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# (Copper–curcumin) β-cyclodextrin vaginal gel: Delivering a novel metal–herbal approach for the development of topical contraception prophylaxis

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ABSTRACT

Delivering a safe and effective topical vaginal contraceptive is the need of present era. We explored the potential of a metal (copper) and herbal moiety (curcumin) for this topical contraceptive prophylaxis. Complex of copper and curcumin (Cu–Cur) was synthesized and the concerns regarding its aqueous solubility was resolved by including it into the hydrophobic cavity of β-cyclodextrin (β-CD) as (Cu–Cur)CD inclusion complex. Dose assessment was made on the basis of in-vitro spermicidal assays and cell cytotoxicity studies. Finally the (Cu–Cur)CD loaded vaginal gel was prepared, characterized and evaluated for in-vitro spermicidal activity and preclinical toxicity studies. Spectral and morphological characterizations confirmed the synthesis of (Cu–Cur) and (Cu–Cur)CD inclusion complex. Spermicidal assays and Hela cell cytotoxic data revealed an optimized 1.5% (Cu–Cur)CD for further studies. 1.5% w/w (Cu–Cur)CD loaded carbopol 974p gel provided 100% motility even at 2-fold dilution and preclinical toxicity studies in Rats and Rabbits revealed its highly safe profile. The hypothesis of considering metal–herbal complex and its cyclodextrin complex has worked and the well planned strategy of including it in (β-CD) cavity provided a preeminent platform for vaginal delivery. In-vitro assays and preclinical toxicity analysis confirmed its potential to be used as highly safe and effective prophylaxis.



## 1. Introduction

An unintended pregnancy is a pregnancy that is mistimed, unplanned, or unwanted at the time of conception (Santelli et al., 2003). Worldwide, 38% of pregnancies were unintended in year 1999 and present scenario is touching the altitude of 41% (Singh et al., 2010). Condoms are considered as the best way to tackle this problem. But its denial, inconsistent or incorrect use and failure favor 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. Bioadhesive polymers based contraceptive gels in connection with condoms could be an alternative to hormonal contraception. Contraceptive gel is an example of such patented technology which combines bioadhesive polymers such as carbomer, polycarbophil with the most widely used spermicide available i.e. nonoxynol-9 (Raymond et al., 2004). But deleterious effects of nonoxynol-9 on vaginal epithelium limit its use (Catalone et al., 2004).

The stake of copper in the form of copper T has been in use for over five decades, touting a greater than 99% pregnancy prevention rate. Focusing on a particular aspect relating the deleterious effect of copper on sperm explains the rationale behind this research work. Copper ions prevent pregnancy by inhibiting the movement of sperm, because the copper-ion-containing fluids are directly toxic to sperm and inhibit the sperm's motility and viability. As mentioned in literature copper ions affect the integrity of sperm in multiple ways. One aspect defines its mechanism by interacting with the cells lipid bilayer, leaking the contents of the cell into the surrounding environment. There are reports conforming the deleterious effect of copper ions on the biochemical structures and confound enzyme structures, making them useless (Ortiz and Croxatto, 2007). Moreover, the studies explaining the overall effect of copper ions revealed that copper causes the uterus and fallopian

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tubes to produce a fluid that contains white blood cells, copper ions, enzymes, and prostaglandins, a combination that is toxic to sperm.

Curcumin (diferuloyl methane) is the second component of our study. Inhibitory effect of curcumin on sperm function, fertilization, and fertility is reported in a study. (Naz, 2011) Curcumin also inhibits human sperm motility, function and fertility both in-vitro and in-vivo. Total loss in forward motility as well as concentration dependent inhibition of capacitation and acrosome reaction provide a strong basis to use it in contraceptive microbicide formulation.

In this research we aimed to explore the potential of Metal Ligand (M–L) complex of Copper (M) and curcumin (L) in the area of vaginal contraception. Copper–curcumin (Cu–Cur) complex had already been synthesized by many researchers (Barik et al., 2005; Chauhan et al., 2013; Krishnankutty and Venugopalan, 1998; Zhao et al., 2010). Aqueous solubility of the Cu–Cur complex presented a serious problem for its therapeutic research. The first task of this research work is to solve the issues of poor aqueous solubility. Here we resolved this problem by including the (Cu–Cur) complex in (β-CD) cavity. Lyophilized (Cu–Cur)CD nano-inclusion complex is evaluated for vaginal epithelial cell cytotoxicity and spermicidal activity. Optimum dose selected on the basis of the cytotoxicity and efficacy data is then incorporated in carbopol-974p gel. (Cu–Cur)CD loaded gel is then explored for vaginal safety aspects by pre-clinical toxicology study on Wistar Rats, Rabbits and vaginal lactobacillus bioflora. Spermicides capable of killing 100% human sperm almost instantaneously at physiological concentrations in vitro are likely to provide adequate pregnancy protection in vivo.

## 2. Materials and methods

Curcumin, cupric acetate, Dimethyl sulfoxide (DMSO), β-Cyclodextrin (β-CD), Tergitol, tetrahydrofuran (THF) and Triethanolamine, Propidium iodide was purchased from Sigma Aldrich, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT), Nutrient Mixture F-10 Ham, Lactobacillus MRS Agar (MRS Agar) was purchased from Himedia Laboratories, Carbopol 974p was procured as a gift sample from Lubrizol (Belgium), *L. acidophilus* and *L. jensenii* were procured from IMTECH Chandigarh.

