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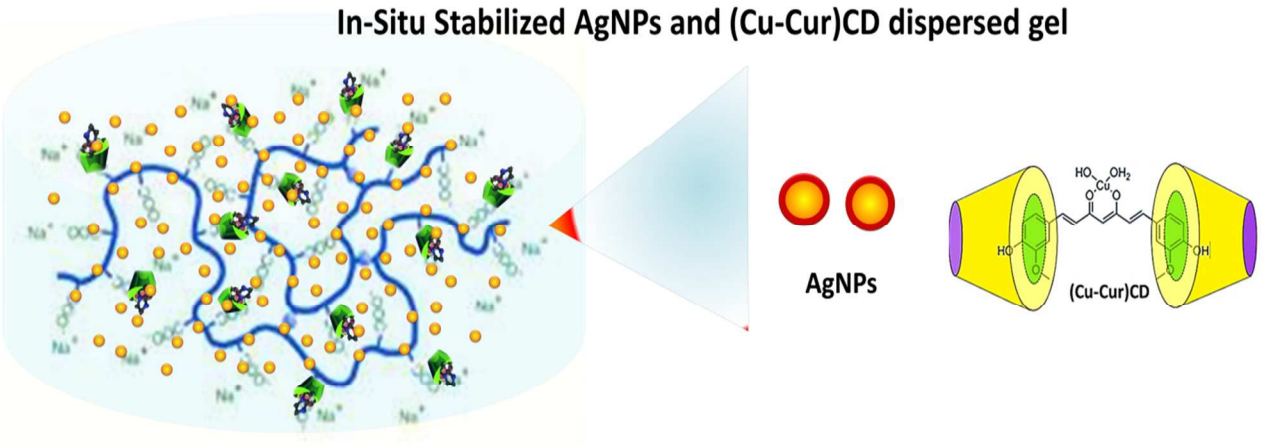
GRAPHICAL ABSTRACT



In-Situ Stabilized AgNPs and (Cu-Cur)CD Dispersed Gel, A Topical Contraceptive- Antiretroviral (ARV) Microbicide

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ABSTRACT

Increasing scenario of sexual HIV-1 transmission and unintended pregnancies demands modern approaches to concomitantly tackle this problem. Dually active strategies with "microbicidal" anti-human immunodeficiency virus (anti-HIV) as well as contraceptive properties constitute one such strategy. Nano-metal and metalo-herbal technology was explored to develop such approach with minimal toxicity. Synthesis of in-situ stabilised silver nanoparticles (AgNPs) 2-24 nm and (copper-curcumin)β-cyclodextrin (Cu-Cur)CD inclusion complex were carried out. Cell viability and activity analysis revealed an acceptable dose of 500µg/ml of silver nanoparticles and 10mg/ml of (Cu-Cur)CD respectively. AgNPs dispersed (Cu-Cur)CD incorporated carbopol 974p gel was prepared and characterized for its pharmaceutical properties. AgNPs, (Cu-Cur)CD and whole formulation was evaluated for in-vitro anti-HIV, spermicidal and antifungal potential. Results revealed that HIV-1 propagation and sperm motility was completely inhibited at folds dilution levels. In-vivo mating studies proved the contraceptive potential of the formulation. Pre-clinical toxicology study assures the acceptability our formulation as an intravaginal product. Novel nano-metalo-herbal strategy was found to possess a potential for topical contraceptive antiretroviral (ARV) microbicide action with an undeniably safe profile to be used as a vaginal product.

Keywords: Contraceptive microbicide; anti-HIV-1; silver nanoparticles; (copper-curcumin)β-cyclodextrin; pre-clinical toxicology.

1. INTRODUCTION

The incessant high rates of the acquired immune deficiency syndrome (AIDS) and the brutal increase of unintended pregnancies specifically in less developed countries, permit the development of novel strategies to help individuals avoid these risks. Unprotected sexual intercourse is the most common mode and accounts for more than 75% of infections worldwide[1-3]. Sexually transmitted HIV infection and sperm fertilization share the same anatomical and functional context, and therefore offer an opportunity for simultaneous intervention [4]. Condoms are considered as the best way to tackle this problem. But its denial, inconsistent or incorrect use and failure favour this misfortune. Further, a large population is not comfortable with oral contraceptives; this creates a thought of having a women oriented approach which can be easily used to avoid this epidemic [5]. Dually active compounds displaying "microbicidal" anti-human immunodeficiency virus (anti-HIV) as well as contraceptive properties is the best strategy to deal concomitantly with this problem. "*Contraceptive Microbicides*" is the suitable term for such approach [4, 6, 7].

Surface active agents, pH buffering agents, receptor blockers and enzyme inhibitors acting on sperm and HIV during primary events, antimicrobial peptides etc. represents the brief categorization of the compounds already targeted for the purpose of dual efficacy [8]. But clinically very few were tested and found to be unacceptable because of either lack of efficacy or safety [7]. Applied intravaginal microbicide, (viral and sperm's membrane disrupting agent) nonoxynol-9 (N-9) have shown contraceptive efficacy but product failed in clinical trials due to the lack of safety, which unfortunately resulted in increased HIV incidents [9]. Exploring compounds having dual characteristics with the advent nanotechnology and herbal considerations will be a new direction. Moreover using combinations with one compound having anti-HIV and other with contraceptive potential can also be a good approach. With this viewpoint the selection of silver nanoparticles (prominent anti-HIV agent) and a pre-synthesised metaloherbal complex (having spermicidal potential) were explored simultaneously.

In this work we explored the potential of nano-metal and metalo-herbal technology for the development of contraceptive microbicide compounds having dual activity profile and minimal toxicity. In our previous research we successfully explored a contraceptive moiety i.e. (copper-curcumin)β-cyclodextrin inclusion complex (Chauhan et al.2014) [10]. Present work is based on the hypothesis to deliver this inclusion complex along with a nanometal i.e. silver nanopartilces (AgNPs). Here cyclodextrin inclusion complex helped to resolve the major problem of aqueous solubility related with this metalo-herbal complex. In-situ stabilized AgNPs were synthesised by regulating the reduction process of tannic acid (a polyphenolic compound derived from plant extracts) mediated reduction of silver salt. Capping of AgNPs with tannic acid residues during this reduction process provides a surface stable silver dispersion to be used for combinational use with dextrin complex.

Mode of action of silver nanoparticles against HIV-1 is not fully elucidated. Mixed judgments from different researchers unable us to stand with one mechanism [11-13]. Studies suggested that AgNPs act at primary stage of viral replication, as a virucidal agent or as an inhibitor of viral entry. Their affinity for gp120 viron receptor prevents CD-4 dependent binding, fusion, and infectivity[14]. Some mechanistic explanations revealed that AgNPs might preferentially interact with the negative cavity of gp120 where the interaction with the two disulphide bonds situated in the carboxyl half of the glycoprotein modifies this viral protein by denaturing its disulphide-bonded domain[15].

In this study, both the moieties were initially evaluated for vaginal epithelial cell cytotoxicity, anti HIV-1 activity and spermicidal activity. Dose selection of both the components was done by analysing the efficacy levels and the toxicity levels (on vaginal epithelial cell toxicity). Finally a hydrogel formulation containing an appropriate dose of both these moieties was prepared and characterised. Nano-herbal microbicidal gel (NHM gel) was then tested for in-vitro pre as well as post exposure prophylactic studies using different tropism of HIV-1 strains. NHM gel was then analysed for its contraceptive efficacy using both in-vitro tests and in-vivo mating challenge in Wistar rats. Supplementary to the above activities NHM gel and its components were tested against a most common sexually transmitted candida infection, as these kinds of infections assist the dissemination of HIV-1 from the vaginal epithelium. Finally its vaginal safety was evaluated by pre-clinical toxicology studies on Wistar rats, Albino rabbits and vaginal lactobacillus bioflora.

2. MATERIAL & METHOD

Silver nitrate, Tannic acid, Mucin, Dulbecco's modified eagle medium (DMEM), RPMI-1640, Fetal bovine serum, Phytohaemagglutinin (PHA), Ficoll-hypaque and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium (MTT), Curcumin, Bovine serum albumin (BSA), β-cyclodextrin, tergitol, triethanolamine was purchased from Sigma Aldrich (Bangalore, India). Carbopol 974p was procured as a gift sample from Lubrizol (Mumbai, India).

R5 HIV-1_{Ba-L} was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, US. Hela Cells were procured from NCCS, Pune while the cell culture exposed in MTS assay (Hela, HEL, VERO, CRFK, MDCK) were included in the study protocol performed at Department of Virology, Leuven, Belgium. *C. albicans*, C. *tropicalis, L. acidophilus and L. jensenii* were procured from IMTECH Chandigarh.

2.1 Synthesis, optimization and characterization of in-situ stabilized silver nanoparticles

AgNPs were synthesized by using tannic acid (a polyphenolic compound derived from plant extracts) dependent reduction of silver nitrate [16]. Molar concentration ratio of tannic acid to silver nitrate was controlled with a constant silver nitrate (3mM) concentration, while varying the concentration of tannic acid (0.0375, 0.075, and 0.15) mM sequence in order to achieve the respective molar concentration ratio patterns of (0.125, 0.025, and 0.05). Further at each molar ratio, three different pH value exposures of tannic acid solution were made (pH 7, pH 8 and pH 9, adjusted with K_2CO_3) for the synthesis of AgNPs.

