBr in a mortar. Samples were then transferred to 13 mm-diameter pellet forming die and very high pressure (1000 kg/cm^2) was applied under vacuum; resulted to a transparent pellet. The pellet was placed on sample holder and scanned from wave number 4000 to 400. Spectral analysis was performed using FT-IR dedicated OMNIC series software on a windows 7 platform.

Encapsulation Efficiency

Encapsulation efficiency was measured by mixing 50μl of nanoparticles and 950μl of 1X PBS (pH5) and incubated for twelve hours on rocker. After incubation, 200μl of 30% Silver nitrate was added and vortexed. 1ml of methanol was added, mixed thoroughly and centrifuged at 16000rpm for 10min at 4°C. After centrifugation supernatant was collected and filtered through 0.2 micron syringe filters and then filtrate was used for estimation by HPLC (Waters) at 270 nm. Mobile phase composition used was acetonitrile: methanol (60:40 v/v) [36]. 10μl of sample was injected at the flow rate of 1ml per min. The encapsulation efficiency (EE %) was

calculated using the following formula

$$\text{Encapsulation Efficiency } (\%) = \frac{M_{total} - M_{lost}}{M_{total}} \times 100$$

Here M_{total} is the total amount of AZT entrapped during AZT-lactonano preparation and M_{lost} is the amount of AZT unavailable after release from nanoparticles.

pH Dependent Drug Release Assay and Percent Release of Drug

600μg of AZT-lactonano were incubated for 12h with 1ml of 1X PBS (pH 2 to 8), SGF (simulated gastric fluid) and SIF (simulated intestinal fluid); 200 μl of silver nitrate was added to precipitate the protein and drug was extracted by adding methanol, centrifuged and supernatant was estimated for AZT using HPLC. To measure the percent release of AZT from nanoparticles, AZT-lactonano were incubated with 1ml of PBS (pH 5.0 and 7.4). At different time interval aliquots were withdrawn, and estimated for the presence of AZT.

In Vitro Stability Study

The *in vitro* stability testing was performed for drug loaded nanoparticles (AZT-lactonano) in PBS (pH 7.4) (NP solution). The stability study was carried out in terms of quantity of drug present in the nanoparticles using HPLC and diameter. Freshly prepared nanoparticles were incubated for various time points such as 0, 1, 2, 4, 6, 8, 10, 12, 16, 24, 48, 72 and 96h at two different temperature (4°C and room temperature). Drug content was quantified using the protocol mentioned in drug release assay section. The size of above incubated nanoparticles were measured using FE-SEM.

Animal Study Design

Male and female Wistar rats of 6 months old were acclimatized for a week at animal house facility, University of Hyderabad under 12h light/dark cycle. For study, animals were divided into seven different groups according to seven time points i.e., 30min, 1h, 2h, 4h, 8h, 16h, 24h. Each group contains 3 males and 3 females and drug was administered orally to all animals. Rats were administered with 10mg/kg body weight of sol AZT and equivalent of AZT-lactonano. Each group was subjected for above mentioned time points. Animals were monitored hourly after oral administration of drugs. No death was observed during the experimentation. After completion of respective time points, animals were euthanized using sodium pentobarbital (50 mg/kg, IP) and blood was collected through heart puncture. Then, tissues such as brain, heart, esophagus, lungs, spleen, liver, stomach, small intestine, large intestine, kidney and bone marrow were collected. Serum was separated from blood by centrifugation at 4° C, 1500g for 10min. Except bone marrow, tissue from all other organs were homogenized; 30% of silver nitrate was added to precipitate the tissue protein. Extraction of AZT from tissue was done by addition of methanol followed by the centrifugation of the whole mixture at 12,000 rpm for 12 min at 4° C. After centrifugation, supernatant was filtered with 0.2 micron syringe filter and estimated by HPLC UV detector at 270nm.

Tissue Sectioning and Safety Analysis

Organs were removed and processed for histopathology. The tissues were observed under microscope for any abnormalities after the treatment with the nanoformulation. Safety analysis was done by using biochemical kits that were commercially available for serum AST, urea, bilirubin and creatinine.

Bone Marrow Micronucleus Assay

Animals were administered orally with 10mg kg^{-1} body weight of sol AZT and equivalent amount of AZT-lactonano. The test was performed using the modified protocol of Schmid [37]. Only three time points (4h, 8h, and 16h) were chosen for the test, based on drug distribution in bone marrow. After the completion of time points rats were sacrificed by cervical dislocation. Both femoral bone were dissected and the bone marrow cells were collected by flushing out with 1ml of fetal bovine serum (FBS) and mixed properly. Then cell suspension was centrifuged for 5min at 200g; supernatant were discarded. Marrow pellet was resuspended in minimal volume of FBS. One drop of cell suspension were placed on a clean dry slide and thin smear was made, air dried and then fixed with absolute methanol. Smear was stained with Giemsa stain for 20min and mounted.

Antiviral Assay

SupT1 Cells (100% viability) with a density of 0.5 million were seeded with RPMI 1640, 0.1% FBS in 24-well plates. 80mg/ml of lactoferrin was taken, and formulation with and without AZT (1μg) were added to the cells and they were challenged with HIV-193IN101 at a final concentration of virus equivalent to 20 nanograms of p24 per ml. The infected cells were incubated at 37°C and 5% CO_2 incubator for 2 h. After 2 h, the cells were pelleted at 350x g for 10 min, cells were washed twice with RPMI 1640 containing 10% fetal bovine serum. The cells were suspended in the same medium and incubated for 96 h. After 96 h supernatants had been collected and viral load was analyzed using p24 antigen capture assay kit (ABL kit). The infection in the absence of compound was considered to be 0% inhibition.

Statistical Analysis

All studies were carried out in triplicates for all groups and results are presented as mean with standard deviation. The significance of differences between treatments was analyzed by one-way ANOVA with age and treatment as factors using Sigma plot. The level of statistical significance (P) was set at $P < 0.05$.

Results

Size and EE% of AZT-Lactonano

Field Emission Scanning Electron Microscopy (FE-SEM) and Atomic force microscopy (AFM) analysis revealed that the particles were spherical and were in the range of 50-60nm diameter (Fig 1). Increase in size of AZT-lactonano (50 to 60 nm) compared to lactonano (21-35nm) suggest that drug loading enhance particle size. Encapsulation efficiency was calculated according to the equation mentioned in materials and methods section and found to be 67%.

FT-IR Analysis of AZT-Lactonano

FT-IR analysis confirmed that AZT was found to be intact after the preparation of nanoparticles (Fig 2). Characteristic bands found in the infrared spectra of Lactoferrin proteins (Pure and nano form) include the Amide I and Amide II. The absorption associated with the Amide I band and Amide II band leads to stretching vibrations of the C = O bond and primarily to bending vibrations of the N—H bond respectively. Amide I bands was positioned around 1645 & 1648 cm^{-1} are usually reflected to be characteristic of alpha helices. Amide II (C-N stretching and N-H bending) and peptide N—H stretching frequency were detected at 1542 & 1539 cm^{-1} and 3418 & 3364 cm^{-1} correspondingly. The C-O-C stretch were observed around 1096 cm^{-1} & 1164 cm^{-1} The locations of both the Amide I and Amide II bands are sensitive to the

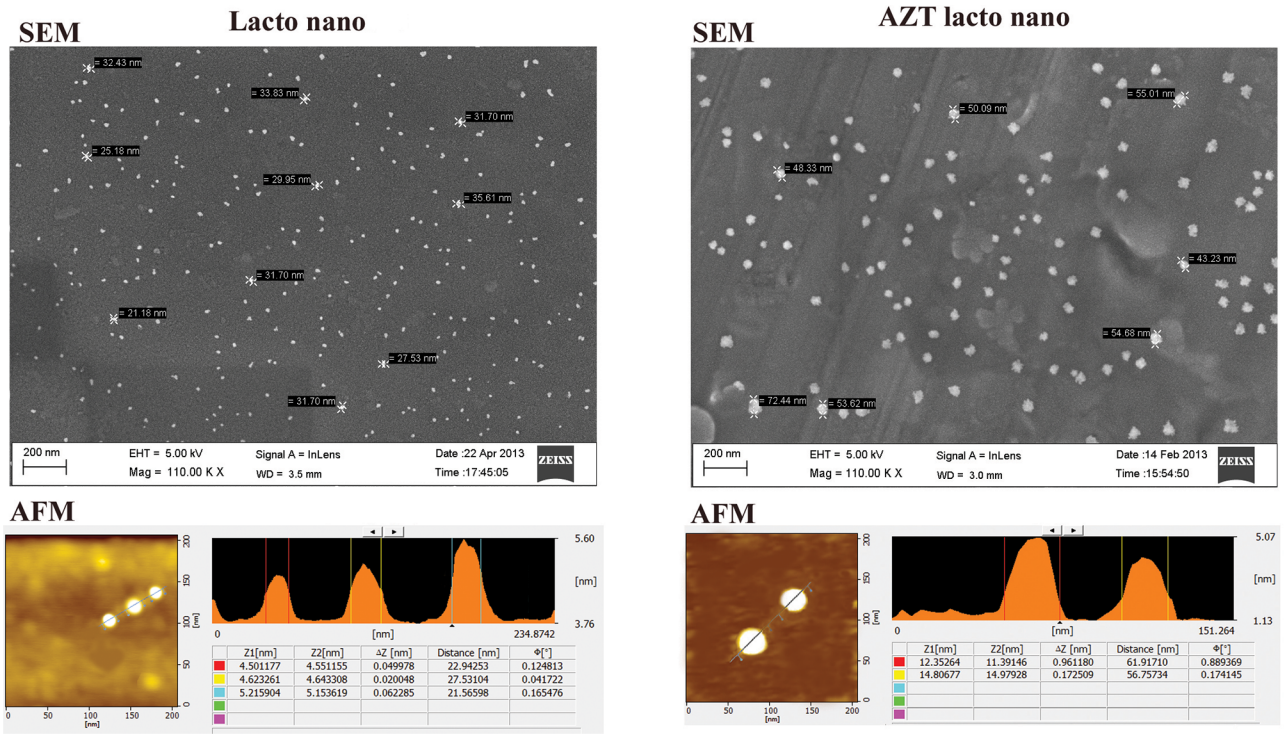

Fig 1. Size of Lactoferrin nanoparticles increases after loading of AZT. AZT containing Lactoferrin nanoparticles were prepared using sol-oil chemistry as described in methods section. The size of nanoparticles was assessed by Field emission scanning electron microscopy (top panel) and Atomic force microscopy (bottom panel). Lactonano: Lactoferrin nanoparticle without any drug loading into it, AZT Lacto nano: Lactoferrin nanoparticle loaded with AZT.

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secondary structure content of a protein. **Assessment spectral analysis of Pure AZT and AZT-Lactonano**: sharp characteristic peaks of carbonyl group (C = O) at 1682 cm^{-1} (Pure AZT) and 1685 cm^{-1} (AZT-Lactonano), Azide group ($N^- = N^+ = N^-$) peaks at 2117 & 2083 cm^{-1} (Pure AZT) and 2115 & 2082 cm^{-1} (AZT-Lactonano), C-O-C stretch belong to 1088 & 1065 cm^{-1} (Pure AZT) and 1089 & 1065 cm^{-1} (AZT-Lactonano), -NH stretching remains at 3460 cm^{-1} (Pure AZT) and 3461 cm^{-1} (AZT Lactonano). Our results of FT-IR spectra proved that there were only slight shifting (may be due to dipolemoment of bond as a result of electrostatic interaction between AZT and Lactoferrin protein) in few stretching vibration but all the major functional group was intact in nanoformulation and didn't take part in any covalent bond formation. It confirmed that AZT is only physically associated (entrapped/adsorbed) with lactoferrin protein.

AZT-Lactonano Release Assay

In case of percent drug release assay, the encapsulated drug present in nanoparticle was considered as 100% at the start of the experiment. The amount of drug released at indicated pH at different time points was estimated and presented as percent release with reference to the drug loaded in the nanoparticles. In vitro analysis of AZT release from the nanoparticles has shown that highest amount of AZT was released at pH-5 (Fig 3A) followed by pH-6 and pH-4. Its release in the presence of simulated Gastric and Intestinal fluids was lesser indicating its stability at extreme pH. Release kinetics at pH 5.0 as indicated in Fig 3B showed a biphasic release, wherein a burst release of 60% of drug within 4 hours, followed by reduced release rate until 10 hours to the extent of 80% then a limited release over 96 hours.

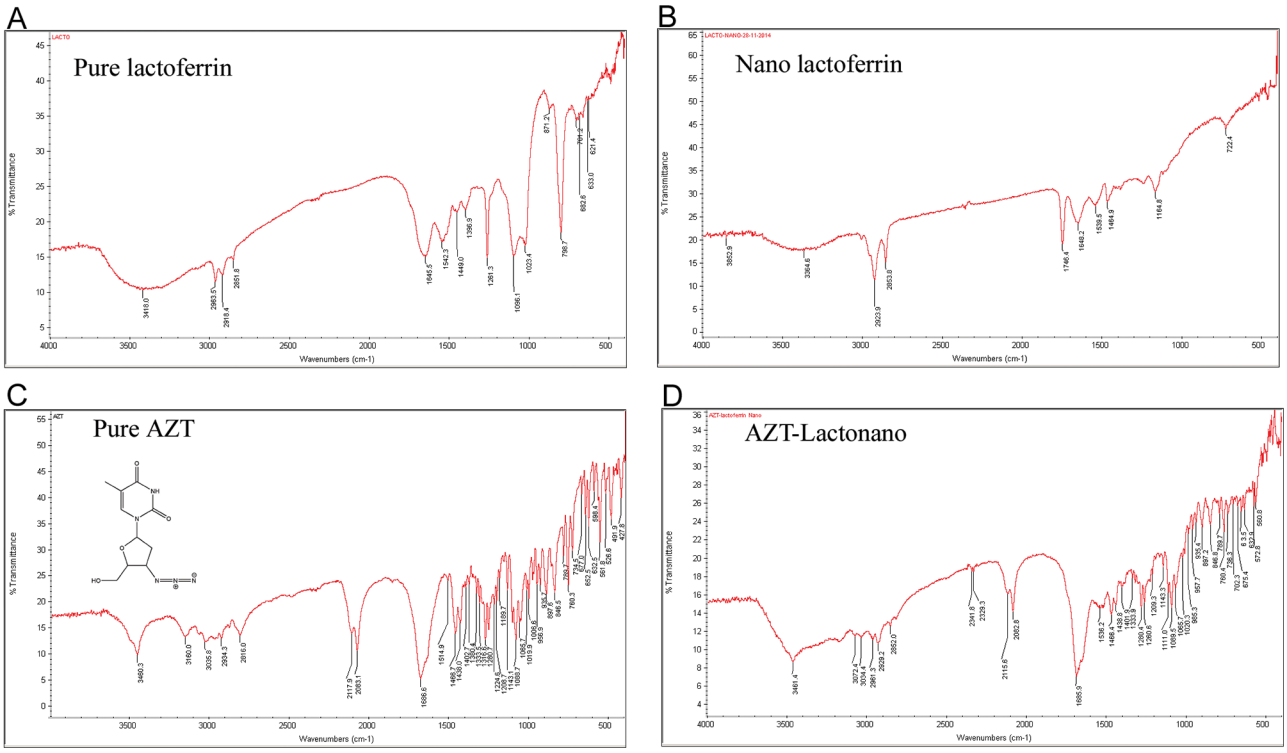

Fig 2. FT-IR spectral analysis. The FT-IR analysis of Pure Lactoferrin proteins (A), Nano Lactoferrin (Lacto nano) (B), AZT molecule (C) and AZT loaded lactoferrin nanoparticle (AZT-Lactonano) (D). It reveals that in AZT-Lactonano, AZT is physically entrapped/adsorbed with the lactoferrin nanoparticles; it didn't take part in any sort covalent interaction.

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In Vitro Stability Studies

Stability of nanoparticles suspended in PBS was analyzed at different time points at two different temperatures. Nanoparticles were found to be quite stable at both temperatures (4°C and room temperature). The drug content of nanoparticles and its diameter was found to remain same from starting to 96hr (Table 1).

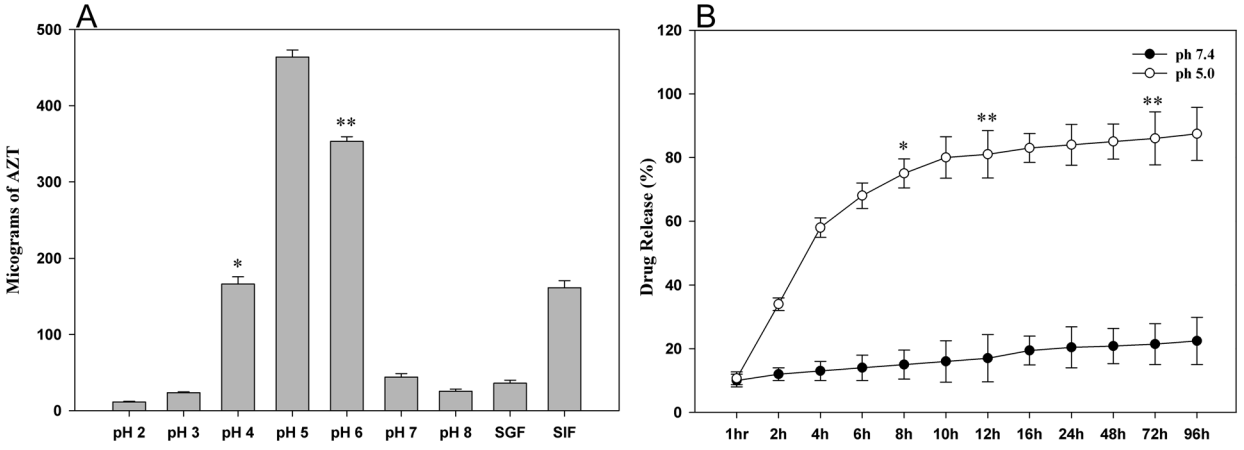

Fig 3. pH sensitivity and time dependent drug release profile of AZT-Lactonano. (A) 600μg of drug was incubated in the buffers of different pH, the release of AZT was maximum at pH-5. This is followed by pH-6 and pH-4. The release was between 1–10% with the remaining fluids. SGF: Simulated Gastric Fluid, SIF: Simulated Intestinal Fluid. (B) Cumulative percentage release profile of AZT-Lactonano at pH 5.0 and pH 7.4. Each data points were taken in triplicate and presented as Mean ± SD. Value of significance, **P < 0.005, *P < 0.05.

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Table 1. *In vitro* stability of nanoparticles.