### *2.1. Synthesis, optimization and characterization of copper–curcumin (Cu–Cur) and copper–curcumin-β-cyclodextrin (Cu–Cur)CD inclusion complex*

25 ml Methanolic solution of cupric acetate (0. 220 g, 4 mmol) was added into the 13.5 ml of methanolic solution of curcumin (0.185 g, 2 mmol). Dark reddish brown precipitates were produced immediately. The mixture was refluxed for 2 h under a nitrogen atmosphere. The solid product was then filtered, washed with cold methanol and water to remove the residual reactants, and then the product was dried in vacuum overnight. The compound is characterized by UV absorption spectroscopy (UV–Visible spectrophotometer, Shimadzu, Japan), IR (FTIR Spectrophotometer, Nicolet-380, Thermo, USA), $^1$H NMR (Bruker Avance II 400 NMR) and DSC (Mettler Toledo DSC 822e). Inclusion complex was prepared by solvent evaporation encapsulation method; with slight modifications as reported in the previous research (Yallapu et al., 2010). (β-CD) 100 mg was dissolved in 20 mL deionized water in a 50 mL beaker containing a magnetic bar. (Cu–Cur) complex in different concentrations, (10, 20, 30, 40 and 50)% was dissolved in 1 ml tetrahydrofuran (THF) was added to (β-CD) solution under stirring at 600–800 rpm. Stirring was done at ambient temperature for 12 h in dark with a perforated aluminum foil covering for THF evaporation. Highly water soluble (Cu–Cur)CD was separated from the supernatant after centrifugation at 1500 rpm and then recovered by lyophilizer (Alpha 1–2 LD plus, Martin Christ, Germany). At constant process conditions (stirring time, stirring speed and temperature), (Cu–Cur) loading inside (β-CD) cavity was analyzed in all the cases and the saturation level was taken as the optimum (Cu–Cur) percentage in complex formation for further studies. (Cu–Cur) loading inside the complex was analyzed using DMSO based extraction method. Optimized inclusion complex was characterized by IR, $^1$H NMR, morphology (Scanning electron microscope, JSM-840 SEM, Jeol, Japan) and DSC.

### *2.2. Spermicidal assays*

#### *2.2.1. 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 milliliter. Spermicidal activity of (Cu–Cur) complex, (β-CD) and (Cu–Cur)CD was evaluated. Briefly, in case of (Cu–Cur) complex, sequential dilutions (5–100 μg/ml) were prepared by dissolving the complex in a minimum volume of DMSO and further diluting it with Ham's F10. Ten sequential dilutions of (β-CD) and (Cu–Cur)CD ranging (2–20) mg/ml 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 50 μl were incubated (37 °C in the presence of 5% $CO_2$) with various concentrations of test ingredients in a final volume of 100 μL. At different time points of 0 s, 30 s, 60 s and 120 s the reaction was terminated with 1.5 mL of Ham's F10. After 10 min centrifugation at 290*g*, the sperm pellet was obtained, which was further resuspended in 100 μL of Ham's F10. Sperm motility was assessed manually under inverted phase-contrast Microscope (MKX-41, Olympus, Japan) (Gupta et al., 2005; Sander and Cramer, 1941).

#### *2.2.2. Hypo-osmotic swelling 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 (Jeyendran et al., 1984). (Cu–Cur)CD treated spermatozoas were exposed to a 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 (MKX-41, Olympus, Japan).

### *2.3. In-vitro cell viability assay*

HeLa cell line (cervical) was obtained from National Center for Cell Sciences (NCCS), Pune, India. Cells were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% (v/v) heat inactivated fetal bovine serum. Cells were maintained in 5% $CO_2$ humidified incubator at 37 °C. During subculture, cells were detached by trypsinization when they reached 80% confluence and split (1:4). Growth medium was changed every 3 days.

Two different assays were performed for the determination of cytotoxicity of the above synthesized components i.e. optimized (Cu–Cur)CD, (Cu–Cur) and (β-CD). Propidium iodide based selective non-viable cell sorting was done by using flow-cytometer. MTS assay cell viability assay was performed to study both specific on Hela cells and non-specific on HEL (Human embryonic lung), VERO, CRFK (Crandell-Rees Feline Kidney cells), MDCK (Madin Darby canine kidney) cells) toxicity. This experiment (MTS assay) was performed in Belgium (Laboratory of Virology and Chemotherapy, Department of Microbiology and Immunology, Rega Institute).

*2.3.1. Nonviable cells sorting assay (Propidium iodide staining)*

Propidium iodide (PI) is a membrane impermeable dye that is generally excluded viable cells from non-viable ones. It binds to double stranded DNA by intercalating between base pairs. PI staining solution was prepared using 10 μg/ml PI in PBS stored at 4 °C in the dark. Hela cells were treated with sequential dilutions of (Cu–Cur)CD, (Cu–Cur) and (β-CD) for 24 h maintaining the final cell concentration of $1 \times 106$ cells/100 μl. After that 100 μl of untreated as well as treated Hela cells were taken in FACS tubes and washed with PBS. Cells were then resuspended in 100 μL of flow-cytometry buffer. 5 μl Of PI staining solution to a control tube of otherwise unstained cells and a gentle mixing is followed by 1 min incubation. Finally dead cell sorting was done by using (BD Accuri C6) flowcytometer.

*2.3.2. Cell viability evaluation (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. The MTS assay is often described as a 'one-step' MTT assay was performed to determine the cytotoxicity of the optimized (Cu–Cur)CD, Cu–Cur and (β-CD) in Hela, HEL, VERO, CRFK, MDCK cell cultures.

*2.4. Preparation and characterization of (Cu–Cur)CD loaded gel*

Nano-herbal gel was formulated using 1.5% w/v of (Cu–Cur)CD in 1% w/v carbopol 974p gel (pH of the gel is maintained at 4.5 using triethanolamine). The developed nano-herbal gel formulation was characterized for macroscopic properties, rheological analysis using (Brookfield Rheometer), mucoadhession, spreadibility using (Brookfield Texture analyzer CT3 10K) (Garg et al., 2003).