At ambient temperature, the flow rate of silver nitrate addition is kept constant (55-60)µl/sec under continuous magnetic stirring. Stirring is stopped immediately after the complete addition of silver nitrate solution and whole reaction is strictly done in the absence of light. Synthesized AgNPs dispersions were optimized on the basis of ultraviolet-visible spectroscopy, particle size and polydispersity index. Optimized AgNPs were then purified and washed by centrifugation at 21913 x g for 30 min at 4°C using distilled water and characterized by ultraviolet-visible spectroscopy using UV-VIS spectrophotometer (*UV-1700, Shimadzu, Japan*), particle size, polydispersity index, surface potential using particle size analyser (Zeta sizer, DLS 4 C Beckman Coulter, Japan), X-ray powder diffractometry using Bruker diffractometer (AXS D8 Advance, Karlsruhe, Germany), thermogravimetric analysis (TG) using Perkin Elmer TG/DTA analyzer (STA 6000, Massachusetts, U.S.A) operating in the range of 40ºC to 740ºC with at temperature rise of 10.00°C/min and morphology using transmission electron microscope (TEM) using (Hitachi H-7500, Georgia ,U.S.A) operating at 100 kV and surface characteristics using atomic force microscopy in contact mode on a multimode scanning probe microscope equipped with a

Nanoscope IV controller at a scan rate of 5.086 Hz (Veeco Instruments, New York, U.S.A) . Stability studies were also performed as per ICH guidelines ***(Supplementary data).***

2.2 Synthesis, optimization and characterization of copper-curcumin (Cu-Cur) and copper-curcumin- β-Cyclodextrin (Cu-Cur)CD inclusion complex

Metal Ligand (M-L) complex of copper (M) and curcumin (L) had already been synthesized by many researchers. Inclusion complex was prepared by solvent evaporation encapsulation method [10]. ***(Work has been published already by Chauhan et al., 2014, also provided in supplementary data).***

2.3 Cell viability Assay (MTS assay), Peripheral blood mononucleated cell (PBMC) toxicity assay) and Haemolytic assay.

2.3.1 MTS assay

The (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) dye reduction assay in the presence of phenazinemethosulfate (PMS), produces a formazan product that has an absorbance maximum at 490-500 nm in phosphate-buffered saline[17]. The MTS assay was performed to determine the cytotoxicity of the optimized AgNPs and MC-40 (pre-optimized metal herbal complex) in different cell cultures. Cell viability assay was performed to study both specific cell toxicity on Hela cells and non-specific toxicity on HEL (Human embryonic lung), VERO, CRFK (Crandell-Rees Feline Kidney cells) and MDCK (Madin Darby canine kidney cell) cultures. Moreover the assay was also performed on CEM-GFP cells (HIV-1 host cells) to assess the direct cytotoxic potential of AgNPs and MC-40 which may results in erroneous conclusion of the anti-HIV data.

2.3.2 Haemolytic assay

Haemolytic assay was done on isolated RBC from Wistar rat blood (as per the ethical guidelines) to assess the irritation potential of the challenged moieties. Varying concentrations of AgNPs and MC-40 (in phosphate buffered saline i.e. PBS 7.4), 0.1% Triton X-100 and PBS-7.4 are used as treated, positive control and negative control, respectively and incubated at 37ºC for 30 min with mild shaking to attain complete haemolysis. The reaction was quenched in ice, the samples were centrifuged at 1500 rpm for 2 min, and the supernatants were analysed by UV-Vis spectrophotometer at 576 nm [18]. The percentage haemolysis was then calculated by the equation-1.

$$\%H = 100\% \ (Abs - Abs_{PBS}) \ / \ (Abs_{tx} - Abs_{PBS}) \quad \text{..... Equation-1}$$

2.4 Anti-HIV activity

Anti-HIV assays were performed on two host cells viz. CEM-GFP cells (expressing CD4, CD8, CCR5 and CXCR4 receptors) and phytohaemagglutinin (PHA) stimulated peripheral blood mononucleated cell (PHA-PBMCs), challenging them against HIV-$1_{NL4.3}$ and HIV-1_{Ba-L} viral strains respectively.

In order to mimic the prophylactic challenge, study was designed to cover two different possible interactions. Pre-interaction (interaction -1 assay) was performed; where the test compounds were interacted with the virus first and later that virus was allowed to infect the cells. Here we tried to elucidate the probable surface receptor blocking or direct virus denaturing mechanism of the test compounds. On the other hand in post-infection (interaction -2 assay); where the virus was allowed to do the job, i.e. the cells were initially infected with the virus first, followed by the addition of test compounds. Here we had tried to inspect the ability of our test compounds to halt the infection after the virus has established an infection. Both these interaction can be better understood from the cartoon presented in **Figure-1.**

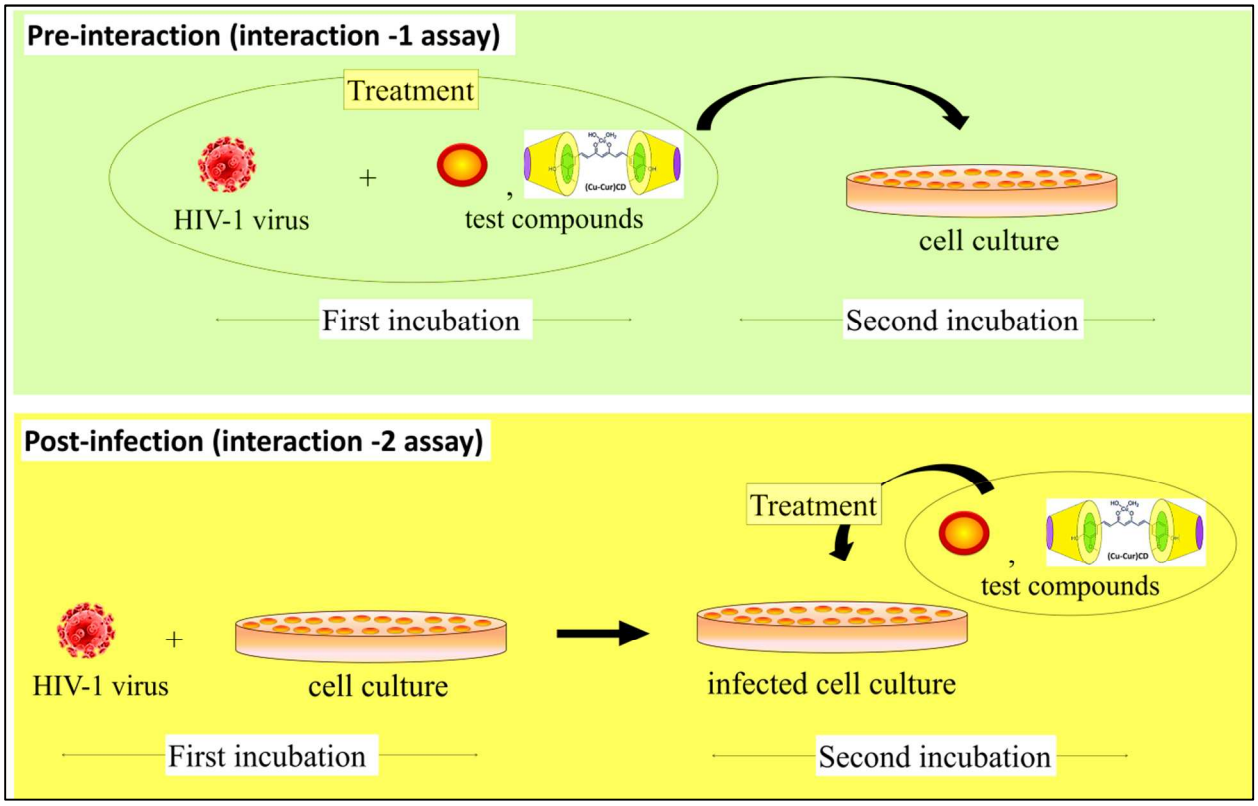

Figure-1 Designing the anti-HIV study on the basis of possible HIV-1 interactions with either formulation components or host cell.

2.4.1 Anti-HIV assay using CEM-GFP cells

CEM-GFP cells in RPMI-1640 medium supplemented with 10% FBS, Penicillin (100 units/ml), Streptomycin (100µg/ml) and Amphotericin B (250 ng/ml). For pre-infection interaction study (interaction-1), different concentrations of AgNPs and (Cu-Cur)CD i.e. MC-40 were allowed to interact separately; with 50% tissue culture infectivity doses ($TCID_{50}$) of HIV-$1_{NL4.3}$ for 4 hours and later added to different wells in 96 well plate seeded with CEM-GFP cells (2.0 x 10^5 cells/ml) and incubated at 37°C and 5% CO_2. While for host- interaction study (interaction-2), different concentrations of AgNPs and MC-40 were incubated with the CEM-GFP cells (already plated in each well of 96-well microculture plate) for a period of 4 hrs. After this incubation, these cells were exposed to $TCID_{50}$ dose of HIV-$41_{NL4.3}$ and incubated at 37°C and 5% CO_2.

After 7th day of incubation of both the incubated challenged cultures, cell free supernatants (100µl each) were collected and investigated for HIV-1 p24 antigen levels using microplate reader (ELISA reader. Percent inhibition of p24 antigen by test compounds was compared to the positive control (infected cells without any test compound) [19-21].

2.4.2 Anti-HIV assay using phytohaemagglutinin-stimulated peripheral blood mononuclear cells (PHA-PBMCs).

Healthy uninfected PBMCs were isolated from blood by Ficoll-hypaque density gradient centrifugation and cultured in RPMI-1640 culture medium supplemented with 20% Fetal bovine serum (FBS), penicillin (50 U/ml), streptomycin (100 µg/ml), PHA (10 µg/ml) and interleukin-2 IL-2 (10 units/ml) for 3 days prior to anti-HIV assay. For selective growth of T cells and over-expression of surface markers like CD4, PBMCs were activated with PHA and IL-2 prior to infection. On day 3, PHA-PBMCs were resuspended in minimal growth medium (RPMI-1640, 10% FBS and IL-2 (10 units/ml)) and plated in each well of 96-well microculture plate at a density of 0.5 x 10^6 cells/ml. In this study, potential of the formulation components were evaluated against R5 HIV-1_{Ba-L} virus.

For pre-infection interaction study (interaction-1), different concentrations of AgNPs and MC-40 were allowed to interact separately; with $TCID_{50}$ dose of HIV-1_{Ba-L} for 4 hours and later added to different wells in 96 well plate seeded with PBMCs and incubated at 37°C and 5% CO_2. For host-interaction study (interaction-2), different concentrations of AgNPs and MC-40 were incubated with PBMCs (already plated in each well of 96-well microculture plate) for a period of 4 hrs. After incubation, these cells were exposed to $TCID_{50}$ dose of HIV-1_{Ba-L} and incubated at 37°C and 5% CO_2.

After 7th day of incubation of both the incubated challenged cultures, cell free supernatants (100µl each) were collected and investigated for HIV-1 p24 antigen levels using ELISA reader. Percent inhibition of p24 antigen by test compounds was compared to the positive control (infected cells without any test compound).