	Diameter of the nanoparticles (nm)		AZT present in mg (%) [#]	
Hours	4°C	Room temp*	4°C	Room temp*
0	55.67± 4.54	53.23± 3.56	6.75 ± 0.69 (100.00)	6.71 ± 0.19 (100.00)
1	54.5 ± 4.30	54.5 ± 2.68	6.49 ± 0.96 (96.15)	6.57 ± 0.82 (97.91)
2	58.6 ± 3.36	59.7 ± 5.13	6.68 ± 0.85 (98.96)	6.66 ± 0.28 (99.25)
4	53.1 ± 4.19	53.6 ± 4.35	6.71 ± 0.52 (99.41)	6.47 ± 0.18 (96.42)
6	52.3 ± 3.63	55.5 ± 4.72	6.58 ± 0.78 (97.48)	6.53 ± 0.36 (97.31)
8	54.7 ± 4.73	57.6 ± 3.81	6.37 ± 0.67 (94.37)	6.54 ± 0.49 (97.46)
10	58.3 ± 6.63	58.7 ± 4.34	6.66 ± 0.78 (98.66)	6.30 ± 0.84 (93.89)
12	53.6 ± 5.13	56.8 ± 3.92	6.48 ± 0.82 (96.00)	6.52 ± 0.92 (97.16)
16	60.2 ± 5.39	54.1 ± 4.77	6.52 ± 0.19 (96.59)	6.50 ± 0.14 (96.87)
24	58.7 ± 4.73	59.4 ± 4.65	6.64 ± 0.35 (98.37)	6.68 ± 0.49 (99.55)
48	55.6 ± 4.73	57.1 ± 2.91	6.51 ± 0.73 (96.44)	6.63 ± 0.19 (98.81)
72	56.7± 5.94	56.7 ± 2.65	6.54 ± 0.37 (96.88)	6.58± 0.27 (98.06)
96	60.8 ± 5.76	58.4 ± 4.39	6.65 ± 0.39 (98.52)	6.35 ± 0.38 (94.63)

All the data (n = 3) were presented as mean ± standard deviation.

[#] Drug present in the particles was estimated by using HPLC, in milligrams. The amount of drug present initially at zero hour at 4°C and room temperature are considered as 100% drug present.

* Room temperature: The temperature used here was an average equal to 23°C.

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Pharmacokinetics and Tissue Distribution of AZT Lactonano

AZT levels in blood and other organs were located after a single dose of nanoparticles loaded with AZT (10mg/kg AZT equivalence) administered in rats orally. Drug levels were observed at seven different time point up to 24h in different tissue (Fig 4). The concentration of AZT in the blood after 2 h was determined to be around 50μg when administered with nanoformulation and around 10μg in the lack of any carrier particle. It proves the relative stability of AZT in nanoformulation against plasma clearance. Nanoformulation does not cause any organs related toxicity due to the accumulation of AZT in various organs, in contrast AZT without any carrier molecule leads to toxicity. It is followed by liver and small intestine which have shown accumulation above 5μg. Less than 5μg of the drug was found in the others remaining organs such as Kidney, Heart, Spleen, Lungs, Brain, Stomach, Esophagus, large intestine and Bone marrow. On the other side we can detect a simultaneous decrease of AZT level in serum; in liver there was an increased level of AZT when treated with AZT-lactonano at 8hr post injection. All pharmacokinetic parameters were assessed by Kinetica v 5.0 software are shown in Table 2. The AZT-lactonano showed an improvement in pharmacokinetic profile with more than 4-fold increase in AUC and AUMC in male and female rats in serum. The serum C_{max} for AZT-lactonano was increased by 30%. Similarly, there was more than 2-fold increase in T_{max} and $t_{1/2}$ (Table 2). These results show that the nanoformulation provide higher bioavailability than sol formulation.

Safety Profile of Nanoformulation

The safety profile of AZT-lactonano was compared with sol AZT and the results show no significant change in serum AST in nanoformulation versus soluble form, while bilirubin was lower in case of AZT-lactonano compared to sol AZT in female rats (Fig 5). Serum urea was significantly low when AZT-lactonano was administered suggesting low kidney toxicity when

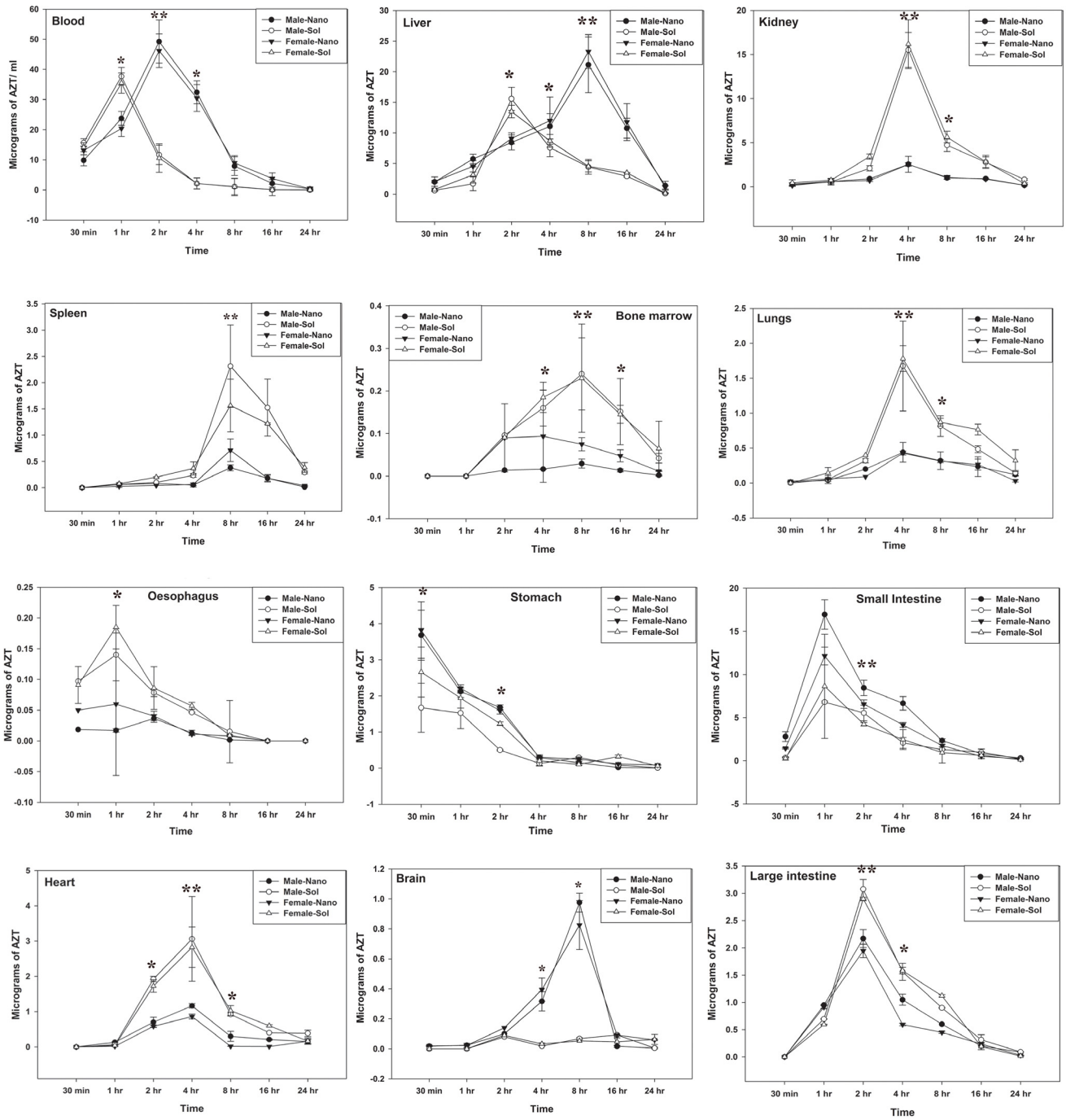

Fig 4. Tissue distribution of AZT. Single dose of sol AZT and equivalent weight of AZT-lactonano (10mg/kg body weight) was orally administered to Wistar rats. After completion of indicated time points, rats were sacrificed under proper anesthesia. AZT was extracted and estimated in blood, liver, kidney, heart, spleen, bone marrow, lungs, brain, oesophagus, stomach, small intestine and large intestine. Male-Nano and Female-Nano denotes the AZT concentration (delivered via AZT-lactonano) present in Male rats (n- = 3) and female rats (n = 3) respectively. Same nomenclature has been followed for Male-Sol and Female-Sol. Differences between groups were assessed by ANOVA. Data were presented as Mean ± SD. Value of significance, **P < 0.005, *P < 0.05.

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Table 2. Pharmacokinetics profile of AZT-lactonano in male and female rats.

AZT		Male		Female	
		Nano	Soluble	Nano	Soluble
AUC	(h)*(μg/ml)	251.57	63.48	254.974	58.74
AUMC	(h)^2*(μg/ml)	1270.3	139.73	1485.24	126.737
C_{max}	μg/mL	49.198	37.67	46.17	35.45
T_{max}	hr	2	1	2	1
$t_{1/2}$	hr	3.07	1.759	3.27	1.92

Values in the parenthesis designates the concentration of AZT in micrograms per ml of blood.

Pharmacokinetic parameters.

AUC: The integral of the concentration-time curve (after a single dose or in steady state).

AUMC: Partial area under the moment curve between t start and t end.

C_{max}: The peak plasma concentration of a drug after oral administration.

T_{max}: Time to reach C_{max}.

$t_{1/2}$: The time required for the concentration of the drug to reach half of its original value.

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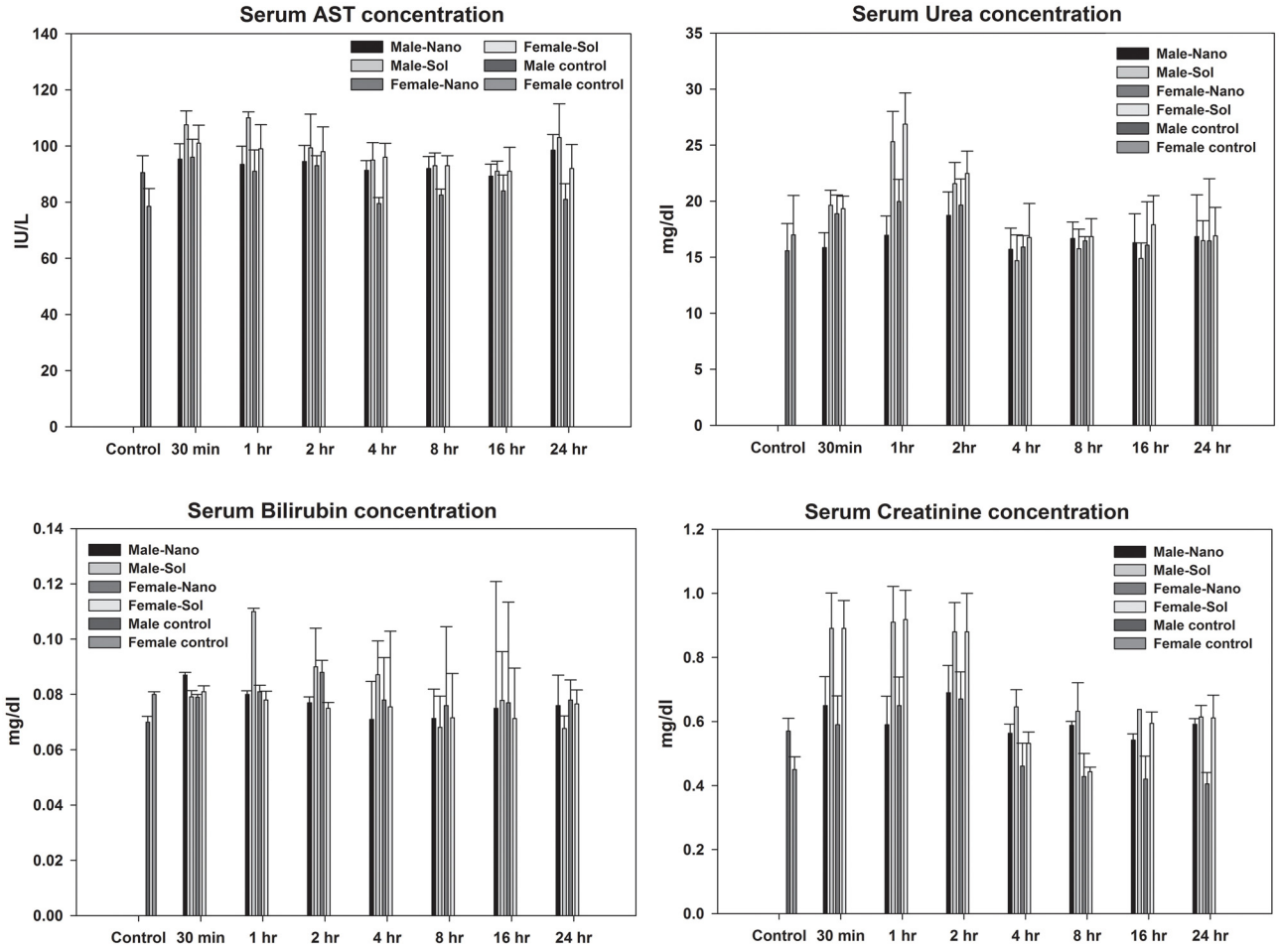

Fig 5. Biochemical safety analysis profile. Safety analysis was done using biochemical kits after oral administration of nano and soluble AZT (10mg/kg) in both male and female rats. Liver damage was estimated by Bilirubin and AST level whereas Kidney toxicity was checked by Urea and creatinine level. AZT-lactonano showed no toxicity to both liver and kidneys on the other hand it exhibited minimal urea levels when compared to the soluble AZT.

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nanoformulation was used. Nanoformulation showed improved creatinine levels compared to soluble drug form suggesting low liver toxicity. Tissue sections of heart, liver, spleen, kidney, stomach and intestines (small and large) have shown that there were no toxicities in those tissues when administered with nano forms (Fig 6).

Micronucleus Assay

Bone marrow smears were visualized using Olympus Magnus BX51 microscope under 100x magnification with oil immersion objective. Micronuclei (MN) were identified in form of RBCs (i.e., polychromatic erythrocytes as PCEs). Approximately 1000 cells were scanned per

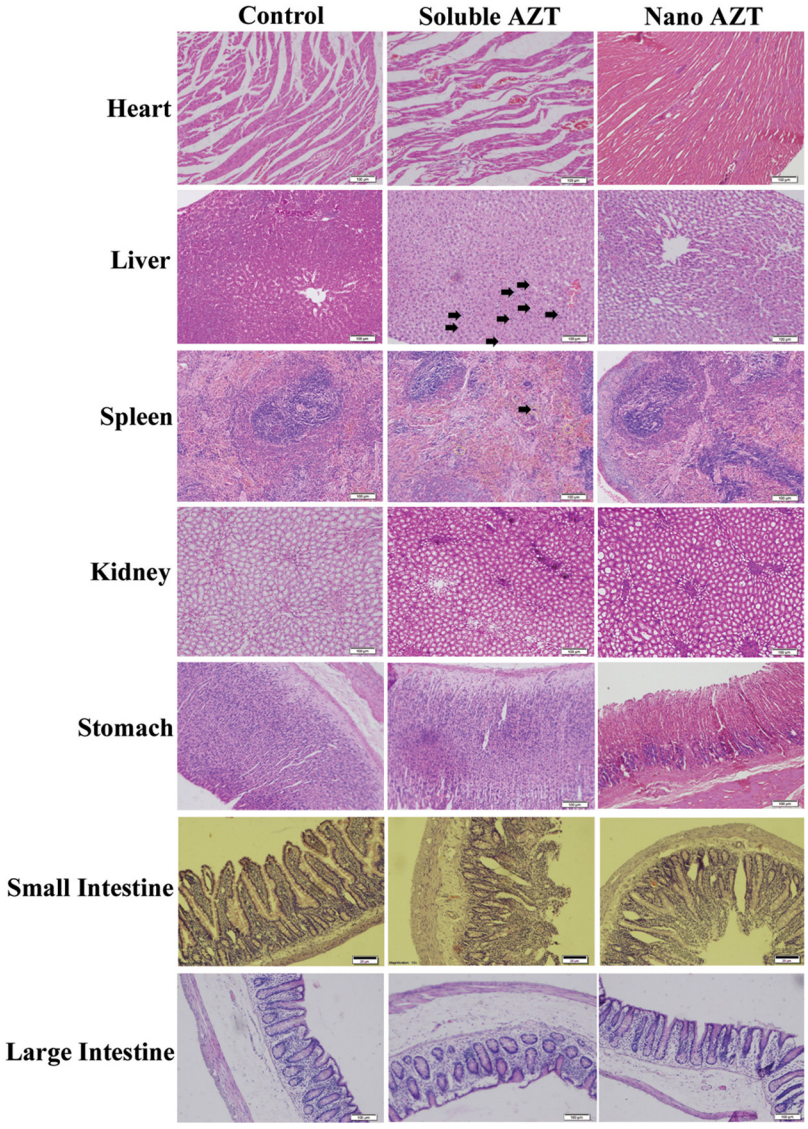

Fig 6. Histopathological analysis of tissues. Rats were orally administered with sol AZT and AZT-lactonano (10mg/kg body weight), after completion of 24hr time point, organs were removed and processed for cryo-sectioning followed by Hematoxylin and Eosin (H&E) staining. It revealed that no toxicity was found in above indicated organs, when AZT was delivered via nanoparticle form as compared to its sol form. Lesion or any abnormalities present was denoted by arrow. Scale bar is equal to 100μm.

doi:10.1371/journal.pone.0140399.g006

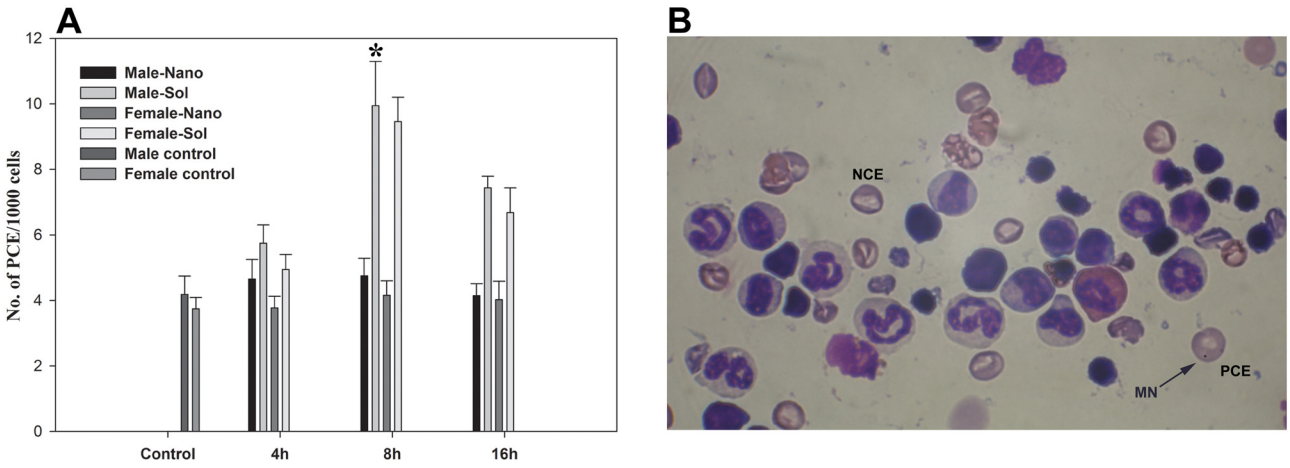

Fig 7. Bone marrow toxicity profile. (A) It shows the frequency of polychromatic erythrocyte (PCE) in bone marrow cells after oral administration of sol AZT and AZT-lactonano at 4, 8 and 16h. Data were presented as Mean ± SD. Value of significance, **P < 0.005, *P < 0.05. **(B)** Bone marrow cells (after 8h of treatment) showing the presence of enucleated Normochromatic erythrocyte (NCE) and nucleated polychromatic erythrocyte (PCE) cells. One PCE holds a micronucleus (MN); indicated by arrow. This images was captured at 100x under oil immersion objective.