*2.4.1. Rheological analysis*

Rheology of gels will affect both their distribution and retention in the vaginal cavity. The study is carried out using R/S-CPS + Rheometer, Brookfield. This study is performed under the thixotropic protocol, shear rate was set in the range of 100–500 (1/s) for a total time interval of 10 min (5 min for both ascending and descending mode) using C75-1 measuring system at 37 °C.

*2.4.2. Mucoadhession and spreadibility*

Mucoadhession and spreadibility was evaluated using Brookfield Texture analyzer CT3 10K. Mucoadhession study was performed on a special testing assembly with TA10 cylinder probe (12.7 mm D, 35 mm L) and maintains the temperature of 37 °C. Fresh got vaginal epithelium was placed both inside the mucoadhession assembly and on the bottom of the probe. 1 ml Gel was placed in the cavity of assembly and the test was run in the compression mode setting the target of 100 g and holds time of 15 s and the trigger load of 3 g. Test and return speed was kept 0.5 mm/s. Spreadibility is assessed by using a male and female cone assembly setup. Male cone was completely filled with the gel formulation and the test was run at ambient temperature. Test was run in the compression mode setting the target of 4 mm and holds time of 2 s, trigger load of 3 g, test and return speed was kept 0.5 mm/s.

*2.4.3. Modified Sander-Cramer assay (sperm motility inhibition assay) for 1.5% (Cu–Cur)CD gel*

The study was performed in similar manner as described above for (Cu–Cur)CD. 4 Serial dilutions of 1.5% (Cu–Cur)CD gel in Ham's F10 were tested for the spermicidal activity.

Tergitol NP-9 was used as positive control while medium containing no test compounds was employed as negative control. Following the similar procedure the sperm motility was assessed manually under inverted phase-contrast Microscope (MKX-41, Olympus, Japan).

*2.5. Pre-clinical toxicology study of 1.5% (Cu–Cur)CD gel*

Study was performed in 18 female Wistar rats and 6 female Albino rabbits according to the protocol recommended by the US Food and Drug Administration (US-FDA) for products meant for vaginal use (Stone, 2009; Talwar et al., 2008).

*2.5.1. On female Wistar rats*

Animals were divided in 3 groups G-1, G-2 and G-3. Each group consist 6 animals with avg. weight in the range of 160–220 g. G-1 animals were treated with the 1.5% (Cu–Cur)CD gel while G-2 was treated with placebo gel and G-3 was kept as control group. 300 mg Of intravaginal application was done twice a day for 21 days in G-1 and G-2. Vagina was examined macroscopically for signs of irritation, inflammation, ulceration, vaginal histology and hematology (Stone, 2009).

*2.5.2. Standard rabbit vaginal irritation test*

Animals were divided in two groups each comprised of 3 animals. One group is treated with 1.5% (Cu–Cur)CD gel and other with placebo once daily for 10 days. A standard procedure and scoring system was used to assess the irritating properties of the formulation in the rabbit model. Vagina was examined macroscopically for signs of irritation, inflammation and ulceration (Dhondt et al., 2005).

*2.5.3. Lactobacillus toxicity screening*

This study is a part of toxicity screening, for the acceptability of any intra-vaginal product (Fichorova and Anderson, 1999; Fichorova et al., 2001a,b). Using two lactobacilli strains i.e. *L. acidophilus* and *L. jensenii*, anti-lactobacillus effect of 4 serial dilutions of 1.5% (Cu–Cur)CD gel was seen. Firstly, lactobacillus strains were cultured on sterilized MRS broth. Then 50 μl of broth was transferred to sterilized plates containing MRS agar. After 30 min, wells were made and 4 serial dilutions of 1.5% (Cu–Cur)CD gel were transferred to these wells. Plates were incubated at 32 °C, after 48 h. Anti-Lactobacillus activity was expressed in terms of diameter of zone of inhibition (in mm).

*2.6. Statistical analysis*

Each experiment was repeated three times using sperm sample from three different donors and the data were analyzed by one-way analysis of variance using the GraphPad Prism software (Version 3.0). *P*-value less than 0.05 were considered statistically significant.

## 3. Results

*3.1. Synthesis, optimization and characterization of copper–curcumin (Cu–Cur) and copper–curcumin-β-cyclodextrin (Cu–Cur)CD inclusion complex*

Reddish brown colored (Cu–Cur) complex was obtained and this synthesized complex was characterized for various parameters. UV spectral study in DMSO revealed a single peak for Cur at 432 nm and two peaks for Cu–Cur complex at 433 and 456 nm. IR (KBr) $cm^{-1}$: spectral data of curcumin and (Cu–Cur) reveals the formation of new bands at 535 $cm^{-1}$ and 477 $cm^{-1}$ in (Cu–Cur) spectra indicating the interaction between copper(II) and oxygen (O) atom of curcumin (Kolev et al., 2005). $^1$H NMR (DMSOd):

spectral data of curcumin and (Cu–Cur) revealed the absence of characteristic peaks of curcumin (6–8 ppm) in the spectra of the metal complex, indicating the chelation with the metallic ion. Metal ion interaction with the keto-enolic region of curcumin moiety is an evident observation from new IR peaks; moreover the absence of some key hydrogen peaks justified the tampering of keto-enolic group. DSC thermogram showed a sharp peak for the melting of (Cu–Cur) around 197.3 °C, which indicates the formation of crystalline metal complex.