2.5 Spermicidal activity

2.5.1 Modified Sander-Cramer assay (Sperm motility inhibition assay)

Human sperm were obtained from consenting three healthy donors after 72 h of abstinence. The samples were washed once in Ham's F10 containing 0.1% human serum albumin (HSA) and centrifuged. The sperm were resuspended to give a concentration of 60 million motile sperm per millilitre. Spermicidal activity of AgNPs and MC-40 was evaluated. Sequential dilutions of AgNPs and MC-40 were prepared in Ham's F10. Tergitol NP-9 was used as positive control, while medium containing no test compounds was employed as negative control. Aliquots of sperm 50µl were incubated (37°C in the presence of 5% CO_2) with various concentrations of test ingredients in a final volume of 100µL. After 120 sec, the reaction was terminated with 1.5 ml Ham's F10 and centrifuged for 10 min at 290 × g to obtain the sperm pellet, which was further resuspended in 100µL of Ham's F10. Sperm motility was assessed under inverted phase contrast Microscope (MKX-41, Olympus, Tokyo, Japan) [20].

2.5.2 Hypo-osmotic swelling (HOS) test

Hypo-osmotic swelling test is based on the loss of semi-permeability of the intact cell membrane, after an exposure to membrane attacking moiety. Effective concentrations of AgNPs and (Cu-Cur)CD treated spermatozoas were exposed to HOS solution (75 mM fructose and 20 mM sodium citrate) for at least 30 min at 37°C to detect changes in the sperm membrane integrity. The number of spermatozoa showing characteristic tail curling or swelling was counted under inverted phase contrast microscope [22].

2.5.3 Sperm viability test (fluorescent staining)

Dead and alive sperms were differentiated by two different dye's staining using fluoresced red propidium iodide which stains dead spermatozoa only, while fluoresced green SYBR-14 dye stains alive only. AgNPs (250 µg/ml) and MC-40 (5mg/ml) treated human spermatozoa's were dual stained with SYBR-14 and propidium iodide to distinguish green-fluorescing live from red-fluorescing dead spermatozoa in contrast with the dual stained untreated spermatozoa's. Sperm count was taken from 200 spermatozoa under a phase contrast microscope [23].

2.5.4 Apoptotic and necrotic changes in plasma membrane of human spermatozoa

Mechanism of sperm cell death was further studied by dual fluorescent labelling with fluorescein isothiocyanate (FITC)-Annexin V (to study the expression of phosphatidylserine on apoptotic sperm cells) and propidium iodide (to study the enhanced membrane permeabilization of necrotic cells). Highly motile human sperm (2×10^6) were incubated with AgNPs (250 µg/ml) and MC-40 (5 mg/ml) in Ham's F10 media. After incubation, sperms were washed in 1% BSA in Tyrode's buffer and labelled with fluorescent dyes. Apoptotic study was done using a detection kit and the percentages of cells positive for FITC and PI were determined using flow cytometer (BD Accuri C6, CA, U.S.A). While identification, the unstained cells were marked as viable, FITC stained

were marked as apoptotic, both FITC and PI labelled were marked as late apoptotic cells and only PI stained were marked as necrotic [24].

2.6 Anti-Candida Activity

Candida infection is most commonly seen sexually transmitted infection which can prominently assist vaginal HIV-1 transmission. Minimum inhibitory concentration (MIC) was assessed for *C. albicans* and *C. tropicalis* using serial dilution assay using 96-well flat-bottomed microtiter plate [25]. ***(Detailed study protocol is given supplementary data).***

2.7 Formulation and characterization of Nano-herbal microbicidal gel (NHM gel)

Dose selection for AgNPs and MC-40 was done on the basis of selectivity index (IC_{50} / EC_{50}) which provides a safe and effective range to work with. Nano-herbal gel was formulated using optimized concentration of AgNPs and MC-40 (i.e. 500µg/ml of AgNPs and 10 mg/ml MC-40) in 1% w/v carbopol 974p base. In vessel #1 carbopol 974p gel was hydrated in distilled water and triethanolamine was added drop wise to make it a clear gel and incubated for 12hrs. MC-40 was dissolved in 5ml distilled water and added dropwise to the vessel #1 with constant stirring at 500 rpm. Finally AgNPs suspension was added dropwise to vessel #1 with a mild stirring at 100 rpm until the homogeneous gel was obtained. The developed nano-herbal gel formulation was characterized for pharmaceutical aspects including macroscopic properties, pH, thixotropic/rheological properties and texture analysis using Rheometer (R/S CPS, Brookfield, Middleboro, U.S.A.) and Brookfield texture analyzer (CT3 10K, Brookfield, Middleboro, U.S.A.) respectively[26, 27].

2.7.1 In-vitro challenge study for the pharmaceutical parameters of NHM gel

Box-Behnken statistical screening design was used to statistically evaluate the vehicle's behaviour in vaginal simulated conditions (during sexual intercourse) with respect to the pH changes, viscosity, mucoadhesion and spreadibility alterations. A 3-factor, 3-level design used is suitable for exploring quadratic response surfaces and constructing second order polynomial models with Design Expert®, Version 9.0.3.1 (Stat-Ease Inc., Minneapolis, U.S.A). A design matrix comprising of 15 experimental runs was constructed. The dependent and independent variables selected are shown in table-1. Physiological ranges for vaginal fluid secretions (using vaginal simulated fluid "VSF"), semen secretions (using semen simulation "SS") and application quantity of formulation (that can vary in population) were taken as independent variables to mimic the physiological challenges. Different challenges as per the design were evaluated for NHM gel behaviour with respect to pH, viscosity, mucoadhession and spreadibility variations**.** ***(The experiment was detailed in supplementary data)*****[28]**.

2.8 Pre-infection interaction (Interaction-1) anti-HIV study of NHM gel's

On CEM-GFP cells: Different dilutions of NHM gel were allowed to interact separately with 50% tissue culture infectivity doses ($TCID_{50}$) of HIV-$1_{NL4.3}$ for 3-4 hours (*pre-infection interaction*), and later added in different wells; prior fed with CEM-GFP cells and incubated at

37°C and 5% CO_2. On day 7 of culture, the cell free supernatants were harvested and investigated for HIV-1 p24 antigen (Ag) levels in the supernatant by ELISA. All the experiments were performed in duplicates and percent inhibition of p24 Ag by test formulation was compared to the control/placebo group (infected cells only).

On PBMCs: Similar experiment as mentioned above was run, challenging PBMCs with HIV-1_{Ba-L} virus. Percent inhibition of p24 Ag by sequential formulation's dilutions was compared to the negative control/placebo group (infected cells only).

Table-1 Independent and dependent variables considered during in-vitro challenge study for of NHM gel using Box-Behnken design.

Factor **Independent Variables**		**Levels used** **Low (-1)**	**Medium (0)**	**High (+1)**
X_1	VSF (mg)	500	3000	5500
X_2	SS (ml)	2	4	6
X_3	NHM gel (g)	1	2	3
Dependent variables (at 37 ºC)		**Observation Basis**		
Viscosity		An exploratory study to analyse the behaviour of vehicle gel in simulated challenged conditions		
pH				
Mucoadhession				
Spreadibility				

2.9 Spermicidal activity of NHM gel (Modified Sander-Cramer assay)

The study was performed in similar manner as described above for individual formulation component. Different dilutions of NHM gel in VSF were observed for the in-vitro spermicidal activity. 5% Tergitol NP-9 was used as positive control while medium containing no test compounds was employed as negative control. The sperm motility was assessed manually under inverted phase-contrast microscope.

2.10 Challenge mating study in rats

Eighteen female Wistar rats having regular and nearly similar four day estrous cycle were selected in this evaluation. The animals were divided into three groups, each consisting of six rats. First group (G-1) was left untreated; the second group (G-2) was treated with placebo, and the third group (G-3) received NHM gel. In second and third groups, 100µl dose was

administered intra vaginally in each animal (twice a day) for seven days. Animals were allowed to cohabit with males of proven fertility at a ratio of 1male:2 females on the 8th day. Animals were examined the following morning for evidence of successful copulation. Animals with spermatozoa in vaginal smears or mucus plug were separated from male partners and this was considered day one of pregnancy [29, 30].

2.10 Efficacy of NHM gel against candida infections

C. albicans and C. tropicalis fungal cells strains were used in the present study. Sterile molten potato dextrose agar (PDA) was poured into sterile petri dishes with (a) different NHM gel's dilutions (X, 2X and 4X with VSF) and (b) without treatment (as control). These test plates containing X, 2X and 4X dilutions (250µl) were inoculated with 50µl cell suspension ($1x10^5$ cells/ml) of *C. albicans* (in one set of triplicate) *and C. tropicalis* (in other set of triplicate) using a sterile inoculating loop. Plates were incubated at 25-30°C in an inverted position keeping the agar side up with increased humidity. Control plates were incubated in similar manner for 24 hr. The number of colonies and their size were measured at the end of the experiment [31].

2.11 Pre-clinical toxicology study of nanoherbal gel

Toxicology studies were conducted in both rodent (viz. Wistar rat) and non-rodent (viz. Albino rabbit) species. All the procedures were conducted in accordance with the guidelines approved by the institutional ethics committee of ISF-CP Moga with approval no. TAEC/M4/CPCSEA/P64/2012. Study was performed in 24 female Wistar rats and 12 female Albino rabbits according to the protocol recommended by the US Food and Drug Administration (US-FDA) for products meant for vaginal use. Animals were maintained under light and temperature control (complying with standard husbandry conditions) with food and water adlibitum [32, 33].

2.11.1 Pre-clinical toxicology study on female Wistar rats

Animals were divided in three different groups, untreated "control" (G-I), placebo (G-II) and NHM gel (G-III). Each group consist 8 rats with average weight in the range of 160-220 g. 100µl of intravaginal application was done twice a day for 21 days. Individually observation for overt clinical signs (vaginal swelling, redness, and discharge including bleeding) was done daily. The change in the regularity of estrous cycle was observed by microscopical examination of vaginal lavage [33].

2.11.1.1 Effect on local tissues, vaginal tissue proliferation, and in situ apoptosis (After 21 day protocol)

Animals from each group were sacrificed to obtain the vaginal tissues by opening the slit ventrally between the urethral orifice and fornix. Macroscopic examination of excised vaginal tissue was done for surface defects, marks of inflammation and anykind of ulceration. After that, representative samples of the proximal, middle and distal portions were collected and fixed in

10% neutral buffered formalin fixative. Fixed tissues were embedded in paraffin, sectioned at thickness of 5µm, stained with hematoxylin and eosin, and examined under inverted phase-contrast microscope. The vaginal tissue sections were observed for epithelial ulceration, edema, leukocyte infiltration, and vascular congestion [34].