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slide for the presence of micronucleus (MN) in immature PCE. Fig 7A represent that, at 8h of post application of drug a significant increase in the number of MN-PCEs was observed in case of sol AZT and at the same time AZT-lactonano showed low bone marrow toxicity. MN could be characterized as round and darkly stained (Fig 7B) nuclear fragment that indicate chromosome damage.

Antiviral Activity of AZT Lactonano

Antiviral activity of lactoferrin alone and AZT loaded in nanoformulation was analyzed to ascertain if active form of the drug is intact. Here 80mg/ml of soluble lactoferrin and equivalent of nanoform of lactoferrin has showed 70 and 73% antiviral activity respectively. Further, the activity of AZT at one microgram concentration is similar to that of soluble AZT with more than 90% of inhibition of HIV-193IN101 replication in Sup-T1 cells. It suggests that activity of encapsulated AZT remain stable in nanoformulation. (S1 Fig).

Discussion

Various formulations have been employed previously to improve the oral bioavailability of AZT. These include controlled [38] and extended [39,40] release matrices, microspheres [41], nanoparticles [42] and liposomes [43–45], which have been proposed for the delivery of AZT. Even though various routes like intranasal, intravenous and transdermal routes have been tried for AZT delivery. Peroral route of administration is the most preferred one because of frequent dosage and patient compliance. Absorption of AZT is reported to be quick and rapid when administered orally and undergoes first pass metabolism before giving an average systemic bioavailability of more than 60% [46]. Based on physiochemical characteristics, the aqueous solubility, pKa and LogP of AZT was reported as 29.3 g/l, 9.68 and 0.06 respectively [47]. Zidovudine typically exhibits a 1-compartment model in plasma during its oral administration followed by an elimination phase that is biexponential. It is relatively a lipophilic molecule where 25% of it binds to albumin [48,49] and gets metabolized in the body mainly by hepatic 5'-glucouronidation forming a stable metabolite which gets excreted in the urine. In order to maintain the required therapeutic concentration of AZT, frequent doses have to be given which may

lead to elevation to toxic levels in the blood resulting in severe side effects like granulocytopenia and anemia. Greater focus has been given for targeting AZT to lymphoid and reticuloendothelial cells in previous formulations since the delivery to these cells is utmost important as they constitute major viral reservoirs and a sterilizing cure for AIDS is impossible unless and until these are eliminated completely.

Since the encapsulation mechanism involves a process of partition of lipophilic-lyophilic AZT and lactoferrin in water and oil phases. Such phase transitions may induce protein-protein associations that entrap drug in intermolecular core as well as intramolecular cavities of proteins. This lead to a defined percent of drug (67%) associated with the protein nanoparticles based on the log P value of the drug, viz., AZT (log P, 0.06). This can be supported by the observation that AZT is released in biphasic kinetics with burst (60%) when protein surface of particles exposed to pH 5, which could be due to change in orientation of protein monomers, this may follow a release of drug molecules localized at various cavities in the protein over time (to the extent of 20%). In spite of biphasic release, 80% of loaded drug was released within 10 hours, making the availability of active drug for inhibiting target enzyme, revise transcriptase activities. Furthermore, oral absorption of nanoparticles at low pH (<3.0) and circulatory pH 7.4, allow particles intact with the loaded drug. When these particles reached the target cells (lymphocytes etc.), they enter through receptor-mediated endocytosis followed by fusion to endosome, a transient pH change to 5.5 in endosome will allow significant drug release in target cell and make effective concentrations of drug reaching at the site of action. Thus, stability of particles at pH below 3.0 and at 7.4 make these particles attractive for oral delivery in vivo.

In vivo studies showed that the AZT-lactonano showed has improved pharmacokinetic profile with more than 4-fold increase in mean serum AUC and AUMC in both male and female rats. The serum C_{max} for AZT-lactonano was increased by 30% whereas more than 2-fold increase was observed in T_{max} and $t_{1/2}$ for both male and females. It suggests that the AZT in the nanoparticles gets released slowly leading to this significant increase in the pharmacokinetic parameters. At the same time, this nanoformulation has not shown any abnormal concentrations in different organs leading to toxicity. The safety profile of nano and Sol AZT was compared and the results show no significant change in serum AST in nano versus soluble form, while bilirubin was lower in case of nano when compared to soluble form in female rats. Serum urea was significantly low when AZT-lactonano was administered compared to soluble AZT suggesting low kidney toxicity when nanoformulation was used. AZT-lactonano showed no apparent differences in creatinine levels compared to soluble form suggesting low kidney toxicity. In addition H&E staining of all the tissue sections has not revealed any abnormal morphology for both of the formulations employed.

Bone marrow suppression is the main reason to discontinue AZT based therapy [50] because the hematopoietic progenitor cells are heavily damaged. Micronucleus (MN) test is very reliable and fast in vivo assay to determine any marrow cells alteration [51]. MN are minute extra-nuclear bodies formed during anaphase stage [52]. Generally two forms of MN are found in RBCs, polychromatic erythrocytes (PCE) and Normochromatic Erythrocytes (NCEs) [53]. Our results show that AZT-lactonano is not involved in any MN formation but at the same time Sol-AZT is two-time more genotoxic.

As the free drug is reported to have lower penetration into the infected cells, the above formulation selectively targets and delivers AZT to cells that express lactoferrin receptors on their surface through receptor-mediated endocytosis by which the therapeutic index of AZT can be improved. The amphiphilic nature of AZT results in low entrapment and significant leakage when packed in conventional liposomal vesicles as it gets partitioned between lipid bilayers and the core aqueous environment. The lesser size of the nanoparticles with up to 67% encapsulation of AZT makes the current formulation to overcome the above problem. Currently,

oral dosed HIV nanoformulation was not available for patients. The quality of patient's life can be improved by simplifying the AZT dosage schedule by less frequent administration of a sustained-release formulation since HAART regimens that combine multiple agents lead to severe side effects. The advantage in employing lactoferrin as a carrier is its ability to interfere with virus binding to DC-SIGN of dendritic cells by its interaction with the V3 loop of gp120 and coreceptors [54]. Further, the same formulation can be employed to improve the brain delivery of AZT since the lactoferrin is reported to cross the blood-brain barrier [55].

Conclusion

The Present study shows the applicability of protein -based nanoparticles formulation of AZT through oral delivery. The nanoparticles were prepared using sol-oil protocol. AZT-lactonano showed a biphasic drug release profile and releases its maximum payload at pH 5. *In vivo* studies concludes that the physical encapsulation of AZT in lactoferrin nanoparticles makes the formulation safer and efficacious. Nano-formulation enhances the various pharmacokinetics profile like AUC, AUMC, C_{max} and $t_{1/2}$ while keeping the antiviral activity of AZT intact. Further AZT-lactonano is found to be two times less genotoxic as compared to sol AZT.

Supporting Information

S1 Fig. Antiviral activity of lactoferrin alone (soluble & nano) and AZT (soluble & nano). Antiviral activity of AZT was found to be intact in case of nanoformulation. The p24 level was measured as viral load. Here 80mg/ml of lactoferrin and equivalent concentration of nanoform was taken. The nano-AZT showed more than 85% antiviral activity at a final concentration of 1μg.
(TIF)

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Author Contributions

Conceived and designed the experiments: PK AKK. Performed the experiments: PK YSL. Analyzed the data: KG YSL. Contributed reagents/materials/analysis tools: KG. Wrote the paper: AKK BC.

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RESEARCH PAPER

Triple Drug Combination of Zidovudine, Efavirenz and Lamivudine Loaded Lactoferrin Nanoparticles: an Effective Nano First-Line Regimen for HIV Therapy

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ABSTRACT

Purpose To enhance efficacy, bioavailability and reduce toxicity of first-line highly active anti-retroviral regimen, zidovudine + efavirenz + lamivudine loaded lactoferrin nanoparticles were prepared (FLART-NP) and characterized for physicochemical properties, bioactivity and pharmacokinetic profile.

Methods Nanoparticles were prepared using sol-oil protocol and characterized using different sources such as FE-SEM, AFM, NanoSight, and FT-IR. *In-vitro* and *in-vivo* studies have been done to access the encapsulation-efficiency, cellular localization, release kinetics, safety analysis, biodistribution and pharmacokinetics.

Results FLART-NP with a mean diameter of 67 nm (FE-SEM) and an encapsulation efficiency of >58% for each drug were prepared. *In-vitro* studies suggest that FLART-NP deliver the maximum of its payload at pH5 with a minimum burst release throughout the study period with negligible toxicity to the erythrocytes plus improved *in-vitro* anti-HIV activity. FLART-NP has improved the *in-vivo* pharmacokinetics (PK) profiles over the free drugs; an average of >4fold increase in AUC and AUMC, 30% increase in the C_{max}, >2fold in the half-life of each drug. Biodistribution data suggest that FLART-NP has improved the bioavailability of all drugs with less tissue-related inflammation as suggested with histopathological evaluation

Conclusions The triple-drug loaded nanoparticles have various advantages against soluble (free) drug combination in terms of enhanced bioavailability, improved PK profile and diminished drug-associated toxicity.

KEY WORDS drug delivery · HIV · nanoparticles · pharmacokinetics · zidovudine

ABBREVIATIONS

3TC or LMV	Lamivudine
ART	Antiretroviral therapy
ARV	Antiretroviral
AZT	Azidothymidine or Zidovudine
DL	Drug loading
DMSO	Di methyl sulfoxide
EE	Encapsulation efficiency
EFV	Efavirenz
FLART-NP	First-Line ART Nanoparticles
FT-IR	Fourier transform infrared spectroscopy
HPLC	High Performance Liquid Chromatography
HR	Hemolysis rate
IC_{50}	50% Inhibitory concentration
Lf	Lactoferrin
NP	Nanoparticles
NTA	Nanoparticle tracking analysis
PBS	Phosphate-buffered saline
sol	Soluble or free

INTRODUCTION

A 15.8 million estimate of population were on ART treatment in June 2015 (1), came true with 17 million people receiving ART at the end of 2015 (WHO HIV/AIDS Fact sheet, July 2016). An effective and uninterrupted antiretroviral (ARV) treatment can control the viremia in infected persons, further with reduce risk in the frequency of HIV transmission with improved quality of life (2). In current scenario, there is

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no vaccine available for HIV prevention; all the candidates being studied are in the underlying phase of their development (3). In the early 1990s the HIV treatment began as monotherapy then shifted to the dual drug regimen, trailed by three-layered drugs combination treatment. At the point of virus multiplication, the new copies formed contain mutation, hence forth the progenies are somewhat different from original parent virus. Mono-drug and dual-drug therapy could develop resistance in HIV. This requires the necessity of treatment which consolidates drugs having a diverse method of activity against HIV (2,4,5). There are 26 drugs affirmed by US Food and Drug Administration (FDA); the highly active antiretroviral therapy (HAART) has improvised the health of infected individuals and lowered the morbidity and mortality (6). Generally, a combination of antiretroviral (ARV) drugs are suggested during the HAART treatment. Each class of ARV drug-targeted to a particular site in HIV replication cycle providing multi-targeted action against HIV. The recommended first-line HAART is a combination of one NNRTI (Non-Nucleoside Reverse Transcriptase Inhibitors) plus two NRTI (Nucleoside/Nucleotide Reverse Transcriptase Inhibitors) (7). A combination of Zidovudine (AZT), Efavirenz (EFV) and Lamivudine (3TC) is one of the frequently used first line regimen. Long term use of the combination of ARV drugs reported various toxicity; cardio-toxicity, and erythrocyte toxicity is being frequently limit their use (8). The most important hurdle in the ARV treatment is that patient needs to take the medication on consistent schedule, it may lead to various side effects and other health complications (9). As nanoparticles (NP) are the system that delivers the drugs in a sustained manner and possess long drug retention time inside the body; directly relates to the reduction in the dosage schedule as well as other side effects. NP have proven as an effective drug delivery system for a wide range of disease complications such as cancer (10), AIDS (11,12), Parkinson's disease (13) and much more. NP possess a variety of properties such as biodegradable (14), sustain drug release (15) and improve the efficacy of poorly bioavailable drugs (16) etc. The pharmacokinetic behavior and tissue distribution of NP are dependent mostly on its size and it surfaces properties (17). Our current research describes the applicability of lactoferrin based nanoparticles for HIV/AIDS treatment. Lactoferrin is a pleiotropic molecule having a broad spectrum functional activities viz. anti-HIV, anti-bacterial, microbicidal etc. (18,19). Our previous studies demonstrated that lactoferrin can serve as an effective delivery vehicle for cancer treatment (20). Indeed AZT loaded lactoferrin nanoparticle indicated higher bioavailability of drug with improved safety (21). The primary objective of present study is the development of a novel targeted drug delivery system composed of a triple drug (AZT, EFV, and 3TC) loaded lactoferrin nanoparticle. While the secondary objective is the evaluation of the advantages of the nanoformulation for improved *in-vivo* pharmacokinetics profile as well as with reduced side effects, when compared to the free drugs. This will furnish a formulation with enhanced therapeutic utility in terms of reduced dose of drugs, minimum health complications with increased therapeutic index.

MATERIALS AND METHODS

Materials

Zidovudine, efavirenz and lamivudine were used are of pharmaceutical grade. Lactoferrin was purchased from Symbiotics, USA. Purity of drugs and lactoferrin was confirmed by HPLC and Mass spectrometry. All reagents used were of analytical grade. Olive oil was procured from Leonardo.

Nanoparticles Preparation

Nanoparticles (NP) were prepared using previously established protocol (22). Briefly, an equal amount of drugs viz., Zidovudine (AZT) (3.33 mg), Efavirenz (EFV) (3.33 mg) and Lamivudine (3TC) (3.33 mg) were dissolved separately in 100 µl of DMSO. Further, in four separate tubes containing different concentrations of Lactoferrin (Lf), such as10, 20, 30 and 40 mg were dissolved in 500 µl of 1X PBS (pH 7.4). Drugs and protein solutions were incubated on ice for a period of 60 min. The same were mixed with 25 ml of olive oil followed by sonication for 15 min at 4 ^{o} C with a narrow stepped titanium probe of an ultrasonic homogenizer. After sonication, the samples were immediately transferred into liquid nitrogen for 15 min, followed by incubation on ice for 4 h. Particles formed were centrifuged at 6000 rpm for 30 min. The supernatant that contains oil was discarded and the pellet was extensively washed with ice-cold di-ethyl ether to remove any oil traces left. Further, the pellet was suspended in PBS and used for the later experiment. The same protocol was followed for the preparation of blank lactoferrin nanoparticles or nanoparticles without any drug (lacto-nano).

Morphological Characterization (FE-SEM and AFM Microscopy)

Structure, size and morphology of NP was characterized using FE-SEM (Field Emission Scanning Electron Microscope) and AFM (Atomic Force Microscopy). For FE-SEM, the freshly prepared NP (NP suspension) was dried on a sterile glass slide; particles were coated with gold and analyzed. For AFM imaging, the NP was spin coated on a clean glass slide. For all the imaging techniques, manufacturer's instructions were followed for the data collection as well as analysis.

Particle Size and Size Distribution: Nanoparticle Tracking Analysis (NTA)

Nanoparticles size distribution was performed by NTA using Nanosight NS500. This instrument determines the size of nanoparticles based on the Brownian motion of particles present in the samples. Samples were diluted in Milli Q and experiments were done in triplicate. The camera setting was adjusted to minimize the background noise and video was recorded for 60s. After capturing the video, it was processed and analyzed using dedicated software (NTA analysis software version 2.0). Optimum visualization of maximum number of nanoparticles were done using manual focusing with combination of high shutter speed (600) and gain (250). Following setting has been followed during the analysis, Frames Processed: 1498 of 1498, Frames per Second: 24.98, Calibration: 141 nm/pixel, Blur: Auto, Detection Threshold: 10 Multi, Min Track Length: Auto, Min Expected Size: Auto, Temperature: 24.80°C, Viscosity: 0.89 cP.

FT-IR Study of Nanoparticles

The FT-IR spectral analysis was performed using kBr pellet method. The 0.1–1.0% of lyophilized nanoparticles were mixed with approximately 200 mg of KBr followed by transferring of mixture to a 13 mm diameter pallet forming die under a very high pressure of 1000 kg/cm^2. A transparent pallet was formed. This was then transferred to the sample holder followed by scanning in a range of 400 to 4000 cm^{-1}. Finally the analysis of spectra was done using OMNIC series software.