(Cu–Cur)CD inclusion was prepared according to the procedure mentioned and further it was optimized on the basis of loading of (Cu–Cur) inside the β-CD hydrophobic cavity. (Cu–Cur) loading was estimated for each batch of inclusion complex. Table 1 explains the preparation and optimization of inclusion complex on the basis of (Cu–Cur) inclusion. Optimization study revealed that lyophilized batch (MC-40) and (MC-50) have shown comparatively higher as well as identical (Cu–Cur) loading (around 24 μg/mg of complex) and % yield (88–90 mg). This clearly indicates the saturation of (Cu–Cur) inclusion inside the available β-CD cavities after 40% (Cu–Cur) trial. Rationally, formulation (MC-40) was considered as optimum for further characterization and other studies.

**Table 1**
Optimization table of (Cu–Cur)CD complex ($n = 3$).

| Batch | β-CD (mg) | (Cu–Cur)% | Yield (mg) (Cu–Cur)CD | Loading of (Cu–Cur) per mg of (Cu–Cur)CD (μg) |
|---|---|---|---|---|
| MC-10 | 100 | 10 | 63 | 6.2 ± 1.4 |
| MC-20 | | 20 | 76 | 7.8 ± 0.4 |
| MC-30 | | 30 | 81 | 17.4 ± 1.1 |
| MC-40 | | 40 | 88 | 24.2 ± 2.6 |
| MC-50 | | 50 | 90 | 24.5 ± 1.7 |

(Cu–Cur)CD complex (MC-40) appears as light greenish yellow colored fluffy powder. This fluffy appearance points toward its amorphous nature. Aqueous saturation solubility of the (MC-40) at ambient conditions was found to be around 38.5 ± 1.4 mg/ml. UV spectra in deionized water showed an absorption maxima at 265 nm.

IR (KBr) $cm^{-1}$ spectra revealed the major existence of (β-CD) specific peaks with a slight shift to higher/lower wave numbers while only few characteristic peaks of (Cu–Cur) were observed. $^1$H NMR spectra again displayed the same dominance as (MC-40) showed all the peaks relevant to (β-CD). But inclusion of (Cu–Cur) results in the shifting of proton signals to high field region.

DSC thermograms are indicative of complexation of (Cu–Cur) within cyclodextrin cavity. DSC thermograms of (MC-40) complexes showed a substantial reduction in the intensity of the peak, broadening of peak and a shift to lower temperature (136–143 °C) in comparison to sharp melting point of pure (Cu–Cur). Marked reduction in intensity accompanied by broadening and shift to a lower temperature of the endotherm indicates the substantial inclusion of (Cu–Cur) in the (β-CD) cavities. Scanning electron microscopy clearly justifies the above observation of having a highly amorphous nature of (MC-40). Fig. 1 displays a descriptive view of the FT-IR, $^1$H NMR, DSC analysis of (Cu–Cur), (β-CD) and (Cu–Cur)CD as well as UV spectral and SEM image of (Cu–Cur)CD.

### 3.2. Spermicidal assays

#### 3.2.1. Modified Sander-Cramer assay (sperm motility inhibition assay)

Scoring the motile and immotile sperm under the phase contrast microscope revealed that (Cu–Cur) and (Cu–Cur)CD complex presented both concentration and time dependent effect as mentioned in Fig. 2. (Cu–Cur) potentially immobilized human sperms irreversibly at minimum effective concentration (MEC) of

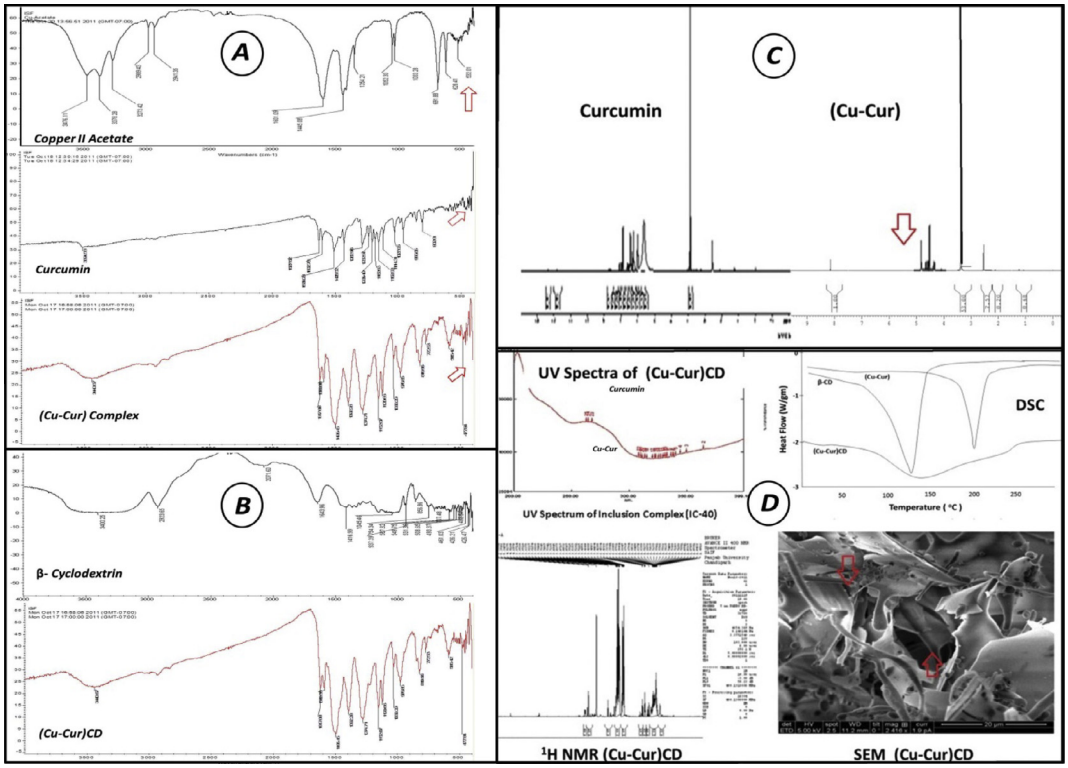


**Fig. 1.** Characterization of copper–curcumin (Cu–Cur) and copper–curcumin-β-cyclodextrin (Cu–Cur)CD inclusion complex. (A) IR spectral overlay of copper II acetate, curcumin and (Cu–Cur), (B) IR spectral overlay of β-CD and (Cu–Cur)CD inclusion complex, (C) $^1$H NMR spectras of curcumin and (Cu–Cur), (D) characterization parameters for (Cu–Cur)CD including UV spectra, DSC, NMR, $^1$H NMR spectras and SEM picture.