2.11.1.2 Haematology study: Study was performed to predict the chances of systemic infection when compared with the placebo and control group. After 7 days blood samples were collected from the rats and CBC (complete blood count) and DLC (differential leucocyte count) using flow cytometer as well as standard microscopical analysis.

2.11.1.3 Organ Distribution study

Because AgNPs possess lower therapeutic safety window as compared to MC-40 (as determined in in vitro results) systemic effects were observed by studying the AgNP's organ accumulation. After 21 days, rats were sacrificed and visceral organs (kidney, lungs, liver, brain and heart) of the dissected rats were removed and washed to remove any adhered debris and dried using a tissue paper. Isolated organs were weighed separately and homogenized in PBS pH 7.4 using tissue homogenizer. They were centrifuged at 6000 rpm for 30 minutes. The supernatant was separated, filtered and AgNPs quantification was done in the supernatant using AAS spectroscopy (Shimadzu AA-7000, Japan).

2.11.2 The standard rabbit vaginal irritation test

Animals were divided in three different groups, untreated "control" (R-I), placebo (R-II) and NHM gel (R-III) each consisting 4 rabbits. Once a day intrvaginal application of placebo and NHM gel (250µl each) was made on (R-II) and (R-III) groups respectively for 7 consecutive days. Vagina was examined daily for the macroscopic signs of irritation, inflammation and ulceration [35].

2.11.3 Lactobacillus toxicity screening

This study is a part of toxicity screening, for the acceptability of any intra-vaginal product. Using two lactobacilli strains i.e. *L. acidophilus and L. jensenii,* lactobacillus toxicity was determined for different formulation dilutions [35, 36]. Sterile, molten (45-50°C) Lactobacilli MRS agar was poured into sterile petri dishes with (a) different NHM gel's dilutions (X, 2X and 4X with VSF) and (b) without treatment (as control). These test plates containing X, 2X and 4X dilutions (250µl) were inoculated with 50µl cell suspension ($1x10^6$ cells/ml) of *L. acidophilus and L. jensenii* (in respective set of triplicates) using a sterile inoculating loop. Plates were incubated at 37°C in an atmosphere containing 5% CO_2 and 95% air for a period of 72 hr. The number of colonies and the size were determined at the end of experiment.

2.12 Statistical analysis

All biochemical observations were based on 3 independent experiments. Data were expressed as mean±SEM or percentage. The results were analyzed by 1-way analysis of variance (ANOVA), and chi-square test, as applicable, using the Microsoft excel (Microsoft office 10, Washington, U.S.A) and Graph Pad Prism 3.0 software. (GraphPad Software, Inc.CA, U.S.A).

3. RESULT AND DISCUSSION

3.1 Synthesis, optimization and characterization of in-situ stabilized silver nanoparticles

Nanoparticle dispersion was yellowish brown in colour. **Table-2** represents the effect of molar concentration ratio (TA/$AgNO_3$) and pH of tannic acid solution on average particle size, particle size distribution (PDI), and λ max. Investigation revealed that at molar ratio 0.025 (TA/$AgNO_3$) and pH-9 (tannic acid solution) showed least average particle size around 17nm with optimal polydispersity. Particle size distribution data **(figure-2)** at these conditions (molar ratio 0.025) revealed that, AgNPs ≤ 20nm were present in 14% higher concentration when compared with the distribution of nanoparticles at molar ratio 0.05. This climb was 9% when concentration of particles ≤ 30nm was compared.

Optimization study revealed that, size of AgNPs at lower molar ratio (0.0125) for all pH values was found to be undesirably large (i.e. > 40 nm). A considerable reduction in size was observed when the molar concentration of tannic acid is doubled to 0.075mM (i.e. at molar ratio 0.025). Among the various pH values, the smallest size was found to be 17.4 ± 0.2 nm at pH-9. Overall studies revealed that at each molar ratio, pH-9 was providing relatively smaller size than pH 7 and 8. Increasing the pH of tannic acid solution above 9, results in abrupt increase in particle size.

Beauty of this synthesis procedure is the in-situ stabilization of synthesized silver nanoparticles **figure-2**. Glucose moiety is one of the hydrolyzed product of tannic acid, and it act as a weak reducing agent at room temperature but a strong stabilizing agent at alkaline pH. There exist an ideal requirement for the partially hydrolysis of tannic acid to produce optimum quantity of gallic acid (for the reduction of $AgNO_3$ to AgNPs) and glucose (for stabilization of AgNPs in their smallest possible size). pKa of tannic acid is around 10, which means that pH >7 can easily hydrolyse tannic acid to an appreciable extent [37]. But experimental results showed that pH-9 provided an optimum extent of hydrolysed tannic acid for reduction and stabilization process.

Table-2 Concomitant optimization of molar concentration ratio of tannic acid to silver and pH of tannic acid solution. [n=3]

Sr.no	Molar ratio (T.A:AgNo3)mM	pH	P-SIZE (nm)	PDI	λ max
1		pH-7	47 ± 3.1	0.320 ± 0.105	424 ± 2.7

	0.0125 (0.0375 : 3)	pH-8	51.2 ± 2.2	0.410 ± 0.127	419 ± 1.5
		pH-9	40.1 ± 2.6	0.391 ± 0.016	418 ± 2
		pH-10	78.5 ± 6.4	0.644 ± 0.27	427 ± 0.7
2	**0.025** (0.075 : 3)	pH-7	32.8 ± 1.8	0.307± 0.12	418 ± 3.6
		pH-8	22 ± 1.3	0.321± 0.163	413 ± 1.8
		pH-9	**17.3 ± 0.2**	**0.311± 0.146**	**413± 1.2**
		pH-10	55.9 ± 1.7	0.487± 0.52	425 ± 0.4
3	**0.05** (0.15 : 3)	pH-7	31.1 ± 2.4	0.330± 0.053	420 ± 2.9
		pH-8	25.4 ± 1.4	0.245± 0.119	418 ± 3.4
		pH-9	19.6 ± 0.2	0.203± 0.068	412 ± 2.9
		pH-10	63.2 ± 2.7	0.148± 0.083	426 ± 1.3

Concept of molar ratio optimization can be easily understood on the basis of an effective reduction and stabilization requirements. At molar ratio < 0.025, concentration of glucose (hydrolysis product of tannic acid), was not enough for the effective stabilization of small sized AgNPs. Understandably, large surface area and thus high requirement of glucose for an effective surface stabilization of small sized growing nanoparticles could be the possible reason behind the increased size. Moreover at smaller molar ratio, incorporation efficiency of atoms into nuclei/particle (growth) will be higher per collision resulting in higher growth rates and thus size increase. At molar ratios > 0.025, increasing reagent's concentration i.e. (tannic acid) results in the increased reaction rates, that further lead to higher monomers (hydrolysed products) concentration. Increased monomers concentration may facilitate the effective nucleation (growth step) to such an extent, which resulted in increased particle size. It could be understood that apart from reducing and stabilizing role, tannic acid was also acting as organizer for facilitating nucleation [38]. In contrast, at molar ratio 0.025, the incorporation efficiency of atom into nuclei/particle (growth) as well as in-situ stabilization, both take place in such an effective and timely manner that results in smallest sized and stabilized AgNPs ranging 2 to 24 nm.

3.2 Characterization of Synthesised AgNPs.

Optimized AgNPs suspension with least size 17.3±0.2nm and polydispersity index of 0.311± 0.146 possess the UV-VIS absorption maxima at 413±1.2nm. Since there was only one transverse peak, it indicates that the nanoparticles are isotropic in nature. This absorbance maxima corresponds the observed particle size justifying the Mie's relationship between surface plasmon

resonance and AgNPs size (< 20nm) (Mastro et al., 2008, Yang et al., 2003). Zeta potential value comes out to be -21.03±0.1mv, clearly relating the colloidal stability of nano-metal suspension. PXRD studies showed the sharp intense peaks of AgNPs (specifically at 2θ values of 38.15 and 49.3 degree) confirming the crystalline nature of AgNPs. Thermogravimetric (TG) analysis of synthesised AgNPs is presented in ***figure-1s (in supplementary data)*** revealed a substantial weight loss only in the temperature region of 260-480ºC signifying the loss of associated water and degradation of surface anchored stabilizing components. TEM analysis further confirmed the diametrical range and polydispersity of the synthesised AgNPs (between 2 to 32nm) with spherical shape and no definite signs of agglomeration. Further AFM studies provided a fine level of surface smoothness with appreciably regular circles with uniform edges. This can be attributed to the synchronized $AgNO_3$ reduction and stabilization process at optimized conditions. This eventually provided an undeviating platform for the upstream nano-assembly course. Morphological symmetry was the result of in-situ stabilization control provided by the concomitantly available glucose flux during the nucleation process. **Figure-3** illustrates all the above discussed characterization parameters.