In-Vitro Studies

Drug Loading (DL) and Drugs Encapsulation Efficiency (EE)

EE was defined as the ratio between the amount of drug encapsulated and the drug used initially in the preparation of NP. The extent of drugs entrapped in Lf-NP was quantified using HPLC. Freshly prepared NP was incubated with 1 ml of PBS (pH5) at room temperature under rocking condition for 24 h. As drugs were getting released into the solution, 30% of silver nitrate (100 µl) per ml of sample was added to precipitate the protein content. The drug was further extracted by adding 1 ml of HPLC grade methanol. The mixture was centrifuged at 12,000 rpm for 20 min and drugs were quantified in the supernatant. Experiments were done in a set of three. A standard curve was established using different known concentrations of AZT, EFV and 3TC estimated using HPLC. Drug loading and Encapsulations were calculated using the following formula.

$$\text{Drug Loading (DL)} = (\text{Mass of drugs in NP}/\text{Mass of NP}) \times 100$$

$$\text{Encapsulation Efficiency (EE)} = (\text{Existent amount of drugs in NP}/\text{ Initial amount of drug used in NP preparation}) \times 100\,\%$$

pH Triggered Drugs Release and Percent Cumulative Drug Release from FLART NP

600 µg of freshly prepared FLART NP was incubated with 1 ml of PBS of different pH (1–9), SIF (simulated intestinal fluid, pH7.4) and SGF (simulated gastric fluid, pH1.2) for 12 h. After the completion of incubation, the proteins content was precipitated by adding 200 µl of 30% silver nitrate (in water) followed by adding of 1 ml of methanol to extract the drug. Mixtures were centrifuged at 12,000 rpm for 15 min and supernatant were quantified for the presence of drugs. For the estimation of percent release of drug, the FLART NP was incubated with one ml of PBS (pH 5.0 and 7.4) for various time points such as 1, 2, 4, 6, 8, 10, 12, 16 and 24 h. Aliquots were removed at mentioned time points and drugs were estimated using HPLC.

Erythrocytes Toxicity Assay

The hemolytic impact of first-line drugs either in sol or nano form on the rat erythrocytes were conducted according to previously set protocol (23). The 2% erythrocytes stock dispersion was prepared as follows. Initially, the blood samples were collected in heparinized tubes. Blood was washed three times using 0.9% NaCl saline solution. After every wash, the cells were centrifuged for 5 min at 150x*g*; supernatant was discarded. The final pellet dilution was done (v/v) 1:9 in 0.9% NaCl saline solution followed by dilution in Dulbecco's phosphate buffer saline (D-PBS), pH 7.0 (v/v) 1:24 (24). The assay was performed by mixing 100 µl of erythrocytes stock dispersion with 1 ml of (1 mg/ml or 2 mg/ml) of FLART sol or FLART NP. The resultant mixture was incubated at 37°C under shaking condition for various time points. Saline solution and distilled water were considered as negative (0% lysis) and positive control (100% lysis) respectively. After incubation samples were spin down for 5 min at 1200 rpm to remove the debris and integral erythrocytes. Then 100 µl of supernatant was mixed with 2 ml of ethanol-HCl mixture (39:1; 99% ethanol [v/v], 37% hydrochloric acid [w/v]) followed by final centrifugation for 3 min at 750 g. The resultant supernatant was spectrophotometrically measured at 398 nm. The hemolysis rate was measured using

the formula; HR% = [(D1-D2)/ (D3-D2)] x 100. Here D1, D2 and D3 are the absorbance of tested samples, negative control and positive control respectively.

Analysis of Cellular Localization of Nanoparticles by Confocal Study

The cellular localization study of NP was performed in the macrophages cell line (U-937). As the ARV drugs were not fluorescent; the 30 mg of lactoferrin NP was tagged with 30 µg of Rhodamine-123 green fluorescent dye. Approximately 100% viable cells with a density of 10^4 were incubated for different time viz. 1, 2, 4, 6 and 8 h. After completion of the time points, cells were collected and washed thrice using PBS (pH 7.4) and fixed on clean glass slide. The fluorescence was estimated using laser confocal microscope (Carls Zeiss) by keeping the florescence setting according to rhodamine123 (Excitation – 505 nm and emission – 530 nm). Cells without any treatment were considered as control.

Anti-HIV Assay

Anti-HIV activity of FLART NP and their soluble counter parts were analyzed individually and in combination using HIV-1_{NL4-3} clone. Initially the individual drug loaded NP has been tested for their anti-HIV activity. For individual drug anti-HIV assay, 0.1million of SupT1 cells having 100% viability were infected with HIV-1_{NL4-3} for 12 h in RPMI 1640 media containing 0.1% fetal bovine serum. After 12 h, the cells were washed thoroughly to remove any unattached virus particles then resuspended in fresh media plus 10% fetal bovine serum. After 96 h, level of p24 protein in cell supernatant were evaluated using p24 antigen capture ELISA assay (Advanced Bioscience Laboratories). Increasing concentration of AZT, EFV and LMV (3TC) then have been tested and IC_{50} (50% inhibitory concentration) values were calculated for each drug in sol and in nanoformulation. For combination drug anti-HIV assay, all three drugs were mixed in accordance of their IC_{50} value in various ratio such as 1:1:1, 0.5:0.5:0.5, 0.25:0.25:0.25, 0.10:0.10:0.10 and 0.05:0.05:0.05.

In-Vivo Oral Pharmacokinetics Study

Animals and Treatment

Male and female Wistar rats weighing 0.160 kg to 0.250 kg and approximately six month old were obtained from Sainath agency, Hyderabad. Animals were acclimatized in a hygienic, well ventilated condition at animal house facility, University of Hyderabad for at least 1 week prior to treatment. They were allowed freely to access the commercial rat feed pellets and clean water. Current study was carried out in strict compliance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal experiments were approved by the Institutional Animal Ethical Committee, University of Hyderabad. Total 90 rats were divided randomly into eight groups. One among the eight group containing six rats (three male and three female) were served as control and is treated with saline. Rest of the seven groups were divided in accordance of seven different time point (0.5, 1, 2, 4, 8, 16 and 24 h); each group contains 12 rats (six male and six female). Out of six male/female rats, three males/female rats were treated with sol formulation of drug and rest three were treated with nanoformulation. Dosage schedule were fixed to 10 mg/kg for sol formulation (3.33mgAZT + 3.33 mg EFV + 3.33 mg 3TC) and equivalent amount for FLART NP. Time points of drugs treatment was determined according to the half-life of drugs. All dosages were administered orally with the help of 18SWG (Standard Wire Gauge) stainless steel curved oral gavage, while administration, the drug was suspended in PBS. After administration, all the animals were monitored hourly to determine the effect of NP on their weight and behavior. There were no deaths reported during the treatment. After the completion of all the time points, all animals were euthanized using ketamine (60 mg/kg) + Xylazine (10 mg/kg), IP. Blood samples were collected through heart puncture under terminal anesthesia condition. Final sacrifice was done by cervical dislocation. Organs such as brain, heart, lungs, liver, esophagus, spleen, stomach, small intestine, large intestine, kidney and bone marrow were collected. All the organs, except bone marrow were homogenized in 1x PBS (pH 7.4); 200 µl of 30% of silver nitrate/ml of tissue suspension was added to precipitate the protein. Drugs present in the mixture were extracted by adding the HPLC grade methanol into it; centrifuging at 12,000 rpm for 20 min at 4°C. Supernatant were filtered using 0.2 µm syringe filter and were quantified for the presence of drug using HPLC at respective wavelengths of drugs.

Tissue Safety Profile: Biochemical Safety Analysis and Histopathological Study

The biochemical tissue safety analysis was performed by using the commercially available kits from Span diagnostics or Cayman Chemical Company. After treatment with FLART sol and FLART NP, blood and all major tissues were collected. Serum was isolated from blood. The concentrations of AST (aspartate aminotransferase), bilirubin, urea, creatinine and Lactate dehydrogenase (LDH) were estimated in the serum and plotted against time. A small portion from all tissues were stored in neutral buffered formalin and processed for histopathology sectioning and analysis. Images were captured using light microscope at a magnification of 40X.

High Performance Liquid Chromatography (HPLC) Analysis

The quantitative estimation for all the three drugs i.e. AZT, EFV and 3TC were performed using HPLC (Waters, with 2487 dual detector). Drugs were separated using a reverse phase C-18 column. The mobile phase were set differently for each drugs. The mobile phase for AZT is methanol:ammoniumacetate:sodiumdioctylsulfosuccinate (60:40:4, v/v/v) and wavelength was 265 nm (25). For efavirenz and lamivudine, the mobile phase was selected previous set protocol (26,27). The flow rate of mobile phase was set to 1 ml/min and 10 µl of samples were injected for analysis. All the samples were filtered using 0.2 µm syringe filter before processing.

Statistical Analysis

All experiments were performed in triplicates. Data points were presented as means and standard deviation. Statistical analysis were done using student's *t*-test. P value < 0.05 was defined as significant.

RESULTS

Preparation and Surface Morphology (SEM and AFM) of Nanoparticles

The nanoparticles (blank NP or drugs loaded) were prepared using sol-oil chemistry methods. The FE-SEM and AFM analysis was performed to obtain the information about the particles size and surface morphology. FE-SEM analysis revealed an average particles size of 27-35 nm for blank LF-NP and 67 nm for drugs loaded NP (Fig. 1), the average size of individual drug loaded nanoparticles is found to be 59 nm for AZT (21), 70 nm for EFV and 65 nm for 3TC. While the size of the three drugs loaded NP was found in the range of 67 nm, suggesting that the three drugs are cooperatively interacting with the protein leading to optimal drug-loading in the lactoferrin nanoparticles. AFM data showed surface morphology of nanoparticles of particular type of projection or depression suggesting presence of intact structural features that facilitate promote recognition and binding to the receptor present on the target cell surface.

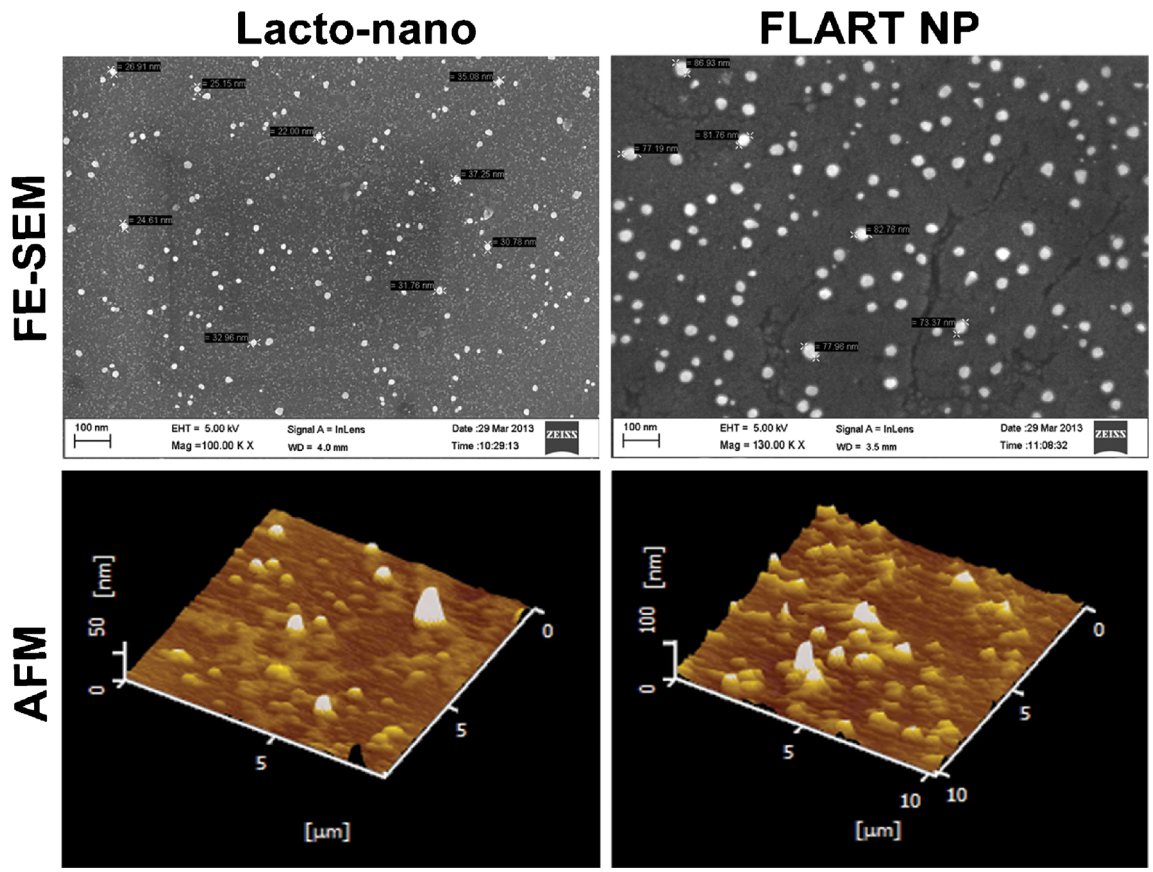

Fig. 1 FE-SEM and AFM analysis of nanoparticles. Upper panel represent the FE-SEM images of blank Lf NP (Lacto-nano) and first-line ART loaded Lf NP (FLART NP). Lower panel shows the AFM images of lacto-nano and FLART NP respectively. Size of NP has been increased up on loading of drugs.

Particles Size Distribution Studies of Nanoparticles

Size distribution of blank NP (Lacto-nano) and FLART NP was measured using NTA method. NTA accurately determines the broad range of NP population ratios. Results suggests an average size of 27 nm for Lacto-nano and 90 nm for FLART NP (Fig. 2).

Assembly of Nanoparticles and FT-IR Spectroscopic Study

Figure S1A shows the possible assembly of drug loaded nanoparticles. Figure S1B and C present the FT-IR spectra of pure lactoferrin and Lacto-nano respectively. Figure S1D and E show the spectra of AZT + EFV + 3TC in physical mixture and in loaded with lactoferrin NP respectively. The FT-IR data of FLART NP (Fig. S1E) shows that all the important functional groups corresponding to each drugs and lactoferrin protein remain intact. A slight shifting of the peaks may be observed in the nanoparticles, it may be due to the effects of dipole moment as the bond characteristics is electrostatic.

Drugs Loading (DL %) and Encapsulation Efficiency (EE %)

NP were prepared for a range of drug to protein ratios, and the results showed different EE for equal ratio of respective drugs (Supp. Table 1). The formulation X3 have shown the maximum EE, hence this ratio of formulation has been used throughout the experiments. The drug loading and encapsulation efficiency for each drugs, AZT, EFV and 3TC were calculated separately according to the already developed formula (28). In Formulation X3, the DL % was found to be approximately 5% for each drug and encapsulation efficiency was found to be in a range of 58 to 71% (Fig. 3a).

In-Vitro pH Dependent Drug Release and Percent Release from Nanoparticles

FLART NP was subjected over a range of various pH conditions starting from pH 1 to 9, SIF and SGF. It was found that maximum amount of drugs were released at pH 5 and 6 (Fig. 3b). The percent drugs release was determined at increasing time points viz. 1, 2, 4, 6, 8, 10, 12, 16 and 24 h under two different pH conditions i.e. pH 5.0 and 7.4. It was found that at pH7.4, only 20% of encapsulated drug was released in 24 h. While at pH 5, the biphasic drug release was observed; approximately 60% of the drugs were released up to 5 h and rest of 40% drugs were constantly released up to 24 h (Fig. 3c). All three drugs are released constantly together with similar kinetics suggesting that they follow the similar mode of drug loading.

Hemolytic Effect of FLART NP

Figure 3d shows the percentage of erythrocytes damage under the influence of FLART sol and FLART NP. A hemolysis

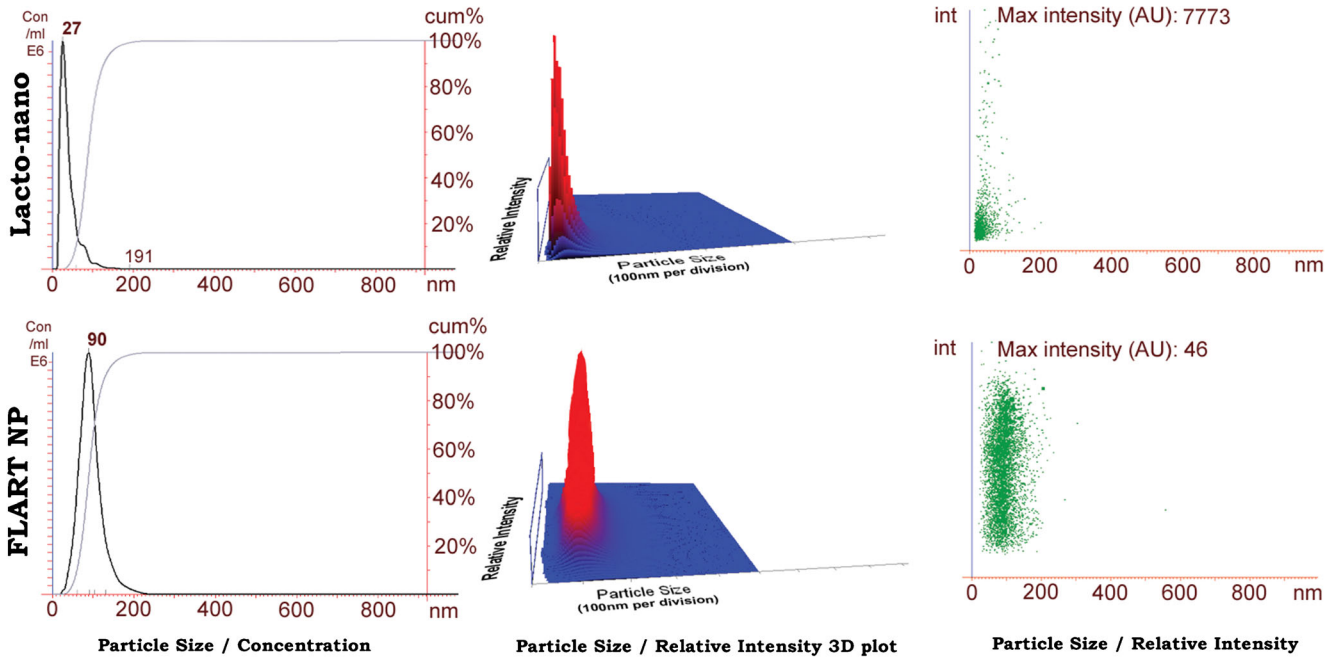

Fig. 2 Nanoparticles size distribution analysis: NTA (Nanoparticle tracking analysis) has been performed using Nanosight NS500. Upper and lower panels represent the lacto-nano and FLART NP respectively. Graph has been plotted between particles size verses concentration/relative intensity 3D plot/relative intensity 2D. Lacto-nano and FLART NP shows a size distribution of approximately 27 nm and 90 nm respectively.