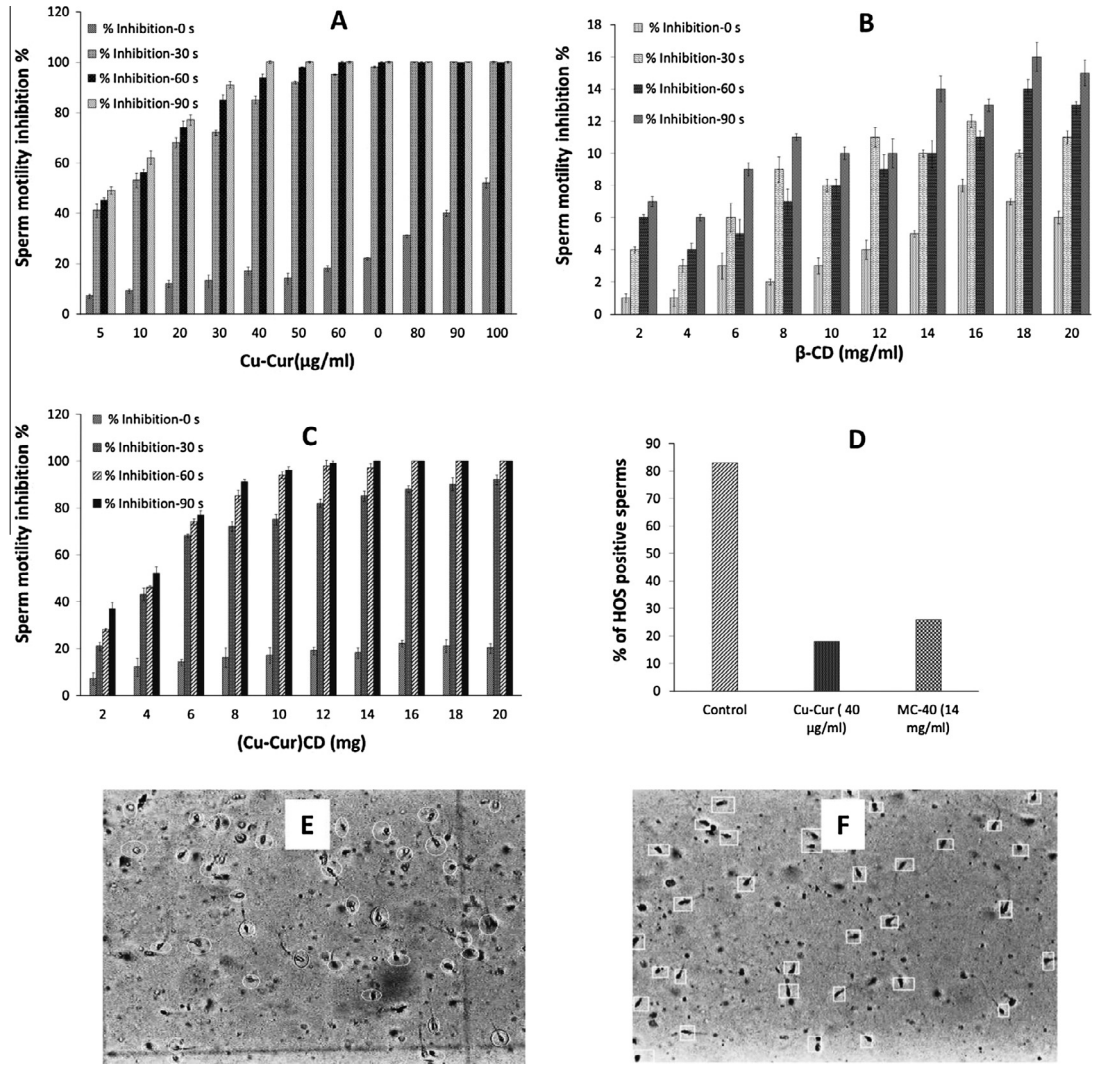


**Fig. 2.** Spermicidal assays reports (A, B and C) displays the sperm mobility inhibition assay data of (Cu–Cur), β-CD and (Cu–Cur)CD respectively. (D) Reports the HOS positive sperms percentage for control (untreated), (Cu–Cur) and (MC-40) treated sperms ($n = 3$).

2.2 ± 0.4 µg/ml and complete immobilization at 40 µg/ml after 90 s. In comparison to the (Cu–Cur), (MC-40) showed relatively higher MEC of 290 ± 15 µg/ml and complete immobilization at 14 mg/ml after 90 s. β-CD displayed MEC of 680 ± 15 µg/ml with only around 15% inhibition at relatively high concentration of 20 mg/ml.