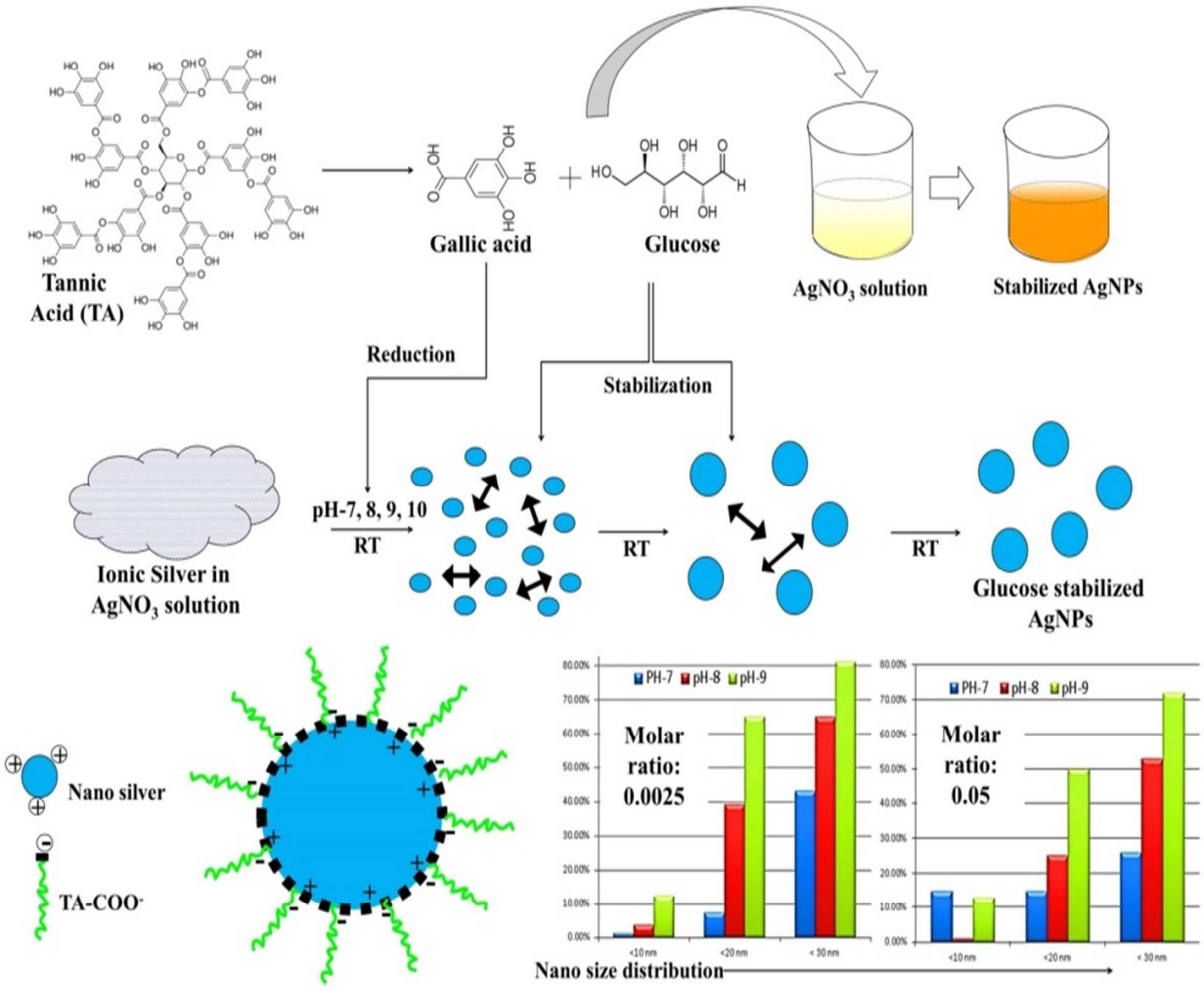

Figure-2 Tannic acid based synthesis and stabilization silver nanoparticles. Figure also signifies the stabilization status of AgNPs and a graphical comparison of the effect of molar ratio and pH variations on the particle size distributions.

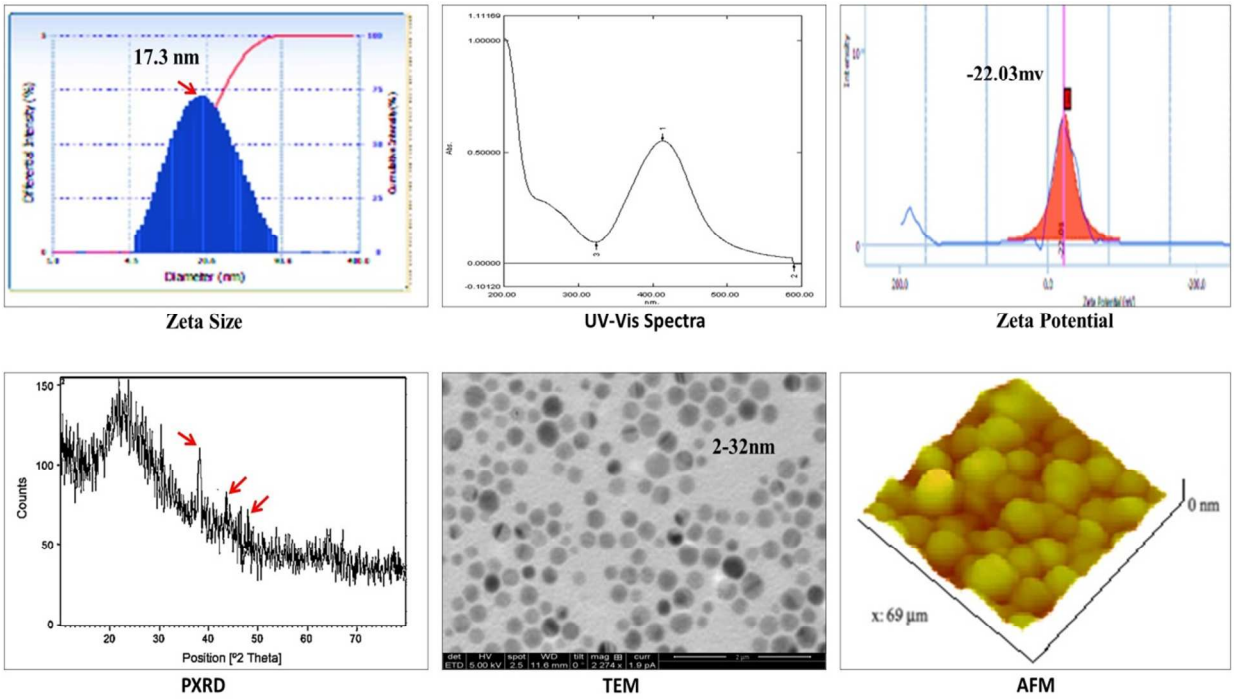

Figure-3 Characterization of synthesised silver nanoparticles.

3.3 Synthesis, optimization and characterization of copper-curcumin (Cu-Cur) and copper-curcumin- β-Cyclodextrin (Cu-Cur)CD inclusion complex

A basic synthesis scheme is illustrated in **figure-4**. Reddish brown colored (Cu-Cur) complex was obtained and (Cu-Cur)CD inclusion was prepared by incorporating (Cu-Cur) inside the β-CD hydrophobic cavity. (MC-40) batch was selected as optimized based on the (Cu-Cur) saturation inside the hydrophobic β-Cyclodextrin cavity. (*Synthesis details are detailed in supplementary data).*

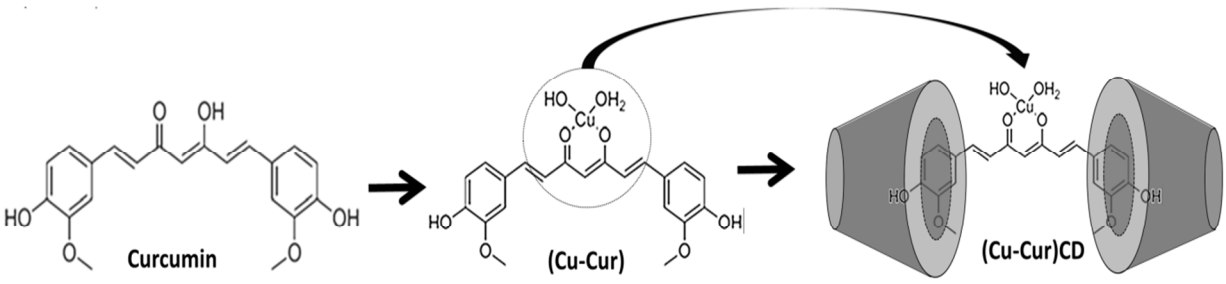

Figure-4 Synthesized (Cu-Cur) and (Cu-Cur)CD. Structure of (Cu-Cur) where metal is expected to bind with 1,3-diketone moiety of curcumin. The probable inclusion of (Cu-Cur) inside the hydrophobic β-CD cavities is shown.

3.4.1 Cell Viability Assay

Minimum cytotoxic concentration (MCC) and cell cytotoxicity 50% (CC_{50}) values revealed the uniform response among all the cell cultures with no sign of specific cell damage as mentioned in **table-3**. Toxicity at cellular level is a characteristic for metal nanoparticles, but synthesised AgNPs presented a safer and uniform cell toxicity response. This signifies the comparatively bio-friendly nature of tannic acid reduced and stabilized AgNPs. Unrelatedly, (MC-40) showed negligible toxicity describing their highly safe nature at both specific and non-specific levels. This can be attributed to the highly biologically safe nature of β-CD shell preventing the direct exposure of Cu-Cur complex.

Table-3 Cytotoxic concentration, as determined by measuring the cell viability with the colorimetric formazan-based MTS assay.

Sample	Hela		HEL		Hep G2		CRFK		U-87 MG	
	MCC	CC_{50}	MCC	CC_{50}	MCC	CC_{50}	MCC	CC_{50}	MCC	CC_{50}
	µg/ml									
AgNPs	6.3± 0.7	431.7± 4.1	4.9± 1.2	396.2 ± 5.7	5.5± 0.3	403.5 ± 2.5	6.8± 0.8	465.2± 7.5	7.1± 2.1	458.1 ± 4.6
MC-40	>100	>100	>100	>100	>100	>100	>100	>100	52.3± 3.9	>100

3.4.2 Hemolytic study

Hemolytic activity of AgNPs and MC-40 was determined by quantifying the released haemoglobin spectrophotometricaly. At 500µg/ml of AgNPs haemolytic effect observed is~28% whereas for MC-40 only 11% haemolysis was observed as the highest concentration 10mg/ml. This reveals a highly safe and non-irritant profile of both the challenged moieties.

3.5 Anti-HIV activity

Both AgNPs and (Cu-Cur)CD i.e. (MC-40) showed dose dependent inhibition of HIV-1 transmission in all the challenged cases as mentioned in **figure-5**. AgNPs with IC_{50} value of 218.2±5.3µg/ml and 295.6±4.7µg/ml during interaction-1 and 2 respectively, in CEM-GFP host cells presented a potent prospective for blocking pre-interaction infection. IC_{50} value for viral dissemination in PHA-PBMCs was 236.9±4.1µg/ml and 362.3±7.4µg/ml for interaction-1 and 2 respectively. Observation laid strong conclusions that, AgNPs possess a better prophylactic potential by blocking the initial viral spread. 500µg/ml concentration showed >93.5% of viral inhibition in both the host cells during interaction-1 while this value declines to 82% while

interaction-2 was studied. On the other hand MC-40 showed an IC_{50} of 6.31±0.15mg/ml and 8.17±0.27mg/ml for interaction-1 and 2 challenges respectively, in CEM-GFP host cells whereas the IC_{50} value in PHA-PBMCs was observed at 6.64±0.08 mg/ml and 8.4±0.19 mg/ml for respective interaction-1 and 2.