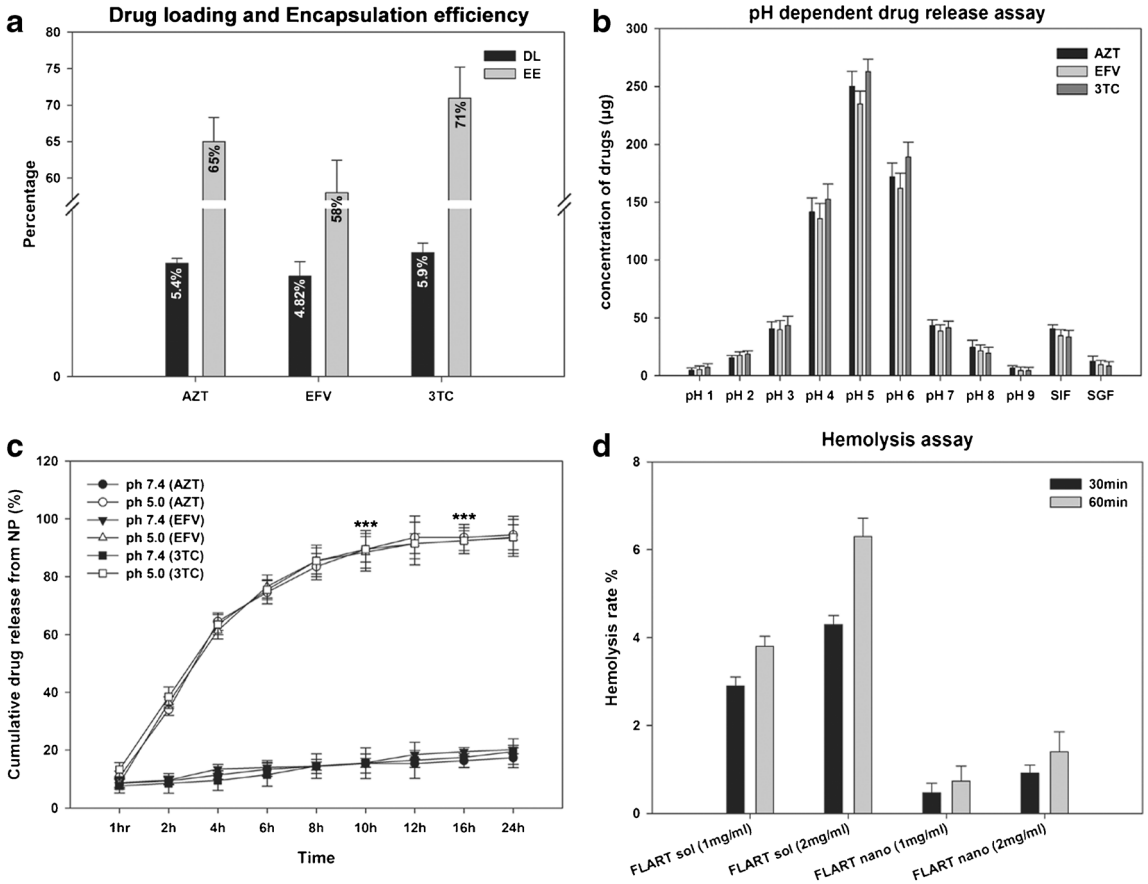

Fig. 3 (**a**) Denotes the DL (drug loading) and EE (encapsulation efficiency) for AZT, EFV and 3TC in FLART NP (formulation code X3). (**b**) pH and simulated fluid dependent drug release from Nanoparticles: 900 μg (300μgAZT + 300μgEFV + 300μg3TC) of FLART NP were incubated in the buffers of different pH, SIF (simulated intestinal fluid) and SGF (simulated gastric fluid). The release of all the drugs was maximum at pH-5 and this is followed by pH-6 and pH-4. (**c**) Percent cumulative Drug release: 900 μg (300μgAZT + 300μgEFV +300μg3TC) of FLART NP were incubated with pH5.0 and pH7.4. The cumulative percentage release profile of each drugs were calculated. (**d**) Hemolysis assay: Erythrocytes incubated with 1 or 2 mg/ml or AZT + EFV + 3TC in soluble or nanoform for 30 or 60 min. HR% (Hemolysis rate) has been calculated and plotted. Data points were taken in triplicate and presented as Mean ± SD. Value of significance, **P < 0.005, *P < 0.05.

percentage of <5% is generally considered as non-toxic (29–31). The hemolysis rate (HR %) when Nano formulation was less than 2.0% at higher concentration, which could be considered as non-toxic, while more than 5% hemolysis was observed with soluble drug.

Cellular Localization of Fluorescent Nanoparticles

Since the ARV drugs are non-fluorescent, we have used rhodamine123 tagged lactoferrin nanoparticles as a tracking agent to understand the cellular trafficking. The results of cellular localization assay (Fig. 4) shows that rhodamine123 tagged lactoferrin nanoparticles enter the cells slowly and reaches at highest level at 4 h. This study also confirms that the slow uptake of dye initially and its maintenance at a constant level for a longer period before elimination from cells, provide longer time for ARV drugs to act against HIV present inside macrophages.

Anti-HIV Activity of Nanoparticles

The *in-vitro* anti-HIV-1 activity of first-line HAART was evaluated as individual and in combination for soluble and nanoformulation. The individual IC_{50} for first-line HAART component are, sol AZT (33.4 nM) & nano AZT (20.5 nM), sol EFV (2.56 nM) & nano EFV (1.1 nM) & sol 3TC (42.57 nM) & nano 3TC (23.18 nM). The percent HIV-1 replication verses drug combination ratio have been plotted (Fig. S2). Further, the anti-HIV activity of individual component and in combination of nanoformulation first-line HAART has been found to be improved over its soluble formulation.

In-Vivo Pharmacokinetics and Tissue Distribution Analysis

All the animals were given a single oral dose of 10 mg (AZT/3.33 mg + EFV/3.33 mg +3TC/3.33 mg) of drugs. Serum was isolated from blood followed by estimation of AZT, EFV

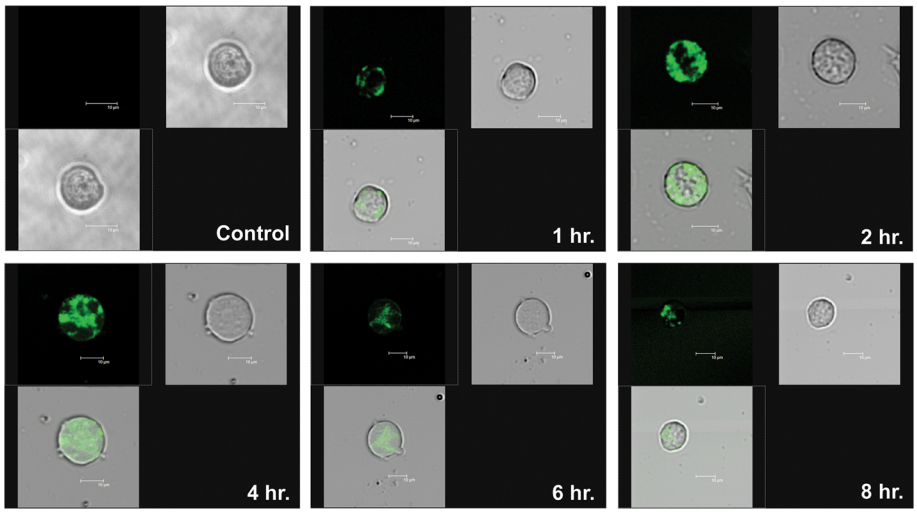

Fig. 4 Cellular localization of Rhodamine-123 tagged Lactoferrin nanoparticles in macrophages (U-937) cells: Cellular uptake of Lactoferrin nanoparticles loaded with rhodamine123 (Lacto-nano-rho) in U937 cells. Time course experiment shows that intracellular retention of NP with time. Scale bar is equal to 10 μM.

and 3TC individually using HPLC. Drug concentrations were calculated and compartmental pharmacokinetics analysis was done using Kinetica software and the PK parameters were presented in Table 1. Comparable pharmacokinetic profile was observed in both males and females. When pharmacokinetic parameters in rats treated with nano formulation versus soluble form are compared, the results show that C_{max} increased by 2.5 fold for AZT, 1.6 fold for EFV and 2.23 fold for LMV. The mean resident time of EFV nanoformulation was improved by a factor of at 2.41fold, while it was 1.2 and 1.29 fold enhancement for AZT and LMV. Nanoformulation has further increased T_{max} by 2 h for all the three drugs (Table 1). The tissues were homogenized and quantified for the drugs available in it. The tissue distribution analysis data were plotted for various tissues and shown in Fig. 5. Based on kinetics of nanoparticle absorption, NP first enters esophagus, stomach followed by absorption through small intestine. Then enters blood followed by liver, heart, lungs, spleen, brain,

Table 1 PK Profile for First-Line Regimen (AZT + EFV + 3TC) Therapy. The Blood Were Collected from the Treated Animals and PK Parameter Were Calculated Separately for AZT, EFV and 3TC

			AUC(tot) (h)*(μg/mL)	F_{rel}	AUMC(tot) (h)^2*(μg/mL)	C_{max} (μg/mL)	T_{max} (h)	$t_{1/2}$ (h)	MRT (h)
Drugs	Sex	Formulation							
AZT	Male	Sol	13.78 ± 2.83	3.824	57.50 ± 13.43	4.41 ± 0.78	1	1.67 ± 0.21	4.17 ± 0.78
		Nano	52.69 ± 11.34		265.39 ± 44.38	11.05 ± 1.97	2	2.40 ± 0.43	5.04 ± 0.91
	Female	Sol	13.14 ± 2.98	3.755	53.853 ± 17.49	4.73 ± 0.83	1	1.57 ± 0.19	4.10 ± 0.83
		Nano	49.36 ± 9.82		227.85 ± 35.59	12.331 ± 1.59	2	2.32 ± 0.49	4.61 ± 1.13
EFV	Male	Sol	0.1878 ± 0.034	4.541	0.4560 ± 0.083	0.0853 ± 0.001	1	1.32 ± 0.14	2.43 ± 0.47
		Nano	0.8531 ± 0.130		4.9954 ± 0.340	0.1369 ± 0.003	2	2.63 ± 0.51	5.86 ± 0.95
	Female	Sol	0.1983 ± 0.042	4.339	0.5221 ± 0.093	0.0899 ± 0.002	1	1.34 ± 0.17	2.63 ± 0.51
		Nano	0.8607 ± 0.158		4.9712 ± 0.428	0.1397 ± 0.003	2	2.50 ± 0.41	5.78 ± 1.18
3TC	Male	Sol	57.37 ± 11.93	3.073	248.87 ± 33.74	12.16 ± 2.77	1	1.35 ± 0.13	4.33 ± 0.92
		Nano	176.34 ± 32.84		989.09 ± 133.42	27.22 ± 3.81	2	3.43 ± 0.63	5.60 ± 1.31
	Female	Sol	38.77 ± 8.98	4.582	150.49 ± 24.63	10.65 ± 2.37	1	1.41 ± 0.20	3.88 ± 0.79
		Nano	177.63 ± 42.47		984.89 ± 163.19	26.57 ± 5.41	2	3.22 ± 0.46	5.54 ± 0.96

Pharmacokinetic parameters abbreviations

AUC (**Area under the Curve**): The integral of the concentration-time curve (after a single dose or in steady state)

Relative Bioavailability (**F_{rel}**): The ratio of $AUC_{Nano}/AUC_{Sol} \times 100\%$

AUMC (**Area Under the first Moment curve**): Partial area under the moment curve between t start and t end

C_{max}: The peak plasma concentration of a drug after oral administration

T_{max}: Time to reach C_{max}

$t_{1/2}$: The time required for the concentration of the drug to reach half of its original value

MRT: Mean Residence Times

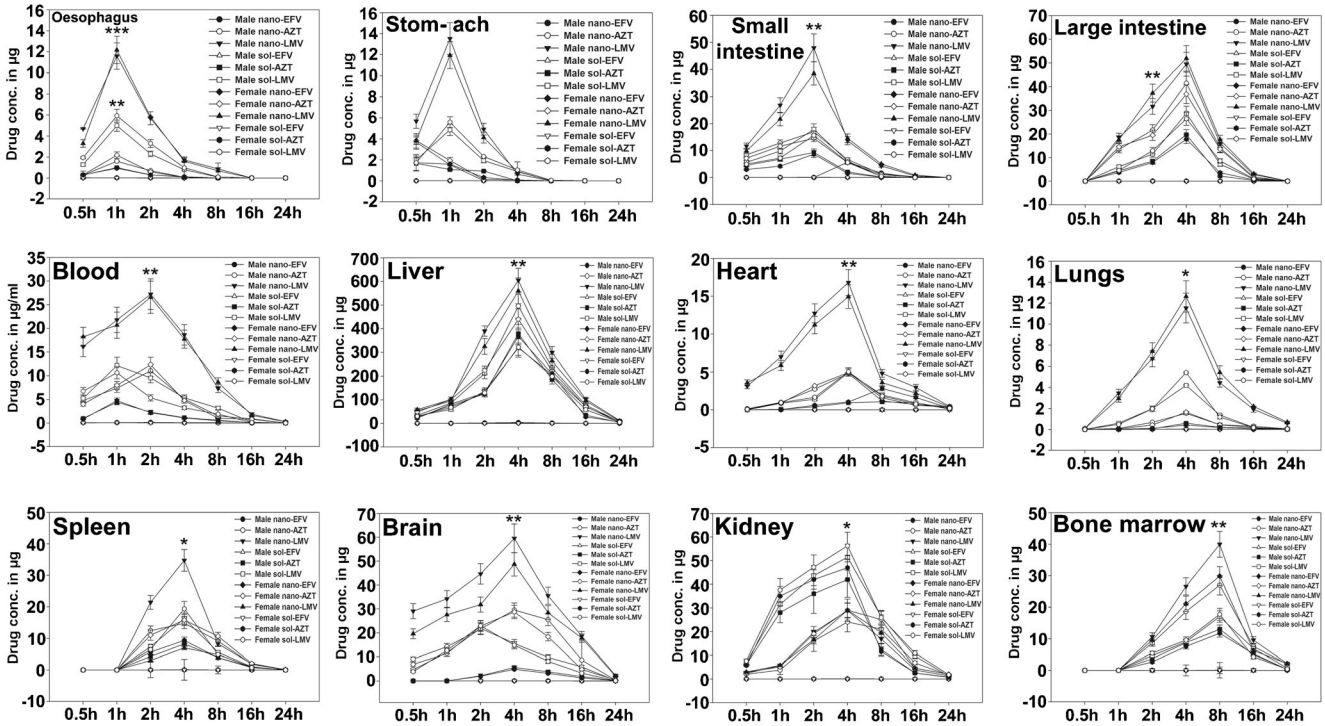

Fig. 5 Tissue distribution of FLART NP: Rats were orally given 10 mg/kg body weight of FLART sol (3.3 mg AZT + 3.3 mg EFV+ 3.3 mg 3TC) and equivalent of its nanoformulation. After different time points such as 0.5, 1, 2, 4, 8, 16 and 24 h the rats were sacrificed and drugs such as AZT, EFV and 3TC were estimated separately and plotted. Each data points were taken in triplicate and presented as Mean ± SD. Value of significance, $^{**}P < 0.005$, $^{*}P < 0.05$. Abbreviation: Male-nano-EFV and Male-sol-EFV: – Concentration of EFV measured separately in male rats, when delivered via FLART NP and FLART sol respectively. Same nomenclature have been followed for other groups.

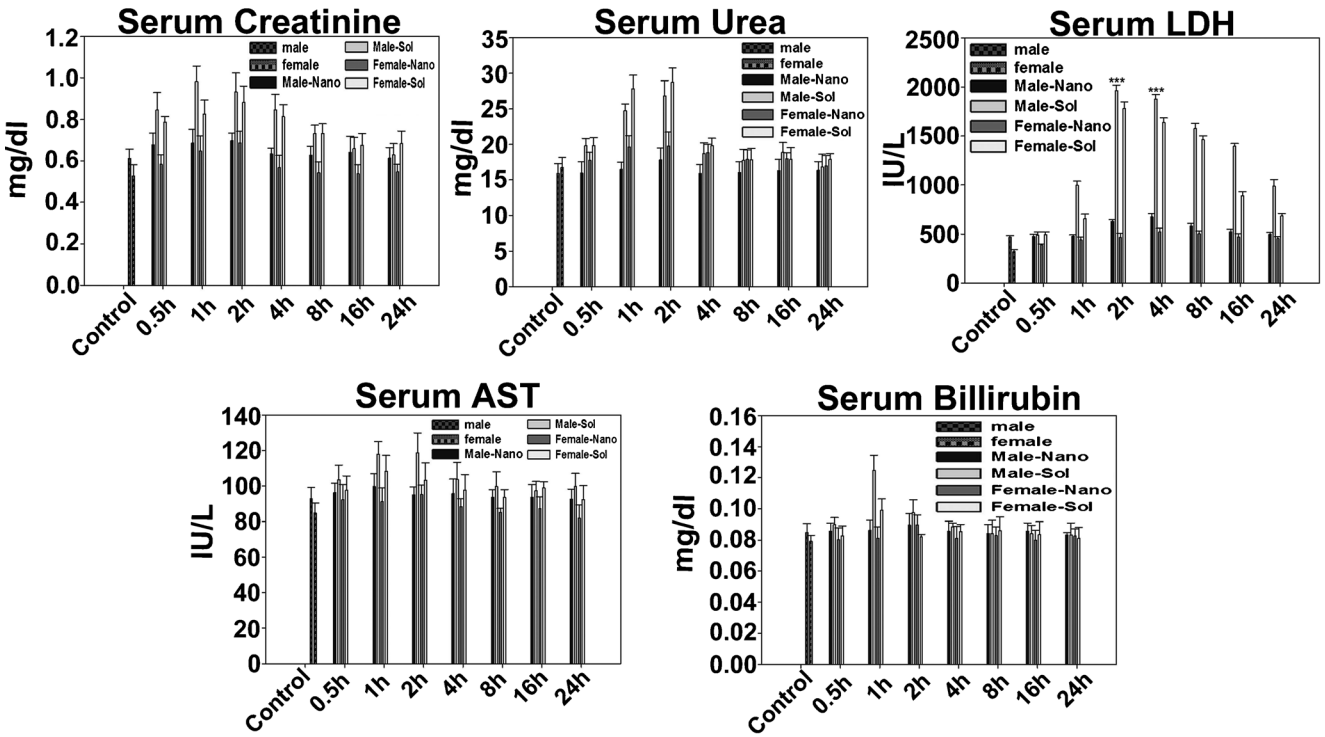

Fig. 6 Safety analysis of FLART NP: Safety analysis was done using biochemical kits after oral administration of FLART sol (10 mg/kg) and equivalent of Nano form, in both male and female rats. Liver damage was estimated by Bilirubin and (Aspartate aminotransferase) AST level whereas Kidney toxicity was checked by Urea and creatinine level. General tissue damage was checked by LDH (lactate dehydrogenase) level. Nano-form has showed no toxicity to both liver and kidneys on the other hand it exhibited minimal urea levels when compared to the soluble form.

kidney then bone marrow. Nanoformulation shows high blood concentration and low clearance as compared to its soluble counterpart. Further strikingly nanoformulation shows enhanced absorption of nano-LMV as compared to its soluble form in all tissues and blood.