*3.2.2. Hypo-osmotic swelling test*

Percentage of tail curling observed in control is quite high (83.8 ± 2.5)%, while after treatment with 40 µg/ml of (Cu–Cur) and 14 mg/ml (MC-40) the tail curling of spermatozoa was significantly reduced to (18.0 ± 1.8)% and (26.0 ± 2.2)%. The loss of HOS responsiveness after these treatments indicated the compromised sperm membrane integrity, suggesting an overall loss of sperm membrane physiology. Fig. 2 shows the HOS responsiveness of the sperm toward all three abovementioned observations, indicated by tail curling.

*3.3. In-vitro cell viability assay*

*3.3.1. Nonviable cells sorting assay (Propidium iodide staining)*

Specific toxicity studies on cervical Hela cells revealed some interesting outcomes. (Cu–Cur) showed potential cell death with MCC (minimum cytotoxic concentration) of 1.8 ± 0.2 and CC50 (concentration for 50% cell cytotoxicity) of 14.5 ± 0.75 µg/ml. While both (MC-40) and β-CD both showed a highly nontoxic nature with MCC of 400 ± 0.20 µg/ml and 750 ± 0.12 µg/ml. Fig. 3 displays the cell death pattern revealed after this study.

*3.3.2. Cell viability evaluation (MTS assay)*

Irrespective of the specificity view the CC50 of 13.4 ± 2.9 µg/ml marks the innate toxicity of (Cu–Cur) on all challenged cells. Unrelatedly, the (β-CD) and (MC-40) showed negligible toxicity describing their highly safe nature at both specific and non-specific levels. Table 2 provides the descriptive view of the MTS assay on five different cell cultures.

*3.4. Characterization of (MC-40) loaded gel*

The prepared gel was homogenous greenish-yellow clear gel, with good lubrication when textured on hand but non-greasy.

*3.4.1. Rheology*

Thixotropic analysis of gel formulation revealed its pseudoplastic flow with a very narrow deformation at the end of the descending protocol. Viscosity values at shear rate of 100–500 (1/s) ranges

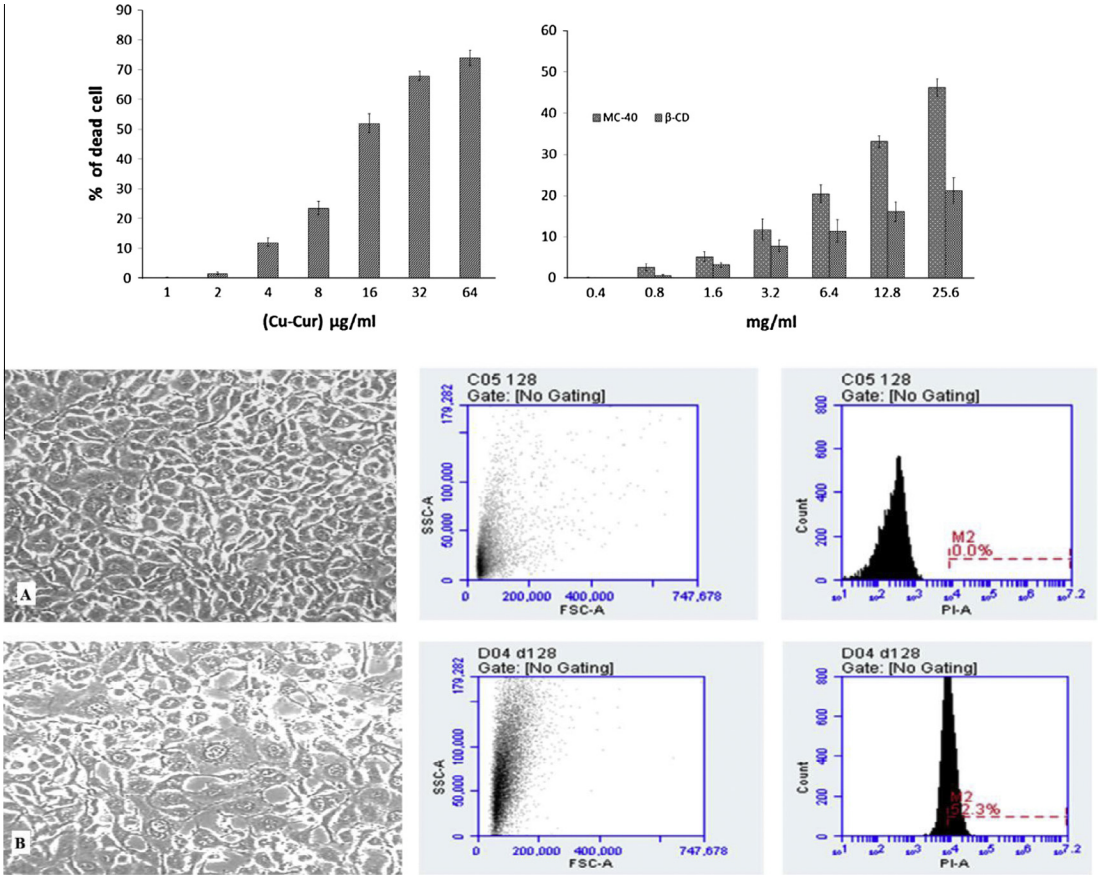


**Fig. 3.** Specific Hela cell cytotoxicity data of (Cu–Cur), β-CD and (Cu–Cur)CD. (Done by flowcytometry based non-viable cell sorting assay ($n = 3$).