Both the moieties reduced the virus spread as estimated by p24 levels in the culture supernatants collected from the infected cells. AgNPs definitely showed a potent prospective as compared to

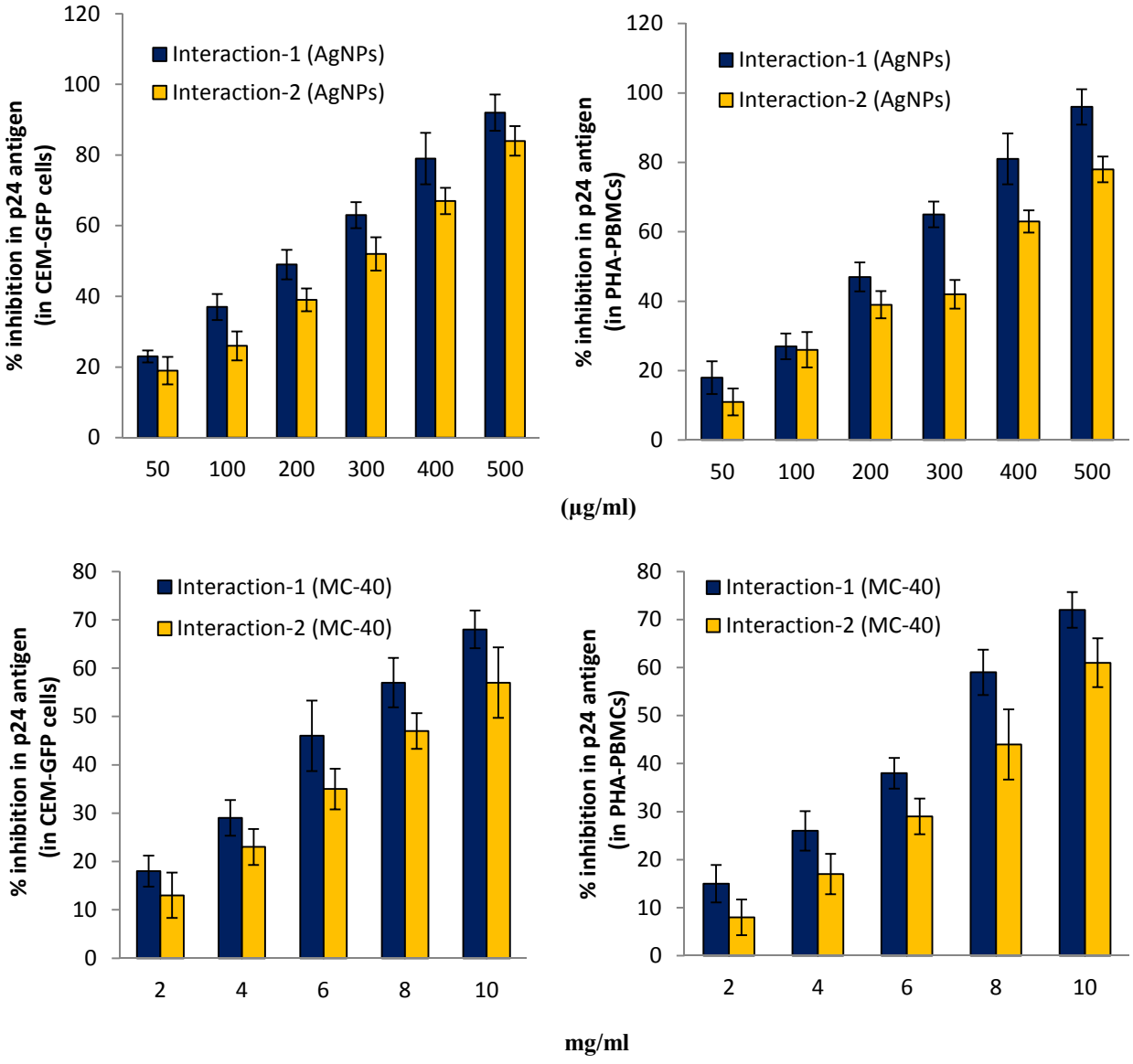

Figure-5 Reduction in virus load by AgNPs and MC-40 using CEM-GFP cells and PHA-PBMCs while exposing to interaction-1 and interaction-2 challenges. Interaction-1 refers to the pre-infection interaction of virus and test sample while interaction-2 states host interaction of the test sample before challenging the virus. Culture supernatant was collected for p24 estimation by ELISA. Levels of p24 antigen by test compounds were compared to the positive control for calculating % inhibition values. p24 value for positive controls while providing CEM-GFP cells

interaction-1 challenge ranges 197.4±2.9 pg/ml while for interaction-2 it is 192.6±0.4 pg/ml, while in case of PHA-PBMCs challenge p24 concentration ranges 164.8±4.7 pg/ml and 142.7±6.1 pg/ml for interaction-1 and 2 respectively. The results obtained were found significant after applying two-tailed Students t' test with P value < 0.05.

MC-40 at both pre and post infection levels. Lower p24 titers, specifically observed during pre-infection interactions, is a possible outcome of direct contact with viral functional molecules (viral envelop, receptors/co-receptors). Moreover interaction-2 (post infection) includes the challenge of both cell free and cell associated virus, where inhibiting cell associated virus presents some serious challenges, like targeting the intercellular viral proceedings. Previous studies predicted the interaction of AgNPs with two disulfide bonds situated in the carboxyl half of the HIV-1 gp120 glycoprotein and finally denaturing its disulfide-bonded domain. Mechanistic basis of MC-40 action can be primarily explained as the direct inhibitory effect of Cu-Cur complex released from the inclusion complex. Multiple mechanisms of HIV-1 inhibition related with both curcumin and copper moieties need more specific studies to define exact mechanism responsible. In addition to the improved Cu-Cur presentation by β-CD, it can also interfere with HIV-1 transmission by disruption of lipid raft functionality or membrane cholesterol balance[39].

3.6 Spermicidal activity

3.6.1 Modified Sander-Cramer assay (Sperm motility inhibition assay)

Scoring the motile and immotile sperm under the phase contrast microscope revealed that AgNPs and MC-40 complex presented concentration dependent inhibitory effect as shown in **figure-6** (left and centre). Terminating the assay after 120sec revealed the irreversible immobilization of sperms by MC-40 at minimum effective concentration (MEC) of 290±15µg/ml and complete immobilization at 5.3±0.1mg/ml. On the other hand AgNPs showed inhibition with MEC of 20±2.5µg/ml and~82% motility inhibition at 450µg/ml.

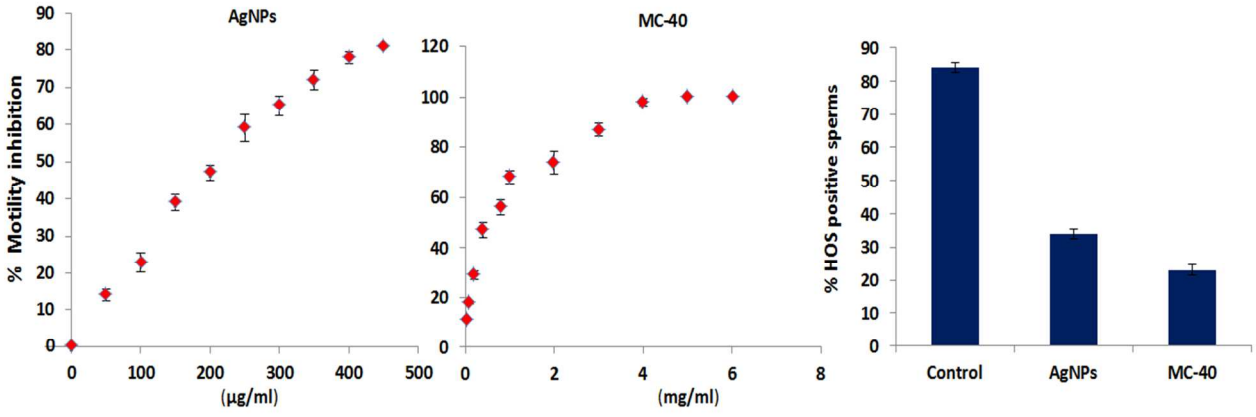

Figure-6 Percentage motility inhibition after AgNPs and MC-40 challenge (left and centre), observed during Sander-Cramer assay. (Right) Percentage HOS positive sperms after the AgNPs and MC-40 challenge.

3.6.2 Hypo-osmotic swelling test

Percentage of tail curling observed in control is quite high (83.8%) while after treatment with 250µg/ml AgNPs and 1mg/ml (MC-40) the tail curling of spermatozoa was significantly reduced to 34.2% and 23.5% as shown in figure-6 (right). The loss of HOS responsiveness after these treatments clearly indicated the compromised sperm membrane integrity in both the challenges. Study revealed an overall loss of sperm membrane physiology which is comparatively prominent in case of MC-40.

3.6.3 Sperm viability test (fluorescent staining)

Viability testing using two different fluorescent stains for live and dead sperms revealed significant sperm death in both the challenged cases. AgNPs and MC-40 treated sperms showed significant uptake of red (PI) dye whereas control group showed only (Sybr-14) green dye uptake **(figure-7 a, b and c).** PI uptake was quantitatively higher in case of MC-40 challenged sperms reflecting higher sperm damage. PI uptake by AgNPs and MC-40 treated cells is a result of sperm death which can also be understood by compromised sperm membrane.