Safety and Histopathology Analysis

The isolated serum was analyzed for the concentrations of creatinine, urea, AST, LDH and bilirubin (Fig. 6). Enhanced level of blood creatinine and urea (related to the kidney impairment) was found when treated with soluble drug combination, which were found to be significantly reduced when nano formulation is employed. Further, increased AST and bilirubin implies the moderate liver damage when treated with soluble combination of drugs at 2-4 h of post-treatment period, is found to be significantly reduced when treated with the nano formulation. These results are further confirmed by the histopathological analysis of tissue sections (Fig. 7) that show minor damage in the liver, heart or lungs tissue sections (indicated by black arrow), while such damage was not detected in these tissue sections when treated with nanoparticle formulation.

DISCUSSION

In the current clinical practice, the first-line HAART regimen is the initial treatment strategy for treatment of persons with HIV/AIDS. World Health Organization (WHO) recommends a fixed dose combination regimen which include one NNRTI with two NRTI. In case of the failure of treatment outcome, it is recommended to change in line of therapy to second line regimen; which includes ritonavir boosted protease inhibitor plus two NRTI (that is not included in first-line treatment) (-32),which also exhibits several toxicity problems.

For effective pharmacokinetic behavior of any drug, the size of a particle should be in a range of 5.5 nm to 100 nm; should show sustained blood circulation and effective target tissue localization and delivery of drug. A comparative analysis by quantum dots showed that particles below 100 nm exhibit low mononuclear phagocyte system (MPS) uptake (33,34). Present study shows that drug loaded NP exhibit a size distribution of approximately 67 nm in dry form and hydrodynamic size of 90 nm with enhanced *in-vivo* pharmacokinetics profile. The AFM microscopic analysis show a characteristic surface projections/depressions retaining structural features of lactoferrin, which may participate in recognition and binding to the receptor on the target tissue. In-spite of having different solubility properties, all the three drugs exhibit an encapsulation efficiency of >58%. EFV has shown comparatively less EE because of its high lipophilicity (log$P$ =5.4) (35). Results in Suppl. Table 1, show that the encapsulation efficiency of nanoparticles decreases at stoichiometric concentration reaches from 3:1 to 4:1 of lactoferrin:drug, suggesting higher protein concentration may enhance protein-protein interaction leading decrease in co-operativity of drug encapsulated protein particles. While drug loading reached maximum at stoichiometric concentrations of 2:1 of lactoferrin:drugs, further indicating drug loading reaches to saturation followed by molar ratio of drug reaching saturation at 3:1, thus saturating drug encapsulation. This also further seen from DL formula, it is clear that as the mass of protein will increase, the mass of nanoparticles (denominator) will increase, thus it may result in the decrease in the DL percentage. Considering saturated encapsulation as complete molar saturation of drug loaded protein particles, formulation ratio as

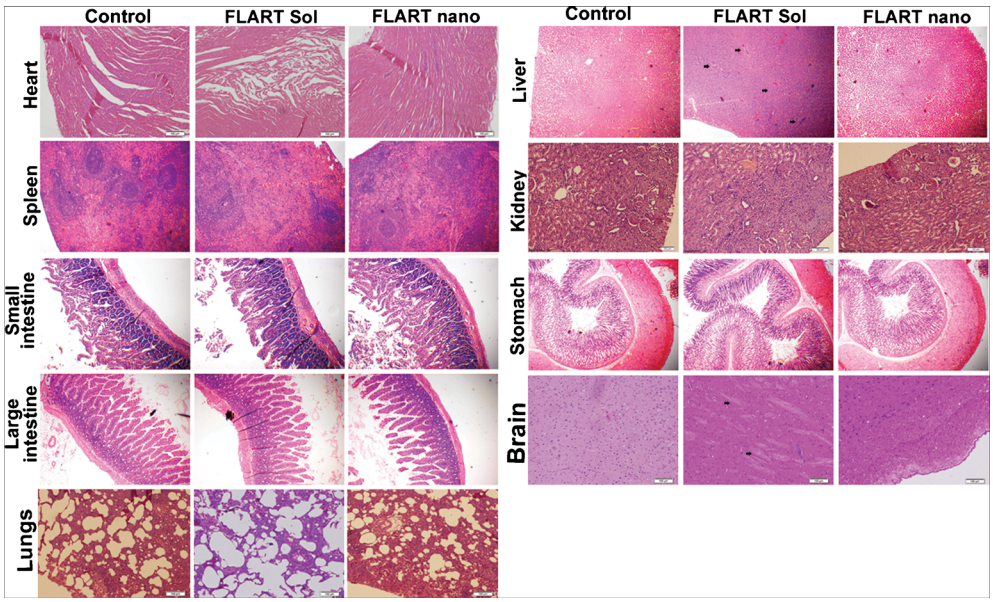

Fig. 7 Histopathology of tissue treated with first-line regimen: Rats were orally administered with FLART sol and FLART nano (10 mg/kg body weight), after completion of 24 h time point, organs were removed and processed for cryo-sectioning followed by Hematoxylin and Eosin (H&E) staining. It revealed that no toxicity was found in above indicated organs, when first-line drugs were delivered via nanoparticle form as compared to its sol form.

per X3 has been considered in this manuscript for nanoparticles preparation. Current lactoferrin NP has found to improve the drug loading capacity in contrast the solid lipid nanoparticles had shown very poor drug loading capacity (36). To make the formulation more efficacious, it is important to deliver the drug to the target tissue. Non-targeted drug delivery leads to undesirable accumulation of drug inside body that results in the tissue toxicity. Intracellular trafficking of nanoparticles were studied in macrophages (U937 cells). The results show that localization of lactoferrin nanoparticles increased exponentially and reaches a maximum at 4 h, followed by a significant decrease over the period of 8 h, thus suggesting that the lactoferrin nanoparticles after release of its payload undergo exocytosis and get secreted out from cells. Thus delivery vehicle would not become burden to the cells. This inference is supported by the results of the pH dependent release assay and percent release assay, where at physiological pH (pH7.4) only 10–20% of drugs was released, while at endosomal pH (pH5.0) approximately 80-90% of drugs release was observed from NP. After oral delivery, it is important that NP should not be able to release the drug in extracellular condition. The results of pH-dependent drug release assay shows that a negligible amount of drugs release was observed in SIF (simulated intestinal fluid) and SGF (simulated gastric fluid), while maximum amount of the drugs were released under endosomal (pH5) conditions suggesting drugs are targeted to endosomes in intracellular delivery. Our current formulation is more superior to other formulation such as chitosan nanoparticles which is found to be unstable at gastric and intestinal condition (37). The drugs could be encapsulated in the core of NP with a non-covalent interaction (electrostatic attraction); supported by FT-IR data as major functional groups are intact. A pH (pH 5) change triggers the protein entities present in NP to change its conformation thus leading to release of drugs from inside of the core. Earlier report suggested that Zidovudine treatment causes severe anemia that leads to cessation of HIV treatment (38). Antiviral data suggest that, the individual drug nanoformulation is found to be more efficacious than its soluble counterpart as indicated by the reduced IC_{50} value. Furthermore, 50% of concentration of the drug combination FLART NP is adequate to confer anti-HIV activity of corresponding soluble combination. Results further suggest that FLART NP exhibits low toxic to the erythrocytes (<2%) even at high concentration, which is acceptable toxicity levels. The *in-vivo* analysis of bioavailability of drugs suggest that FLART NP exhibits an outstanding improvement in the PK profile. Drugs delivered via nanoformulations showed a prolonged exposure of drug in the blood circulation (Fig. S3), as the AUC value was found to be increased by approximately 4-fold. The AUMC (tot) has been increased by 4–10 fold for nanoformulation as compared to the soluble drug combination. The relative bioavailability (F_{rel}) between nanoformulation and soluble formulation for each of the three drugs, AZT, EFV and 3TC were found to be >3, suggesting that drugs delivered through lactoferrin nanoparticles exhibits higher systemic circulation and exposure when compared to its soluble form. Further, it reflects that increased fraction of soluble drugs are getting metabolized before reaching to the systemic circulation as compared to its nanoformulation. It clearly indicates that the first pass effect was found to be minimized when drugs were delivered using nanoformulation. The T_{max} and $t_{1/2}$ have increased by 2-fold. It has been reported that the protein binding capacities of AZT and 3TC are 34–38% and 16–34% respectively, while for EFV it is >99%, thus pointing out to differences in bioavailability of these drugs in different tissues. The observed increase of serum biochemical profile for creatinine, urea, AST and bilirubin shows that liver and kidney impairment in the case of soluble drug combination treatment was completely abolished with FLART Nano formulation confirming advantage of targeted delivery of drugs using Nano formulation. These results are further supported by the Histopathological analysis.

In conclusion, a novel protein based nanoformulation which successfully encapsulated the three HIV ARV drugs, has been tested and the results show significant improvement in efficacy, safety and pharmacokinetic behavior, thus facilitating reduction in frequency of dosage schedule for HIV treatment.

CONCLUSION

In conclusion, we have successfully encapsulated three anti-HIV drugs (Zidovudine, efavirenz and lamivudine) together in the nanoparticles. These first-line ART drugs loaded nanoparticles were well dispersed stable colloidal solution. These protein based nanoparticles are easy to prepare, long lasting, biodegradable and non-toxic to erythrocytes. The *in-vitro* data suggest that these nanoparticles are able to release drugs intracellularly in controlled and sustained manner. Drugs loaded nanoparticles have improved the pharmacokinetics profile for each drugs without causing any toxicity to the major organs. So we can conclude that FLART nanoformulation provide a deep insight in the area of novel drug delivery systems.

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PK (orcid.org/0000-0003-1038-6149) is UGC-NET fellow, YSL is ICMR-SRF fellow. This work was supported by Department of Science and Technology under Nano mission (#SR/NM/NS-1127/2011). AKK is recipient of FRPS BSR UGC one time grant. PK and AKK conceived and designed the experiments. PK and YSL did the experiments and analyzed the data. AKK and PK wrote the manuscript. The authors report no conflicts of interest in this work

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ORIGINAL RESEARCH

An oral formulation of efavirenz-loaded lactoferrin nanoparticles with improved biodistribution and pharmacokinetic profile

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Objectives

Efavirenz (EFV), a non-nucleoside reverse transcriptase inhibitor, is a drug that is frequently included in highly active antiretroviral therapy for treatment of HIV infection. Decreased bioavailability and increased toxicity limit its use. We report a formulation of efavirenz-loaded lactoferrin nanoparticles (lacto-EFV-nano) for oral delivery which exhibited significantly improved pharmacological properties coupled with reduced toxicity compared with its free form.

Methods

Lacto-EFV-nano was prepared using the Sol-oil protocol and characterized using various sources of characterization. *In vitro* and *in vivo* studies were performed to test the stability, safety, efficacy, biodistribution and pharmacokinetics of lacto-EFV-nano.

Results

The nanoparticles prepared for the present study had an average size of 45–60 nm as revealed by field emission scanning electron microscope measurements. Further, dynamic light scattering data showed a hydrodynamic radius of 103 ± 5.3 nm, a zeta potential of −23 ± 1.2 mV and a polydispersity index of < 0.341. Lacto-EFV-nano was found to be stable as assessed using differential scanning calorimetry and Fourier-transform infrared spectroscopy. Cell viability studies showed that lacto-EFV-nano was at least 2-fold less toxic to peripheral blood mononuclear cells, Jurkat T cell and B16-F10 cell lines than free EFV. Furthermore, lacto-EFV-nano [50% inhibitory concentration (IC_{50}) < 1.1 nM] showed > 2-fold enhanced anti-HIV-1 activity compared with free EFV (IC_{50} = 2.56 nM). Lacto-EFV-nano exhibited improved oral bioavailability and an improved *in vivo* pharmacokinetic profile, with a > 3–4-fold increase in the area under the plasma concentration–time curve (AUC), a 6–7-fold increase in the area under the first moment curve (AUMC), a > 30% increase in the peak plasma concentration of the drug after oral administration (C_{max}) and a 2-fold increase in the time to reach C_{max} (T_{max}) and the time required for the concentration of the drug to reach half of its original value ($t_{1/2}$). Furthermore, lacto-EFV-nano did not show any organ-related toxicity. A significant decrease in the concentrations of various parameters, elevated concentrations of which are markers of reduced safety, were also observed in rats treated with lacto-EFV-nano.

Conclusions

Compared with free EFV, lacto-EFV-nano is a promising oral nanoformulation with enhanced bioavailability and efficacy of EFV and improved safety.

Keywords: efavirenz, HIV, nanoparticles, pharmacokinetics

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Introduction

According to the current epidemiological data sheet, approximately 35 million of the world's population are living with HIV/AIDS [1]. Progression to AIDS is determined primarily on the basis of a low CD4 count, which may eventually lead to immunological failure [2,3]. The Food and Drug Administration (FDA), USA has approved six classes of drugs for treatment of HIV infection: (1) non-nucleoside reverse transcriptase inhibitors (NNRTIs), (2) nucleoside reverse transcriptase inhibitors (NRTIs), (3) integrase inhibitors, (4) protease inhibitors (PIs), (5)

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fusion inhibitors and (6) co-receptor antagonists [4]. Treatment of AIDS started with mono-drug therapy in the late 1990s, subsequently shifted to combination therapy with two drugs, and is currently triple-drug combination therapy. Triple-drug combination highly active antiretroviral therapy (HAART) includes two NRTIs plus one NNRTI [4–6]. Efavirenz (EFV) is highly lipophilic with a partition coefficient (LogP) of 5.4 [7], having very poor aqueous solubility (8.3 μg/mL) [8]. It is a very commonly prescribed combination drug in first-line antiretroviral regimens for AIDS treatment [9]. EFV exhibits very high plasma protein binding (> 99%) [10], which results in very low bioavailability of 40–45%, which in turn necessitates administration of very high doses [11]. The pharmacological activity of a drug is mainly dependent on the unbound fraction of the drug in the blood plasma. Various attempts have been made to improve the pharmacokinetic (PK) profiles of drugs characterized by poor bioavailability, including the introduction of nanotechnology-based delivery systems using nanoparticles (NPs) and nanosuspensions [11–13]. Among these, the NP-based system is one of the most promising drug delivery systems [14]. To deliver the drug in a controlled and sustained manner at the site of action and to maintain the desired therapeutic concentration over a long period, various biodegradable polymers have been used as nanocarriers. Our study was concerned with the development of a lactoferrin (Lf)-based nanodelivery system. Lf is an iron-binding protein present in external secretions such as tears, nasal fluid, saliva, pancreatic and gastrointestinal secretions, urine and reproductive tissue secretions [15–17]. It is a biodegradable multifunctional protein having various properties such as anti-inflammatory, virucidal and antimicrobial properties. These properties make it a more efficient and safer drug delivery system compared with systems based on other molecules [16]. In addition to these qualities, it is easily available at low cost and is thus an ideal material for use in the development of NPs. The aim of the present study was to develop a highly stable Lf-based oral nanoformulation for delivery of EFV with sustained optimum release of the drug along with reduced toxicity and improved pharmacological properties and anti-HIV activity.

Methods

Nanoparticle preparation

Nanoparticles (NPs) were prepared by the Sol-oil method [18] using a fixed concentration of EFV and varying concentrations of Lf (Supporting Information Table S1). Initially, in each of a set of five tubes, 10 mg of EFV was dissolved in 100 μL of methanol. In another set of five separate tubes, an increasing concentration of Lf (10, 20, 30, 40 and 50 mg) was dissolved in 500 μL of phosphate-buffered saline (PBS; pH 7.4). EFV and Lf were mixed in the ratio shown in Table S1 and incubated on ice for 60 min. The resultant mixture was slowly added to 30 mL of olive oil at 4°C with continuous dispersion by gentle manual vortexing. The sample was sonicated for 15 min at 4°C using the narrow stepped titanium probe of an ultrasonic homogenizer. The sonication amplitude was 5 μm and the pulses were 30-S long, with an interval of 1 min between successive pulses. The resulting mixture was immediately frozen in liquid nitrogen for 15 min and was then thawed on ice for 4 h. The particles formed were pelleted down by centrifugation at 2915 × g for 10 min. The supernatant was then discarded and the pellet was thoroughly washed with diethyl ether and dispersed in PBS (pH 7.4). NPs loaded with EFV are referred to as "lacto-EFV-nano" and those without EFV as "blank-NPs" or "lacto-nano".

Nanoparticle characterization

Size measurement of nanoparticles

The diameter of NPs was measured using field emission scanning electron microscopy (FE-SEM; Philips FEI-XL 30 ESEM; FEI, Hillsboro, OR). The sample for FE-SEM was prepared as follows. Freshly prepared NPs were suspended in 1 mL of Milli-Q (Merck-Millipore corporation, Darmstadt, Germany). A drop of suspended NPs was dripped onto a clean sterile glass slide and dried overnight. Particles were coated with gold and were observed under the microscope.

Measurement of the size distribution, polydispersity index (PDI) and zeta potential

The size distribution and PDI of blank-NPs and lacto-EFV-nano in suspension form were measured with dynamic light scattering (DLS) methods using the NP analyser SZ-100 (Horiba Scientific, Kyoto, Japan). The zeta potential (ζ) was measured using specially designed capillary zeta cells.

In vitro experiments

Encapsulation efficiency (EE)

The EE of the NPs (as a percentage of the total amount of drug encapsulated) was evaluated by estimating the concentration of drug released by the NPs [19]. NPs were incubated with 1 mL of 1× PBS, pH 5.0, for 12 h on the rocker. After incubation, 30% silver nitrate (200 μL) was added and the mixture was vortexed. The resultant mixture was combined with 1 mL of high-performance liquid

chromatography (HPLC)-grade methanol to extract the drug. This mixture was kept on the rocker for 2 h at room temperature followed by centrifugation at 24084 × g for 10 min at 4°C. The collected supernatant was filtered through 0.2-μm syringe filters and the drug was quantified using HPLC at 247 nm. The mobile phase consisted of 0.1% formic acid:acetonitrile in a ratio of 25:75 (v/v), with a flow rate of 0.3 mL/min. The EE (as a percentage) was calculated using the following formula:

EE = [(total amount of drug added − amount of free drug in supernatant)/total amount of drug] × 100.

Drug release at different pH values and in simulated body fluids

Three hundred micrograms of lacto-EFV-nano was incubated with 1 mL of 1X PBS at different pH values from pH 1 to 9, including simulated gastric fluid [pH 1.6 (fasted) and pH 3 (fed)] [20] and simulated intestinal fluid [pH 5.4 (fed) and pH 6.5 (fasted)] [20]. After the incubation, the drug was extracted and its concentration estimated by the silver nitrate precipitation method, as described in the previous section.