**Table 2**
Nonspecific cell cytotoxicity data on Hela, HEL, VERO, CRFK, MDCK cells as determined by measuring the cell viability with the colorimetric formazan-based MTS assay. (MCC is the challenged concentration required to cause a microscopically detectable alteration of normal cell morphology and $CC_{50}$ is 50% cytotoxic concentration) ($n = 3$).

| Compound | Hela | | HEL | | VERO | | CRFK | | MDCK | |
|---|---|---|---|---|---|---|---|---|---|---|
| | MCC | $CC_{50}$ | MCC | $CC_{50}$ | MCC | $CC_{50}$ | MCC | $CC_{50}$ | MCC | $CC_{50}$ |
| | µg/ml | | | | | | | | | |
| (Cu–Cur) | 4 | 16.3 | 1.8 | 13.5 | 2.9 | 15.2 | 2.6 | 11.4 | 3.2 | 10.6 |
| (MC-40) | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | 52.3 | >100 |
| β-CD | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | 65.7 | >100 |

from 0.1 Pa s to 0.29 Pa s. Viscosity falls in the range of most of the vaginal products such as KY Jelly® Lubricant), Lacta-Gynecogel® Acidifying gel and Canasten® Antimycotic (Brouwers et al., 2008).

### 3.4.2. Mucoadhession and spreadibility

Mucoadhession detachment force of (Cu–Cur)CD (MC-40) loaded gel was found to be 17.5 ± 0.4 g/cm$^2$. Spreadibility of the loaded gel was found to be 23.5 (gm cm/s).

### 3.4.3. Modified Sander-Cramer assay (sperm motility inhibition assay) for 1.5% (MC-40) gel

Four serial dilutions of 1.5% (Cu–Cur)CD gel in Ham's F10 were tested for the spermicidal activity at different time intervals after exposure. Table 3 represents the percentage of motility inhibition of different dilutions after different time intervals. Study revealed that undiluted formulation is providing complete inhibition of sperm motility within the time span of 30 s; while the two fold dilution i.e. 2× took up to 90 s to render the sperms completely immotile. Increase in dilution have a direct impact on the activity as after 4× dilution the complete inhibition has not been observed even after the time span of 90 s.

**Table 3**
Sperm motility inhibition study of different dilutions of 1.5% (MC-40) loaded gel ($n = 3$).

| Dillution | % Inhibition time (0 s) | % Inhibition time (30 s) | % Inhibition time (60 s) | % Inhibition time (90 s) |
|---|---|---|---|---|
| (No dillution) × | 68 (±8) | 100 | 100 | 100 |
| 2× | 43 (±3) | 78 (±5) | 92 (±2) | 100 |
| 4× | 26 (±2) | 44 (±4) | 72 (±4) | 96 (±6) |
| 8× | 23 (±8) | 37 (±2) | 54 (±12) | 87 (±3) |
| 16× | 22 (±5) | 25 (±7) | 49 (±3) | 81 (±4) |

### 3.5. Pre-clinical toxicology study

### 3.5.1. Pre-clinical toxicology study on female wistar rats

Examining the structural integrity of the vaginal epithelium after 21 days experimental protocol revealed that there was no macroscopic sign of any kind of redness, edema and inflammation,

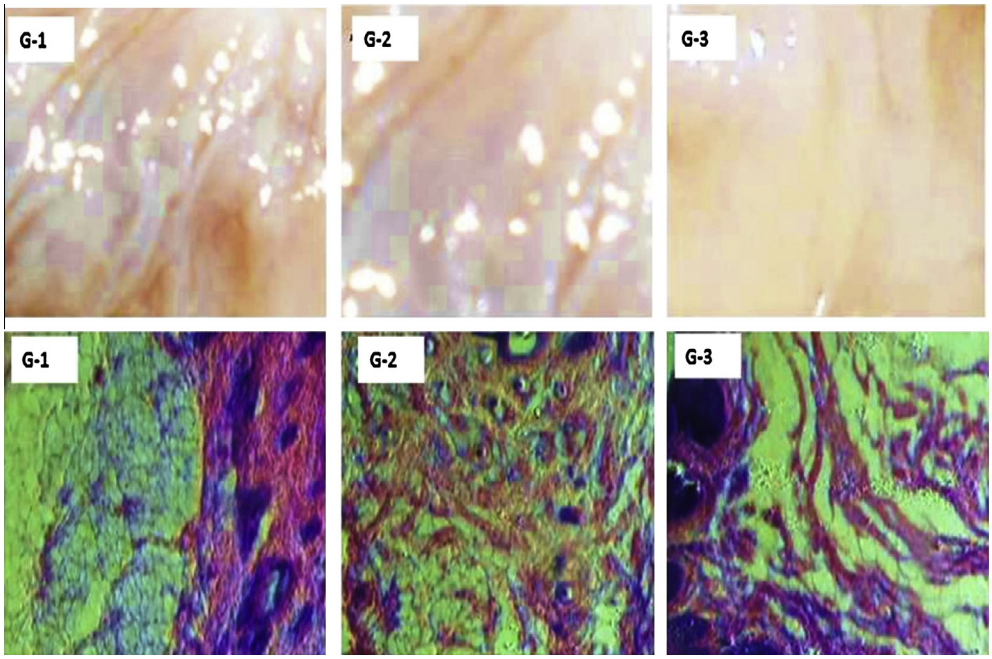


**Fig. 4.** Biopsy and histopathology images of vaginal epithelium. G-1 animals were treated with the 1.5% (MC-40) gel while G-2 was treated with placebo gel and G-3 was kept as control group.

Moreover during the protocol period, no sign of irritation was observed. Microscopic examination of histopathology samples shows the presence of stratified squamous epithelium in all samples with no sign of any type of damage. Fig. 4 shows the biopsy images and microscopic images of histopathology samples (G-1, G-2, G-3 single tan colored tissue). This study revealed the absolute safety of formulation regarding blood chemistry. Haematogram results from animal of each group showed similarity in CBC (complete blood count) and DLC (differential leucocyte count).

*3.5.2. The standard rabbit vaginal irritation test*

No macroscopic alteration in the vaginal morphology was observed. No sign of redness, inflammation and edema confirms the safety of vaginal gel.