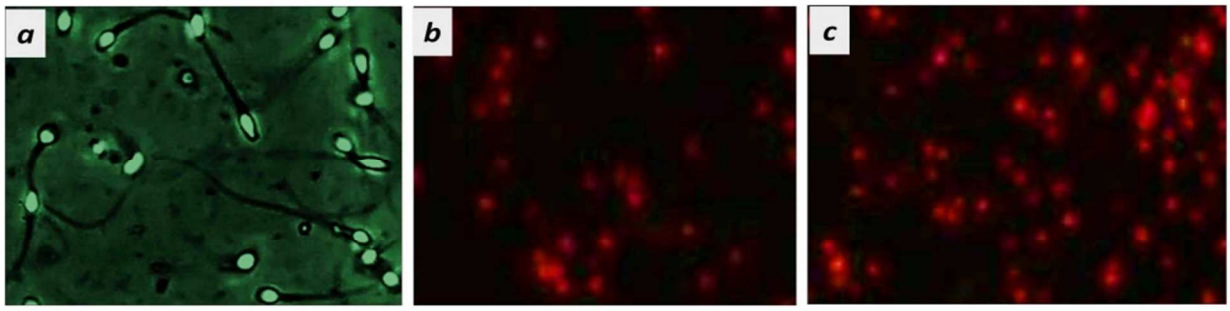

Figure-7 Sperm viability assessment by SYBR-14/PI staining. Control human sperm (a) appears green due to uptake of SYBR14 only, AgNPs and MC-40 treated human spermatozoa (b and c respectively) displaying red fluorescence due to the preferential uptake of PI. Apoptotic/necrotic changes in plasma membrane induction by control.

3.6.4 Apoptotic and necrotic changes in plasma membrane of human spermatozoa

The selective spermicidal action of AgNPs and MC-40 was observed after 3 h incubation at different concentrations**.** By labelling with FITC-Annexin V (for detection of phosphatidyl serine on cell surface) and PI (for cell membrane integrity), cells were identified in different quadrants by flow cytometry. The data from the dual fluorescent labelling with Annexin V- FITC and PI showed that the control sample contained 94.1±0.7% viable, 2.7±0.2% apoptotic, 0.8±0.1% early apoptotic and 2.4±0.2% necrotic sperm. After treatment with AgNPs the number of apoptotic, early apoptotic and necrotic cells rose considerably to 25.4±1.2%, 16.1±0.8% and 3.8±0.1% respectively, while the populations of viable sperm cells reduces to 54.9±1.4%. Treatment with

MC-40 revealed slightly lower apoptotic cells 21.5±0.5% whereas early apoptotic and necrotic cell populations increased to 26.3±0.2%, 5.2±0.1% respectively. Result provides a mechanistic understanding of spermicidal activity of AgNPs and MC-40. Apoptotic cell death is prevalent in case of silver nanoparticles challenge whereas MC-40 displayed the prevalence of apoptotic as well as necrotic events in the challenged sperm cells.

3.7 Anti-Candida Activity

Serial dilution assay revealed significant anti-fungal potential of AgNPs whereas MC-40 displayed much higher MIC values. MIC of AgNPs was found to be approximately three times more than standard clotrimazole drug (MIC of clotrimazole against *C. albicans is* 6.3±0.2µg/ml and *C. tropicalis* 7.1±0.4µg/ml). MIC values of AgNPs against *C. albicans* is (35.2±2.3µg/ml) and *C. tropicalis* is (37.5±1.8µg/ml), while IC_{50} value for both the strains lies around (380±15) µg/ml On the other hand, no appreciable inhibition was seen in case of MC-40. On the other hand, no appreciable inhibition was seen in case of MC-40 with MIC value ranging >250µg/ml. AgNPs may exert an antifungal activity by disrupting the structure of the cell membrane and inhibiting the normal budding process due to the destruction of the membrane integrity.

3.8 Formulation of Nano-herbal microbicidal gel (NHM gel)

Selectivity index for both AgNPs and MC-40 is estimated in **table-4**. AgNPs provided a narrower window due to the deemed toxicity associated at nanoscale. MC-40 on the other hand is effective at comparatively higher concentrations but a very high selectivity index allowed the use of higher concentrations. Finally nano-herbal gel was formulated using 500µg/ml of AgNPs and 10 mg/ml MC-40 in 1% w/v carbopol 974p base.

Table-4 Selectivity index of AgNPs and MC-040 calculated on the basis of their respective anti-HIV-1 potential (approximately calculated for both HIV-1 strains and for both pre and post infection challenges in different host cells), spermicidal potential and specific Hela cell cytotoxicity.

Treatment	HIV-1 EC_{50}	Spermicide EC_{50}	Specific (Hela) Cytotoxicity IC_{50}	Selectivity Index IC_{50}/EC_{50}
AgNPs	214±21.6 µg/ml	62.5±12.2 µg/ml	431.7± 4.1µg/ml	>2
MC-40	7.1±0.39 mg/ml	0.411±5.3mg/ml	--	Extremely High

The prepared gel was non-greasy homogenous oranges-yellow clear gel, with good lubrication when textured on hand. With its pH value of 4.2± 0.1 gel displayed its acidic nature, well-suited for vaginal use. Thixotropic analysis of gel formulations revealed its pseudoplastic flow with a very narrow deformation at the end of the descending protocol. Viscosity values at shear rate of

100-500 γ(1/s) ranges from 0.1 Pa.s to 0.34 Pa.s. Detachment force of gel evaluated while mucoadhession studies was found to be 19.2 ± 0.4 g/cm^2. Spreadibility parameter of gel was found in the range of (25.1 ± 1.7 gm.cm/sec). All these parameters are critical for a vaginal gel's performance and specifically for a contraceptive microbicide which needs uniform spread (depending on viscosity and spreadibility) and mucoadhesive nature to prevent unwanted leakage. These parameters significantly govern product acceptability and the ease of the product use. Viscosity and texture parameters of NHM gel meets some other vaginal products such as KY Jelly® Lubricant) and Lacta-Gynecogel® Acidifying gel.

3.8.1 In-vitro challenge study for pharmaceutical parameters of NHM gel

Contour plots and response surface analysis while Box-Behnken statistical screening, revealed the exact behaviour of the NHM gel in simulated in-vitro challenged conditions. Analysis showed a nominal buffering capacity of gel. Because, maintenance of acidic pH is critical for such prophylaxis, it is not a good choice to depend on the safeguard provided by buffering capacity of formulation. Gel is efficient to provide sufficient viscosity, spreadibility and mucoadhession in challenged conditions to provide comfortable application and to resist the chances of leakage and related discomfort *(detail study is mentioned in supplementary data).* Analysis of the pH and viscosity variations in different simulated runs revealed that quadratic model governs both these responses. On the other hand mucoadhession and spreadibility variations are governed by linear models **(Table-5).**

Table-5 In-vitro challenge study for pharmaceutical parameters of NHM gel using Box-Behnken statistical screening. Table explains the model fit and regression analysis of pH, viscosity, mucoadhession and spreadibility (dependent variable).

Response (Y)	R^2	Adjusted R^2	Predicted R^2	SD	% CV	Model fit	Regression equations
pH	0.96	0.8831	0.3320	0.19	3.14	Quadratic	pH = 5.66 - 0.32A + 0.39B - 0.24C + 0.19 AB - 0.055 AC - 0.058 BC + 0.65 A^2 + 0.064 B^2 + 0.17 C^2
Viscosity	0.81	0.5593	2.0848	0.18	36.16	Quadratic	Viscosity = 0.36 + 0.077A + 0.18B + 0.046C + 0.058 AB - 0.014 AC - 0.22 BC - 0.11 A^2 + 0.17 B^2 + 0.21 C^2
Mucoadhesion	0.93	0.9188	0.8849	2.57	9.51	Linear	Mucoadhesion = 27 + 11.50A - 3.12B - 3.13C
Spreadibility	0.89	0.8650	0.8047	3.09	16.64	Linear	**Spreadibility** = 18.59 + 10.63A – 2.88B - 2.25C

3.9 Pre-infection interaction (Interaction-1) anti-HIV study of NHM gel

Significant reduction in viral propagation was observed during the pre-infection interaction challenge study of NHM gel at various dilutions as presented in **figure-9.** Placebo gel, placed as the control group, offered an inert nature by not interrupting with viral proliferation. With

undiluted NHM gel, around 97.5% inhibition in p24 Ag was observed on both the challenged cells. Effect of VSF dilution was insignificant up to 4X dilution level, where > 90% inhibition in p24 Ag levels assures the prophylactic efficacy of the formulation in such physiological challenges. A significant drop in p24 inhibition was observed after 8X dilution, explained the protection failure during the possible conditions where the dose application is not adequate or there are excessive genital secretions.

3.10 Spermicidal activity of NHM gel (Modified Sander-Cramer assay)

Different dilutions of NHM gel were tested for the spermicidal activity at different time intervals. Table-6 represents the percentage of motility inhibition at different dilutions after different time intervals. Gel showed potential reduction in sperm motility, signifying the contraceptive nature of the product. Similar to the anti-HIV response, dilutions presented an inverse proportionality to the spermicidal profile. Up to 4X dilutions, a complete motility inhibition was noted at 90 sec observation, whereas this inhibition potential fell significantly at next dilution.

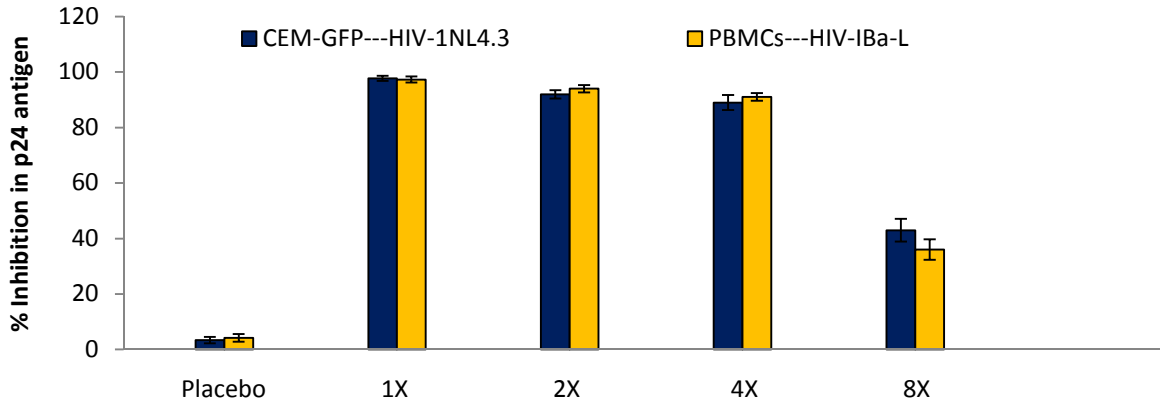

Figure-9 Inhibition in viral propagation/ prophylactic potential of NHM gel at different dilutions with undiluted placebo as control. Challenge in both the viral strains showed 211.6±4.8 pg/ml of p24 titter in placebo group, whereas sequential dilutions of NHM gel showed this titter values of 5.2±0.7 pg/ml (at 1X or undiluted), 14.6±2.1 pg/ml (at 2X), 20.3±1.5 pg/ml (at 4X) and 126±3.8 pg/ml (at 8X). The results obtained were found significant after applying two-tailed Students t' test with P value < 0.05.