In vitro drug kinetics

This experiment was performed to examine the time-dependent cellular uptake of NPs. Briefly, 0.1×10^6 SupT1 cells were suspended in RPMI 1640 with 10% fetal bovine serum and seeded in a 12-well plate. Cells were incubated with 30 μg of soluble EFV (sol EFV) and 30 μg of lacto-EFV-nano for different times: 30 min, 1, 2, 4, 6 and 8 h. After completion of the incubation period, cells were washed in 500 μL of 1× PBS (pH 7.4) twice. The cell pellet was then re-suspended in 500 μL of lysis buffer (0.1% Triton X-100; Sigma Aldrich, Missouri, USA) followed by mild sonication for 20 S. Samples were then mixed with 1 mL of methanol and 200 μL of 30% silver nitrate, and centrifuged at 13548 × g for 10 min. Finally, the concentration of EFV in the supernatant was determined using HPLC.

Anti-HIV-1 assay

Lacto-EFV-nano was evaluated for anti-HIV activity *in vitro*. Briefly, 0.1×10^6 SupT1 cells with 100% viability were challenged with the HIV-1_{NL4-3} virus clone for 10 h. On the next day, the cells were washed thoroughly to remove any unattached viral particles and resuspended in fresh RPMI 1640 plus 10% fetal bovine serum medium. Experiments were conducted at increasing concentrations (0.1–4 nM) of soluble EFV and lacto-EFV-nano. Vehicle control (blank Lf NPs) and zidovudine (ZDV) were used over a concentration range of 0.1–15 μM. ZDV was used as a positive control. The cell supernatant at day 5 was collected and viral replication was estimated in terms of p24 using a p24 antigen capture assay (Lot No. 11111101; Advanced Bioscience Laboratories, Kensington, MD). The p24 level in the absence of drug was considered to represent 0% inhibition. The efficacy of the drug was measured in terms of percent inhibition of HIV-1 replication.

In vivo experiments

Pharmacokinetic and tissue distribution (TD) studies in rats

Studies of the biodistribution of EFV-loaded Lf NPs were carried out in healthy adult albino Wistar rats weighing in the range of 160−250 g. All animals were provided with proper care, food, and water *ad libitum* and kept in well-ventilated spacious cages throughout the study. A total of 66 rats (33 male and 33 female) were used for the PK and TD experiments. The 66 rats were divided into two groups: group 1 (control; saline-treated) contained six rats (three male and three female) and group 2 (drug-treated) contained 60 rats. These 60 rats were further divided equally into five groups, each assigned to a different time-point after administration of the formulation (30 min, 1, 2, 4 and 12 h after administration) at which they would be killed, so each group or time-point was represented by 12 rats (six male and six female). Among the 12 rats at each time-point, six rats (three male and three female) were administered soluble EFV and the remaining six (three male and three female) were administered lacto-EFV-nano. All formulations were given (in a fasted state) at a dose level equivalent to 10 mg/kg body weight. At the respective time-points, the rats were killed by injecting sodium pentobarbital (50 mg/kg intraperitoneally). The tissues collected included the brain, heart, liver, lungs, kidney, spleen, oesophagus, small intestine, large intestine, stomach and bone marrow. From rats killed at the 4-h time-point, a small portion of each tissue was saved in neutral buffered formalin for histopathology. The tissues were washed with saline and homogenized using a rotor homogenizer. Then 1 mL of 30% silver nitrate per 5 mL of tissue homogenate was added to precipitate the protein. The drug was then extracted by adding methanol and centrifuging at 12 000 rpm for 12 min, and the collected supernatant was filtered through a syringe filter and the concentration of EFV was estimated using an HPLC UV detector (Alliance Separations Module e-2695 Waters High Performance Liquid Chromatography) at 247 nm.

Tissue safety and histopathology analyses

After completion of treatments for all the respective time-points, plasma was isolated from all animals. This

plasma was used for the analysis of lactate dehydrogenase (LDH), the lipid profile, creatinine, aspartate transaminase (AST) and alanine aminotransferase (ALT). A safety analysis was performed using commercially available kits. Haematoxylin and eosin (H&E) tissue staining was performed and the tissues were examined microscopically for the presence of any abnormalities.

Statistical analysis

All *in vitro* and *in vivo* studies were carried out in triplicate. Data are presented as mean ± standard deviation (SD). The significance of differences between more than two groups was determined by one-way analysis of variance (ANOVA) and that of differences between two groups was determined using Student's *t*-test. The level of significance was set at $P < 0.05$.

Results

Estimation of encapsulation efficiency of nanoparticles

The EE of lacto-EFV-nano was estimated separately for five different protein concentrations, keeping the drug concentration constant. The EE estimates for different combination ratios are shown in Table S1. The results showed that the EE was maximum for the formulation code F3 (59 ± 1.3) at a 1:3 molar ratio of EFV to Lf. Hence the formulation F3 was used throughout the study unless otherwise stated.

Nanoparticle characterization

Field emission scanning electron microscopy analysis of nanoparticles

The mean diameter of blank NPs (or "lacto-nano") was 19−35 nm (Fig. 1), while after loading of the drug, that of lacto-EFV-nano was 45−60 nm.

Size distribution, PDI and zeta potential of NPs

The DLS results (Fig. 1) indicated the size distribution of blank and drug-loaded NPs. The hydrodynamic radii of blank and drug-loaded NPs were found to be 72 ± 3.4 nm and 103 ± 5.3 nm, respectively. The size of NPs measured by DLS was found to be larger than that obtained using FE-SEM. We infer that the larger size was caused by the water shell present around the NPs. A zeta potential of −23 ± 1.2 mV was found for lacto-EFV-nano (Fig. 1). The PDI of lacto-EFV-nano was < 0.341.

In vitro studies

pH-dependent stability of lacto-EFV-nano

The *in vitro* pH-dependent drug release experiment showed that maximum drug release occurred at pH 5, followed by pH 4 and 6 (Fig. 2a). As compared to pH 5, less drug was released in simulated gastric fluids and simulated intestinal fluids, indicating that NPs were more stable and more of them remained intact during their transport through the gastrointestinal environment, thus suggesting that these intact NPs are absorbed via the oral route.

Cellular retention of lacto-EFV-nano

For the development of any new formulation, cellular drug internalization and sustained retention are essential. Drug kinetics data suggest that EFV was efficiently taken up via Lf NPs. Lacto-EFV-nano showed biphasic internalization of the drug, with a significant linear increase from 0.5 to 4.5 h, then remained constant up to 9 h, followed by a steep drop up to 12 h (Fig. 2b). In contrast, soluble EFV uptake showed an increase up to 2 h followed by a steady decline up to 12 h. The results thus show that lacto-EFV-nano exhibited a longer cellular retention time compared with the soluble form.

Anti-HIV-1 activity of nanoparticles

The anti-HIV-1 activity of soluble EFV and lacto-EFV-nano was evaluated in SupT1 cells infected with HIV-1_{NL4-3} (Fig. 3a). Lacto-EFV-nano showed dose-dependent activity in inhibition of HIV-1 replication, with a 50% inhibitory concentration (IC_{50}) of 1.1 nM, while for soluble EFV the IC_{50} was 2.56 nM. Furthermore, lacto-EFV-nano showed an improved dose-dependent profile. In addition, blank (without drug) Lf NPs also showed dose-dependent anti-HIV-1 activity, with an $IC_{50} < 4$ μM. The results thus suggest that lacto-EFV-nano exhibits anti-HIV-1 activity attributable to both EFV and Lf. The developed nanoformulation lacto-EFV thus works in a dual mode and hence can provide effective dual therapy against HIV-1 (Fig. 3b).

In vivo studies

Oral pharmacokinetics study

A single dose of lacto-EFV-nano or the soluble form of EFV (10 mg/kg) was administered orally to rats. Rats were killed at the time-points indicated in the "Methods" section under anaesthesia and plasma was collected. EFV was extracted using the silver nitrate method and its concentration was estimated using HPLC as described in the

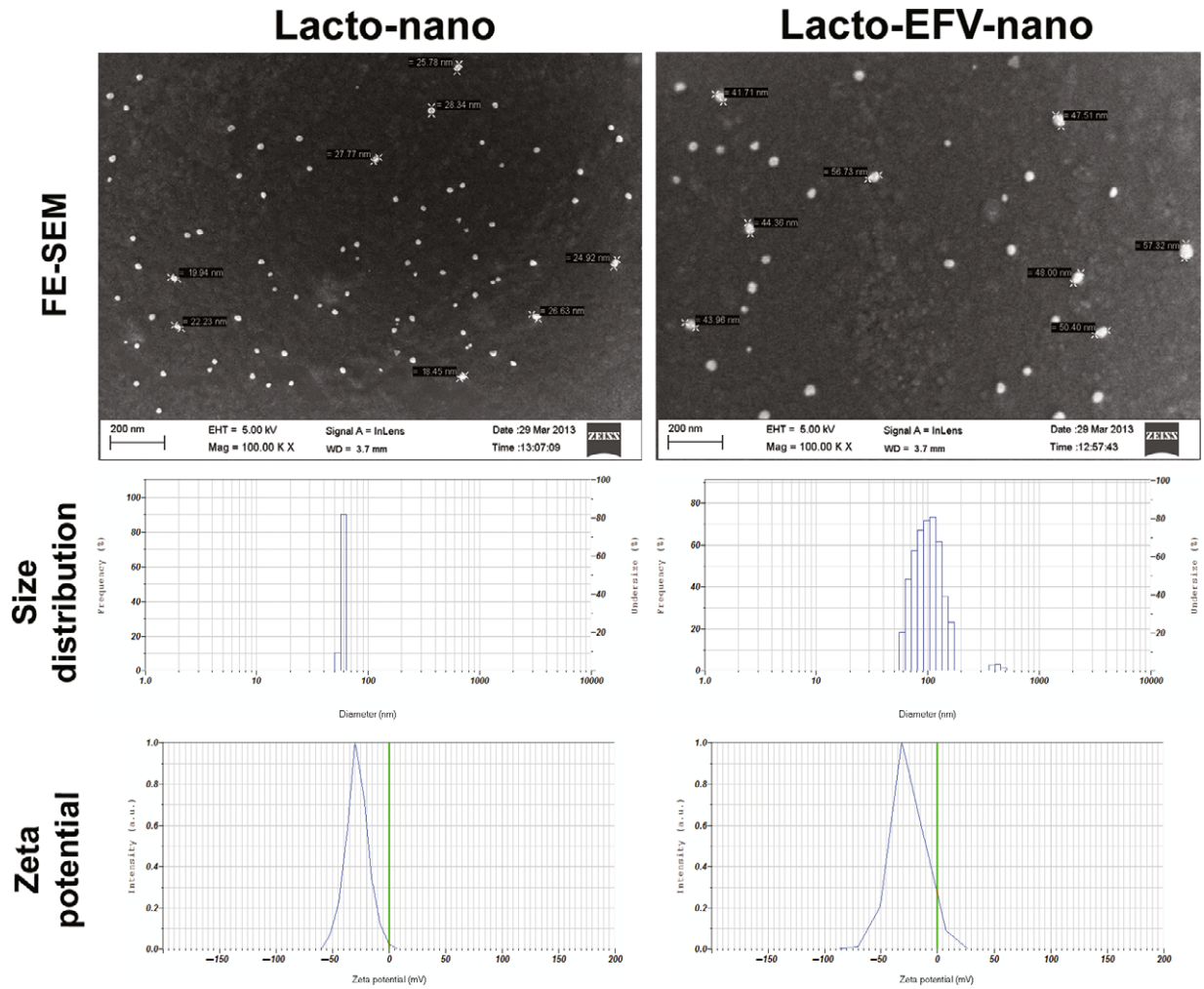

Fig. 1 Characterization of nanoparticles (NPs). The top left and top right panels show results for the field emission scanning electron microscope (FE-SEM) analysis of blank-NPs (lacto-nano) and lacto-efavirenz (EFV)-nano, respectively. The middle left and right panels show the size distributions measured using dynamic light scattering (DLS) analysis of lacto-nano and lacto-EFV-nano, respectively. The bottom left and right panels show the zeta potentials of lacto-nano and lacto-EFV-nano, respectively.

"Methods" section. EFV delivered through lacto-EFV-nano was found to have more localize at 2 and 4 h post administration compared with the soluble form. All PK parameters were computed using KINETICA version 5.0 software (Thermo Fisher Scientific) and are shown in Table 1. An improved PK profile in terms of a 3-4-fold increase in the area under the plasma concentration–time curve (AUC) and a 6-7-fold increase in the area under the first moment curve (AUMC) was observed in rats treated with lacto-EFV-nano compared with those treated with soluble EFV. Further, rats treated with lacto-EFV-nano showed a 30% enhancement of the peak plasma concentration of the drug after oral administration (C_{max}) and 100% increases in the time to reach C_{max} (T_{max}) and the time required for the concentration of the drug to reach half of its original value ($t_{1/2}$). These results suggest that the nanoformulation provides higher bioavailability coupled with an improved PK profile.

Tissue distribution

After administration of the dose of the formulation indicated in the "Methods" section, rats were killed, all the organs were homogenized and drug localization was quantified. The distribution of EFV administered in the soluble or NP form was estimated in various tissues and plotted against time (Fig. 4). Gut tissues, such as the

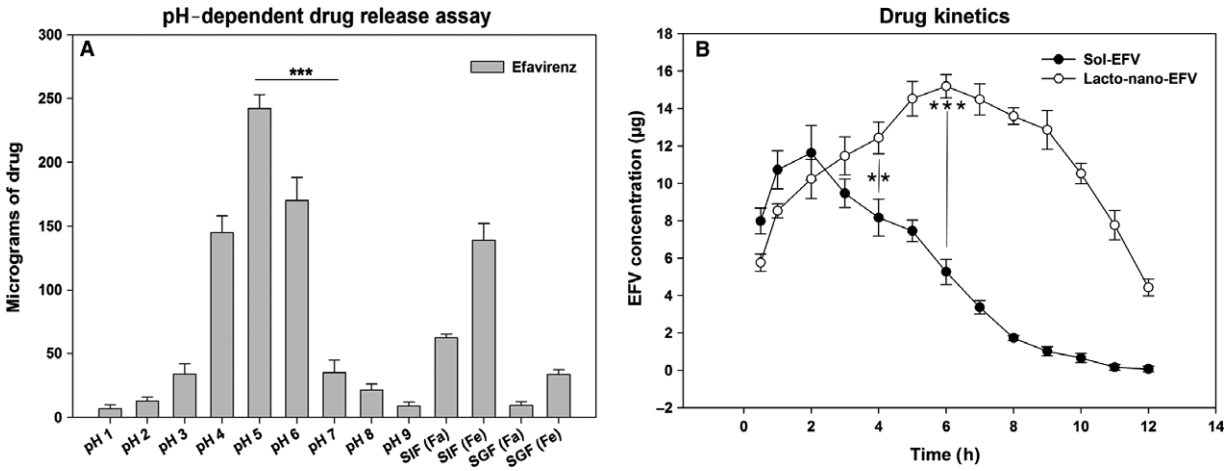

Fig. 2 pH and time dependent drug release profile from nanoparticles (a) pH-dependent release assay measuring the release of efavirenz (EFV) from lacto-EFV-nano. Three hundred micrograms of particles was incubated in buffers of different pH, and the release of EFV was found to be maximum at pH 5, followed by pH 6 and 4. < 20% of drug less than 20% of drug was released in fasted-state simulated fluid. SIF (Fa), fasted-state simulated intestinal fluid; SIF (Fe), fed-state SIF; SGF (Fa), fasted-state simulated gastric fluid; SGF (Fe), fed-state SGF. (b) *In vitro* drug kinetics (cellular uptake) assay of EFV, either in soluble form (Sol-EFV) or loaded in lactoferrin nanoparticles. EFV concentrations were estimated using high-performance liquid chromatography (HPLC) at 247 nm. All data points were measured in triplicate and are denoted as mean ± standard deviation.

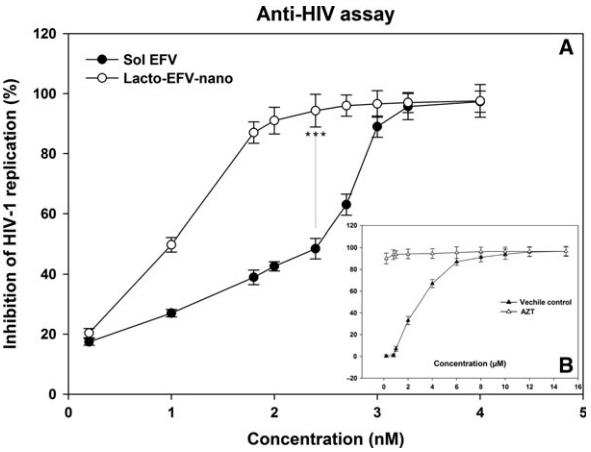

Fig. 3 *In vitro* anti-HIV-1 assay. (a) Comparison between the anti-HIV-1 activities of efavirenz (EFV) in soluble form and in nano form. SupT1 cells infected with HIV-1_{NL4-3} were incubated with increasing concentrations (0.2–4 nM) of soluble EFV and lacto-EFV-nano. (b) Vehicle control (lacto-nano) and zidovudine (ZDV) (negative control) were used at concentrations of 0.2–15 µM. On the fifth day post-infection, the p24 level was measured using an enzyme-linked immunosorbent assay (ELISA). The p24 level in the absence of drugs was considered to represent 0% inhibition. Data are presented as mean and standard deviation. The level of significance is $^{**}P < 0.01$ and $^{***}P < 0.001$ for the difference between lacto-EFV-nano and soluble EFV.

oesophagus, stomach and small intestine, showed higher concentrations of EFV delivered via the nanoformulation compared with the soluble form. The maximum concentration of lacto-EFV-nano was observed at 0.5 h in the oesophagus and stomach, and the next highest concentration was observed in the small intestine at 2 h, in both male and female rats. The peak plasma concentration of the nanoformulation was observed at 2 h followed by a steep decrease, whereas in the case of the soluble formulation the peak concentration was found at 1 h. The results showed that EFV delivered via the nanoformulation had a longer retention time in blood compared with the soluble formulation. In the liver, although both soluble EFV and the nanoformulation showed maximum accumulation at the 2-h time-point, the nanoformulation did not cause any toxicity, as shown in the safety data and histopathology analysis. Further, less accumulation of the nanoformulation was found in the heart, lungs, spleen, brain, large intestine, kidney and bone marrow. Results for drug localization in the kidney showed faster clearance of soluble EFV compared with its nanoformulation.