Table-6 Sperm motility inhibition study of different dilutions of NHM gel) loaded gel [n=3]

Dilution	% inhibition time (0 sec)	% inhibition time (30 sec)	% inhibition time (60 sec)	% inhibition time (90 sec)
(No dillution) X	68.4 (±3.7) %	100%	100%	100%
2X	54.1(±1.3) %	78.3(±1.7) %	96(±0.4)%	100%
4X	41.8(±0.6) %	67.2(±2.3) %	88(±1.5)%	100%

8X	37.2(±1.6) %	48(±2.7) %	64.5(±0.9) %	91(±2.7) %
5% Tergitol NP-9	51.6(±3.4) %	94.3(±2.4) %	100%	100%

3.11 Challenge mating study in rats

Study revealed the potential antifertility activity of NHM gel when compared to placebo treated and untreated rats. All the females mated successfully and no deviation of mating efficiency was observed. 100% absence of fertilization (no positive pregnancy outcome) was observed in NHM gel treated group i.e. G-3. On the contrary, group G-1 and G-2 showed 100% and 66.6% positive pregnancy outcomes respectively. These observations suggest that NHM gel could effectively block sperm potential for fertilization. Mechanistic explanation can be given using the reference of in vitro results. Seized motility and compromised membrane nature by the gel components probably had resulted in disturbing the normal fertilization mechanism. Placebo effect can be taken statistically, where viscosity, pH and ionic strength of the semisolid gel could account to the possibility of interrupted fertilization.

3.12 Efficacy of NHM gel against candida infections

As per the mentioned protocol, antifungal activity of different NHM gel's dilutions was estimated. Although the activity showed inverse proportionality with dilution, but still results showed significant reduction in fungal colonies even at 4X dilution level. Results for reduction in number of colonies of *C. albicans* are; control: 37±0.26 and NHM gel (X: 17.4±1.3, 2X: 24.3±0.73 and 4X: 29.6 ± 0.1). For *C. tropicalis* strain activity pattern follows, control: 33.4±1.71 and NHM gel (X: 21.6±0.9, 2X: 25.8±0.24 and 4X: 27.2±0.4). In accordance with the in-vitro results for individual components, NHM gel showed prominent potential of controlling candida infections, which eventually lessens the chances of viral escape through vaginal epithelium.

3.13 Pre-clinical toxicology study of NHM gel

3.13.1 Pre-clinical toxicology study on female Wistar rats

Examining the structural integrity of the vaginal epithelium during the course of 21 days study protocol, there was no macroscopic sign of redness, edema, abnormal discharge, bleeding and inflammation. Microscopic examination of in vaginal lavage revealed the regularity in estrous cycle.

3.13.1.1 Effect on local tissues, vaginal tissue proliferation, and in situ apoptosis (After 21 day protocol)

Macroscopic examining the excised vaginal epithelium after 21 days protocol revealed that there was no macroscopic sign of any kind of redness, ulceration, edema and inflammation as displays the biopsy images **(figure-10).** Histological examination of excised vaginal tissue showed the

presence of columnar epithelium in all samples with nucleus in basal portion and rest cell shows cytoplasm. The sub mucosa shows an infiltrate of lymphocytes while deeper zone shows muscular layer with no sign of any type of damage. Macroscopic examination and histopathology confirmed no sign of alteration when control, placebo and treated groups were compared. **Figure-10** shows the microscopic images of histopathology samples.

3.13.1.2 Haematology study

Systemic safety conformation was made from haemotogram results from animal of each group. CBC and DLC data showed no significant variations in control, placebo and treated groups as mentioned in table-7.

3.13.1.3 Organ distribution study

Despite of long term vaginal application protocol, kidneys showed maximum AgNPs accumulation of 1.6(±0.2)µg (rest of the distribution data is given in **figure-11**). Results of the organ uptake studies confirmed a minor systemic absorption of AgNPs by vaginal cavity. Results obtained from the in vitro cell cytotoxicity (MTS) assay served as a reference to eliminate the discussion of organ toxicity on consistent use of NHM gel.

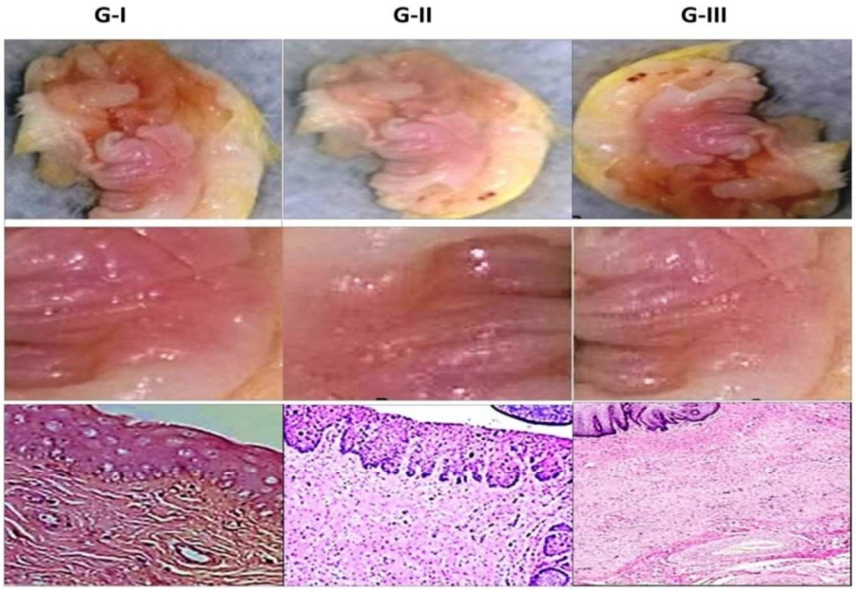

Figure-10 Macroscopic examination of excised vaginal epithelium and histopathology images of vaginal epithelium tissues of "control" (G-I), placebo (G-II) and NHM gel (G-III) treated groups.

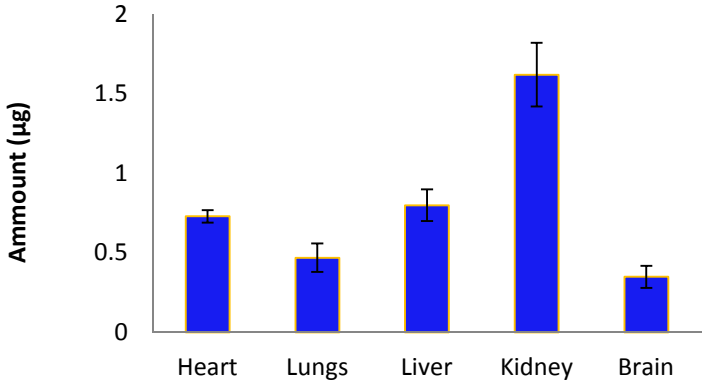

Figure-11 Organ distribution data of AgNPs after 21 days toxicity studies in G-III (NHM gel treated) rats.

Table-7 Haematogram of blood taken from control (G-I), placebo (G-II) and (G-III) treated rats after 21 days in-vaginal application protocol

Investigation	Groups		
	G-I	G-II	G-III
Complete Blood Count			
Haemoglobin (gm/dl)	11.2 (±0.7)	11.3(±0.2)	11.6(±0.4)
Total leucocyte (count /cumm.)	11600(±31)	12600(±18)	10200(±82)
RBC (Millons/cmm)	5.82(±0.1)	6.24(±1.4)	6.11(±0.5)
HCT	38.3(±3.6)	32.4(±1.7)	38.4(±6.8)
MCV	58.8(±3.1)	52(±7.2)	61.7(±4.5)
MCH	22.6(±2.7)	18.1(±2.1)	20.8(±3)
MCHC	38.6(±4.7)	34.8(±1.9)	35.8(±1.6)
Platelet (Lakh/cumm.)	4.12(±0.2)	4.61(±0.6)	3.92(±0.1)
Differential Leucocyte Count			
Neutrophil %	46.3(±1.6)	45.8(±2.7)	46.5(±1.4)
Lymphocyte %	51(±2.1)	51.5(±1.3)	51.9(±0.8)
Eosinophil %	02(±0.4)	02.5(±0.5)	2(±0.3)
Monocyte %	01	01	01
Basophil %	00	00	00
ESR (mm/1st hr)	05(±0.1)	04(±0.1)	05(±0.1)

3.13.2 The standard rabbit vaginal irritation test (after 7 day protocol)

No macroscopic alteration in the vaginal morphology was observed. No sign of redness, inflammation and edema on comparison of control, placebo and treated groups, confirmed the safety of NHM gel.

3.13.3 Lactobacillus toxicity screening: Study was conducted on two lactobacillus strains i.e. *L. Acidophilus* and *L. Jensenii* assure the safety margins of the NHM gel. Study revealed that minor signs toxicity was observed in the undiluted formulation; with dilution the effect followed insignificant mark. Results for reduction in number of colonies of *L. Acidophilus* are; control: 41±0.72 and NHM gel (X: 35.2±3.5, 2X: 38.1±1.5 and 4X: 39.1 ± 1.2). For *L. Jensenii* strain toxicity pattern follows, control: 38.2±2.2 and NHM gel (X: 32.5±0.7, 2X: 36.2±1.1 and 4X: 37.6±0.7). Minor signs of toxicity were observed on both lactobacillus strains but this effect had insignificant presence after dilution.

4. CONCLUSION

Strategic delivery of in-situ stabilized AgNPs and MC-40 showed a promising approach for the development of contraceptive microbicides. In vitro anti HIV-1 studies conformed the potential of AgNPs to interact cell free (pre-host interaction level) as well as cell associated virus (post-host interaction level). Contraception assays proved the potential of MC-40 as well as AgNPs to hinder the conception pathway by disrupting the sperm membrane. NHM gel formulated with rationally selected dose, showed a response harmony with the in-vitro results of its components. Anti-HIV-1, contraception as well as anti-candida studies revealed the potential of the gel at several dilution levels. Pre-clinical toxicology protocol strengthens the candidature of NHM gel as a safe contraceptive microbicide.

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