Safety and histopathology analyses of the nanoformulation

The safety analysis of lacto-EFV-nano was carried out in Wistar rats. Soluble EFV treatment increased the level of LDH in both male and female rats. However, LDH levels decreased when rats were treated with lacto-EFV-nano (Fig. S4). Detailed lipid profiles were obtained in rats treated with the soluble and NP forms of EFV. The results show that treatment with the nanoformulation produced significantly lower elevations in the level of

Table 1 Pharmacokinetic table for efavirenz (EFV)

		Male rats		Female rats	
Parameter	Units	Lacto-EFV-nano	Soluble EFV	Lacto-EFV-nano	Soluble EFV
AUC_{tot}	h*(ng/mL)	2401.43	764.336	2739.81	684.856
$AUMC_{tot}$	h^2*(ng/mL)	11879.8	1944.54	13035.3	1741.96
C_{max}	ng/mL	440.683	315.62	535.12	280.5
T_{max}	h	2	1	2	1
$t_{1/2}$	h	2.927	1.336	2.79	1.359

Pharmacokinetic parameters are as follows. AUC, area under the curve: the integral of the concentration−time curve (after a single dose or in steady state); AUMC, area under the first moment curve: partial area under the moment curve between *t* start and *t* end; C_{max}, the peak plasma concentration of a drug after oral administration; T_{max}, time to reach C_{max}; $t_{1/2}$, time required for the concentration of the drug to reach half of its original value.

total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL) and low-density lipoprotein (LDL). Kidney safety markers such as blood urea nitrogen (BUN) and creatinine were found to be significantly less elevated in nanoformulation-treated rats compared with rats treated with soluble EFV. Finally, AST and ALT levels were estimated and were found to be highly elevated in rats treated with soluble EFV, while in those treated with the nanoformulation of the drug, these levels were significantly less elevated. Tissue sections of the brain, heart, liver, spleen, kidney and gastrointestinal tract showed that there were no toxicities in these tissues when lacto-EFV-nano was administered (Fig 5). Furthermore, lacto-EFV-nano exhibited no drug-related toxicity in any of the tissues, while there were some indications of toxicity exhibited by soluble EFV as assessed by examination of H&E staining images, which showed minor damage in liver and brain tissues (indicated by the printed arrow in Fig. 5).

Discussion

EFV is an NNRTI and is commonly used as the main component of HAART for HIV/AIDS treatment. EFV has a partition coefficient of 5.3 in lipophilic solvents, indicating its high lipophilicity [21]. However, as inferred from its limited aqueous solubility, it exhibits variable bioavailability. In the present study, we investigated the efficacy of lacto-EFV-nano as a therapeutic agent for treatment of HIV/AIDS. Lacto-EFV-nano was prepared using Sol-oil chemistry, which yielded particles having a mean diameter of 45−60 nm with 59% EE. In our study, we prepared NPs using different drug to protein ratios (Table S1). We found that the EE was maximum for the formulation ratio F3, while drug loading was maximum for the formulation ratio F1. As higher encapsulation would provide conditions for higher drug-loaded NPs per unit mass of drug, we preferred to maximize EE rather than drug loading and accordingly the formulation ratio F3 was used throughout the study. Our NP preparation procedure is simple and fast, and does not cause any chemical modification either to the protein or to the drug. Our procedure has an advantage over the other existing chitosan-based NP preparation procedure, as that procedure uses glutaraldehyde, which may affect cell viability and the chemical forms of the components [22]. A recent quantum dot-based study [23] showed that, for renal clearance, the cut-off particle size is approximately 5.5 nm, which means that any particle with a size < 5.5 nm may be excreted from the kidneys rapidly. Our NPs are usually of larger size, and will have slower renal clearance, which in turn facilitates extended blood circulation and results in a higher therapeutic index [23]. In the DLS-based analysis, the hydrodynamic sizes of blank-NPs (72 ± 3.4 nm) and lacto-EFV-nano (103 ± 5.3 nm) were found to be larger than their FE-SEM sizes. DLS measures the NP size in suspension form and in this case the particle size could be affected by the interaction between the water shell and the NP surface charge, leading to an increase in the overall size. A negative surface charge of −23 ± 1.2 mV was found on the NP indicates its stability in the colloidal state. In addition, lacto-EFV-nano has a PDI of < 0.341, suggesting that the particle population is homogenous in nature with a narrow particle size distribution. During lacto-EFV-nano preparation, protein and drugs were incubated together. The presence of aromatic groups such as tyrosine, phenylalanine and tryptophan in protein is responsible for the fluorescence. Fluorescence was found to increase with increased loading of the drug in the protein and was becoming saturated after 60 min (Fig. S1). Lacto-EFV-nano was found to be very stable even at higher temperatures, as shown by the DSC (Differential scanning calorimetry) thermogram in Fig. S2. The thermogram of EFV-loaded Lf NPs shows the stability of EFV in the nanoformulation. Melting of EFV was observed at 138°C. Soluble EFV showed a degradation peak at 260°C, whereas this was totally absent in the nanoformulation. The position of the

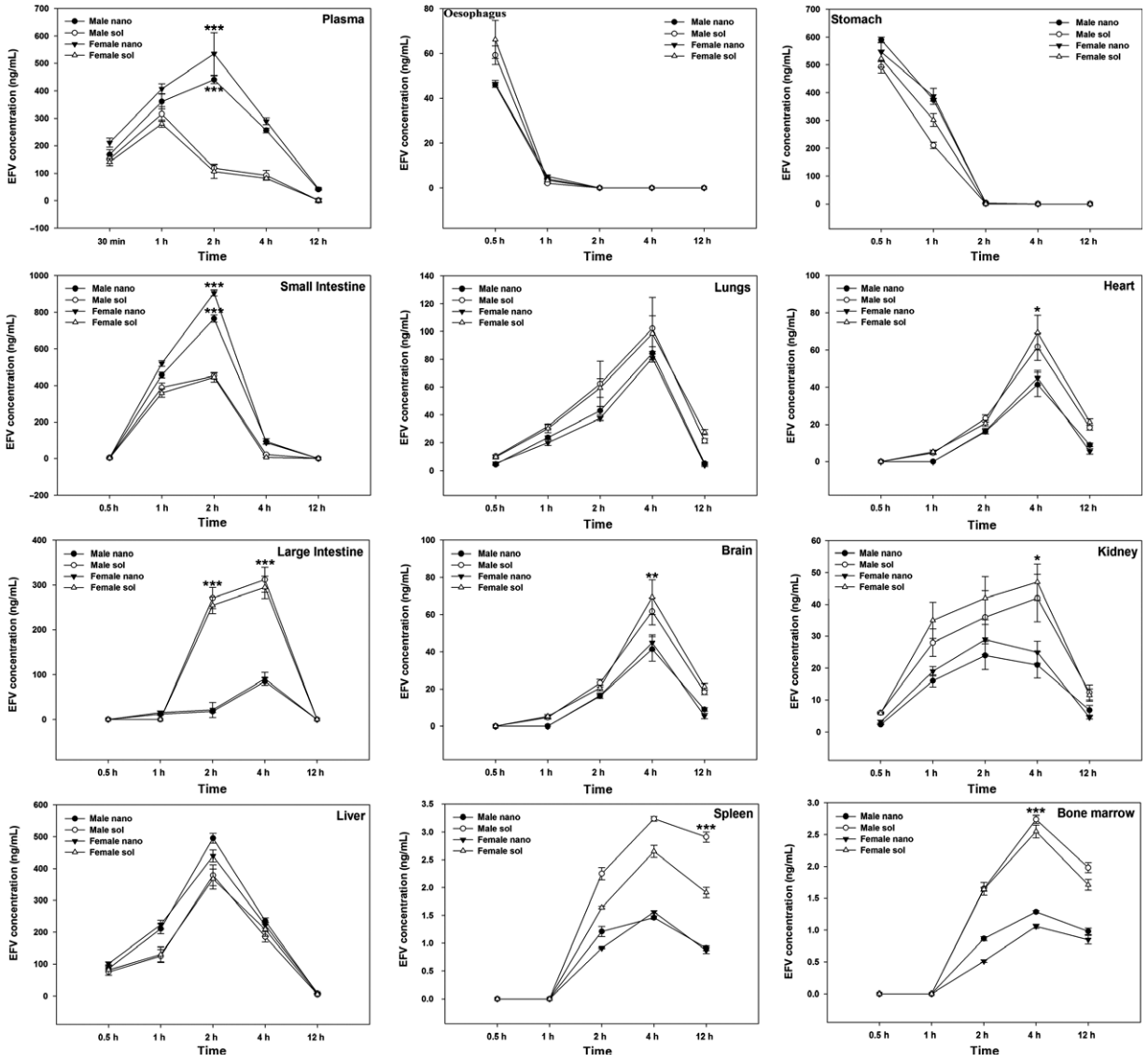

Fig. 4 Efavirenz (EFV) tissue distribution in rats. Oral administration of soluble EFV and lacto-EFV-nano (10 mg/kg body weight) was carried out and tissues were collected at the indicated time-points followed by quantification of EFV using high-performance liquid chromatography (HPLC). "Male sol" and "Female sol" indicate the concentrations of EFV delivered via soluble EFV in male and female rats, respectively. "Male nano" and "Female nano" indicate EFV concentrations delivered via lacto-EFV-nano in male and female rats, respectively. The difference between groups was calculated using one-way analysis of variance (ANOVA); $^{**}P < 0.01$ and $^{***}P < 0.001$.

endothermic peak was found to be quite similar for soluble EFV and the nanoformulation. This suggests a physical interaction between the drug and the protein. Further, the FT-IR (Fourier transform infrared spectroscopy) data show that the featured peaks related to EFV are preserved in the nanoformulation (Fig. S2c,d). A minute variation in the position of peaks was observed in the case of lacto-EFV-nano compared with soluble EFV. This variation could be attributable to the electrostatic interaction in the nanoformulation. Lacto-nano-EFV was found to release its maximum payload at pH 5, corresponding to the endosomal pH. Exposure of lacto-EFV-nano to endosomal pH values could lead to a change in the orientation of the protein monomer, so that drug present in the cavities of the protein is released. In addition, lacto-EFV-nano was found to be stable in gastric conditions, whereas the other oral formulation derived from chitosan NP disintegrates, which may limit the drug delivery potential. [24]. Further, approximately < 10% of the drug was usually released at the circulatory pH value (pH 7.4),

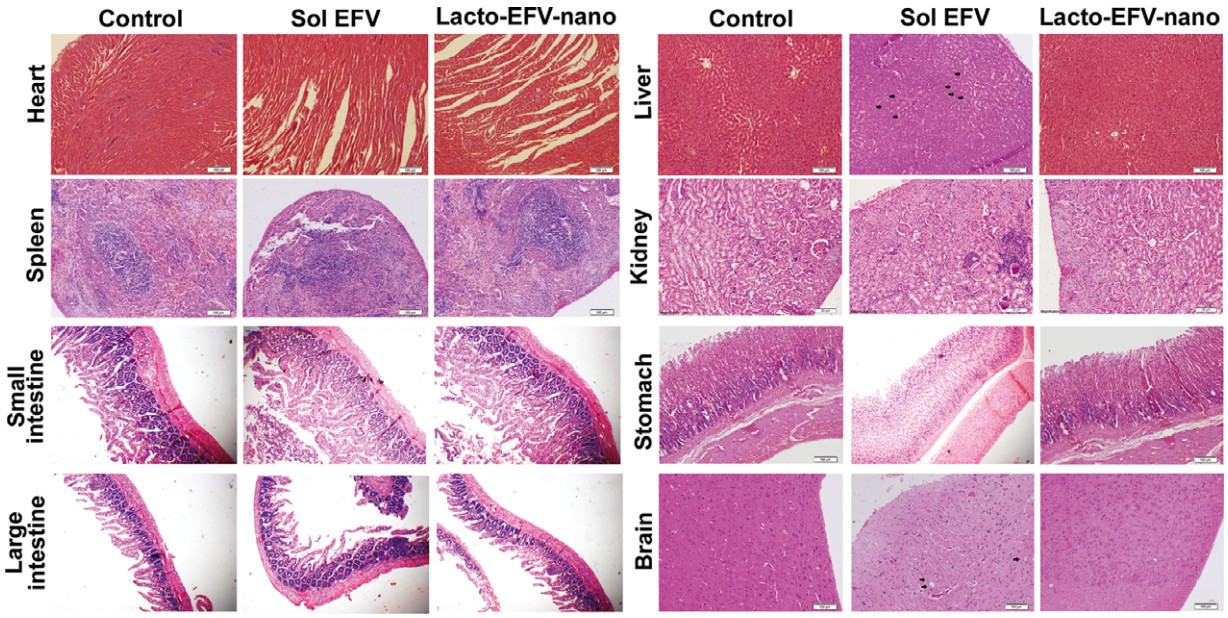

Fig. 5 Histopathological analysis of tissues. Haematoxylin and eosin (H&E) staining revealed that no morphological changes were observed in all tissues when treated with Lacto-EFV-nano. However liver and brain show some abnormalities (indicated with printed black arrow) when treated with soluble efavirenz. The scale bar is 100 μM or 40×, unless otherwise stated.

thus supporting the mechanism of receptor-mediated lacto-EFV-nano entry followed by localization of lacto-EFV-nano in endosomes. These results are consistent with those of our earlier studies on oral and intravenous delivery of doxorubicin and ZDV via Apo-transferrin and Lf NPs [19,25]. Cellular drug kinetics data showed a sustained and long-lasting release of drug from lacto-EFV-nano (Fig 2b). This property makes lacto-EFV-nano superior to other solid lipid NP which releases the drug burstly (100% of drug in < 5 min) [26]. The concentration of soluble EFV attained its maximum at 2 h followed by a steady decrease up to 12 h. In the present study, we showed that lacto-EFV-nano was at least 50% less toxic to a variety of cell lines than soluble EFV; the *in vitro* cell toxicity data support this conclusion (Fig. S3). Toxicity is an important factor to be considered, and it has been reported that soluble EFV therapy was suspended because of severe toxicity affecting the skin [27]. Our results demonstrated that lacto-EFV-nano was 1.5-fold safer than soluble EFV with respect to skin hypersensitivity. This may be seen from Fig. S3, which shows that B16-F10 (a skin epithelial cell line) had a GI_{50} (Growth Inhibition of 50%) of 57.4 and 91.2 μM for lacto-EFV-nano and soluble EFV, respectively (Fig. S3). In terms of the IC_{50}, lacto-EFV-nano was found to be more effective (IC_{50} = 1.1 nM) than soluble EFV (IC_{50} = 2.56 nM) in blocking HIV-1 replication (Fig. 3a). Lf blank-NPs were found to inhibit 50% of viral replication at a concentration of < 4 μM. The anti-HIV-1 activity of lacto-EFV-nano was a combined effect of the activities of EFV and Lf in the nanoformulation. An *in vivo* study was performed in rats of both sexes, as PK results are greatly dependent on gender. The action mechanism of the drug also depends on various parameters such as body weight, hormonal status, cardiac output and body composition [28]. Generally, women experience more adverse events than men as per the reports of the FDA [29]. The nanoformulation developed in our study resulted in an overall improvement in the PK profile when given orally to the rats. A highly significant improvement in the PK profile was observed in terms of the AUC (4-fold) and AUMC (7-fold) for the nano form of the drug compared with its soluble form in both male and female rats, and the PK profile was also better for the nanoformulation than that reported previously for another nanoformulation, which showed an improvement in the AUC of only 2.2-fold [30]. Further, a drug concentration of 200 ng/mL was found in the blood at 30 min, while negligible drug concentrations were found in the intestine. The early presence of the drug in the blood could be attributable to absorption of NPs through some alternative pathways, including the epithelial cells of the stomach [31]. A comparison of male versus female PK profiles showed that the peak values of plasma concentration (C_{max}) of lacto-EFV-nano were higher in female rats (535.12 ng/ml) than in male rats (440.68 ng/ml). Further, the drug exposure

of lacto-EFV-nano was higher in female than in male rats, as indicated by the higher AUC_{tot} increase for females compared with males (female > 4-fold and male > 3-fold). As a higher AUC_{tot} is directly associated with higher systematic bioavailability of the drug, it facilitates a reduction in the dose frequency for patients. In addition, other PK parameters such as $AUMC_{tot}$, T_{max} and $t_{1/2}$ were also found to be significantly improved in nanoformulation-treated rats compared with rats treated with soluble EFV. Increases of 100% in T_{max} and $t_{1/2}$ were found in NP-treated rats (female and male) relative to soluble EFV-treated rats, suggesting a slow and sustained release of the drug via lacto-EFV-nano. In spite of higher accumulation of lacto-EFV-nano than soluble EFV in the liver, less liver toxicity was observed, as shown in histopathology images. Earlier reports demonstrated that EFV treatment leads to liver damage and neurocognitive reactions, such as anxiety [32]. The low hepatotoxicity observed in the case of lacto-EFV-nano could be attributable to protection of EFV by the Lf protein nanoshell, so that further release of the drug into the liver is not possible, thus reducing the possibility of drug-related toxicity. Results of the comparison of the biochemical safety profiles of nano and soluble forms of EFV showed a significant difference in plasma AST and ALT between the nano and soluble forms in both male and female rats. Creatinine and BUN showed significant decrease ($P < 0.001$), suggesting low kidney toxicity, when the nanoformulation was used compared with soluble EFV (Fig. S4). Further, treatment with lacto-EFV-nano did not show any significant differences in lipid profile in male and female rats compare with soluble efavirenz. Any novel drug formulation cannot be used successfully unless and until it fulfils requirements for stability under different physical and environmental conditions. Lacto-EFV-nano developed in the present study was found to be highly stable and effective and had an improved PK profile, compared with the soluble form of EFV, when administered orally.

Conclusions

The present study shows the applicability of a protein-based formulation in increasing the absorption of lipophilic drugs. In this study, stable EFV-loaded Lf NPs were formulated using appropriate proportions of protein and drug. This formulation showed optimally stable physicochemical parameters with higher bioavailability in terms of the values of C_{max}, $t_{1/2}$ and AUC. Further, it was shown that the nanoformulation had enhanced anti-HIV-1 activity and reduced EFV toxicity compared with soluble EFV.

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Conflicts of interest: There are no conflicts of interest to disclose.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1: Encapsulation efficiency optimization of efavirenz loaded lactoferrin nanoparticles.

DEVELOPMENT OF EFFICIENT AND SAFE LACTOFERRIN NANOPARTICLES BASED MPT MICROBICIDAL DRUG - DELIVERY SYSTEM AGAINST HIV - 1

ORIGINALITY REPORT

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SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	Upendhar Gandapu. "Curcumin-Loaded Apotransferrin Nanoparticles Provide Efficient Cellular Uptake and Effectively Inhibit HIV-1 Replication In Vitro", PLoS ONE, 08/22/2011 Publication	%2
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3	Submitted to University of Sheffield Student Paper	%1
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