*3.5.3. Lactobacillus toxicity screening*

Study conducted on two lactobacillus strains i.e. *L. acidophilus* and *L. jensenii* assure the safety margins of the gel formulation. Table 4 reveals the data of zone of inhibition against the two strains. Minor signs inhibition was seen only in the case of undiluted formulation while afterward dilution fails to make any impact on any of the two strains. Keeping the safety aspects in mind this formulation is very much safe for the vaginal contraceptive use.

## 4. Discussion

Spectral characterization confirmed the synthesis of metal–ligand Cu–Cur complex of copper with curcumin. Characteristic two peak UV spectra (as a result of $\pi \rightarrow \pi^*$ electronic transitions and charge transfer) indicated the chelation of copper with the 1,3-diketone moiety (which transform automatically to a more stable keto-enol tautomeric form) of curcumin as explained in Fig. 5(a). IR, $^1$H NMR study further confirms this chelation and DSC provides a conformation of its crystalline nature with melting point slightly higher than pure curcumin.

**Table 4**
Zone of inhibition study conducted on two lactobacillus strains i.e. *L. acidophilus* and *L. jensenii* ($n = 3$).

| Formulation dilution | Zone of inhibition (*L. acidophilus*) | Zone of inhibition (*L. jensenii*) |
|---|---|---|
| No dilution | 1.9 (±0.4 mm) | 1.4 (±0.2 mm) |
| 2× | No-zone | No-zone |
| 4× | No zone | No zone |
| 8× | No-zone | No-zone |

This metal–ligand complex possesses a serious issue regarding its aqueous solubility. Affinity of this hydrophobic chelate toward the hydrophobic β-CD cavity is utilized to render it water soluble. Supramolecular chemistry approach has been utilized for preparing this inclusion complex via solvent evaporation encapsulation technique. Mass transfer process of hydrophobic (Cu–Cur) moiety inside the hydrophobic cyclodextrin cavities was dependent on the concentration of (Cu–Cur) presented during the inclusion process. But the flux for the (Cu–Cur) inclusion and the amount of inclusion is directly proportional to the availability of the inclusion space. At constant process conditions (stirring speed stirring time, temperature and β-CD concentration) and inclining (Cu–Cur) concentration, there must be a saturation level for the further inclusion of (Cu–Cur). Optimization process clarified that 40% (Cu–Cur) is required for the saturation of available β-CD cavities. (MC-40) is thus selected as the optimized batch for further characterization. Absence of (Cu–Cur) specific peaks in IR, $^1$H NMR spectras confirmed the inclusion of (Cu–Cur) inside the β-CD cavities. Lyophilization of the highly soluble (Cu–Cur)CD effects the crystalinity and finally results in a highly amorphous product. Fig. 5(b) explains the probable structure of this inclusion complex and it is given on the supposition that this complex will fits the same conformation as mentioned for curcumin-β-CD complexes. (Yallapu et al., 2010).

The facts emerged in our study points toward the direct effect of (Cu–Cur) and (MC-40) on the intact sperm membrane. The expected depletion in the potency measures is the virtue of limiting the exposure of (Cu–Cur) by including it in dextrin cavity. Comparing the spermicidal potential of inclusion complex with plain β-CD clarified this point. Further HOS test clarifies the impact of both (Cu–Cur) and (MC-40) on disturbing the normal sperm membrane functioning. Literature provides multiple approaches

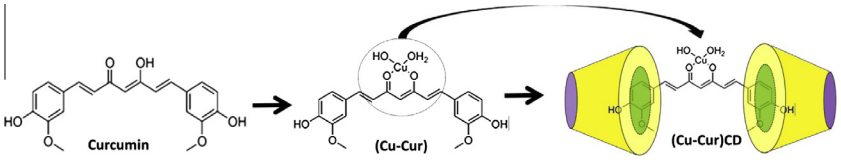


**Fig. 5.** Synthesized (Cu–Cur) and (Cu–Cur)CD. (a) Structure of (Cu–Cur) where metal is expected to bind with 1,3-diketone moiety of curcumin. (b) Probable inclusion of (Cu–Cur) inside the hydrophobic β-CD cavities.

by which curcumin and copper ions displays spermicidal nature. Moreover, reports are also available displaying the effect of β-CD on membrane structure and function of sperm. Thus, exact mechanism responsible or the spermicidal activity of both these moieties needs further studies and explanations.

Toxicity study on Hela cells presents a perfect profile for a product intended to be used in vagina. (Cu–Cur) showed potential toxicity to these cells with CC50 value of 14.5 ± 0.75 μg/ml. From the safety and activity point of view this curcumin chelate is having the toxic concentration lower than the effective concentration. The case is different during the exposure of (MC-40), which the quite similar toxicity profile as compared to β-CD (a GRAS excipient). Non-specific cell cultures responded in similar manner with the CC50 of 13.4 ± 2.9 μg/ml and no detectable alterations of normal cell morphology at 100 μg/ml of both (MC-40) and (β-CD). Finally the highly safe nature of (MC-40) permits its further use in an effective concentration. 1.5% w/w (∼15 mg/ml) concentration of (MC-40) was thus selected for formulation of vaginal spermicidal gel.

Esthetical decency of the (MC-40) loaded gel favor of the real world acceptability of the formulations as a vaginal product. With pseudoplastic flow characteristic, gel exhibits a shear thinning effect when the shear stress will be encountered during sexual intercourse. Mucoadhession and spreadibility behavior of the formulated gel assures an appreciable retention and spreading inside the vaginal cavity which is very much required for a topical vaginal contraceptive. Spermicidal assay at different dilutions of (MC-40) gel provides significant efficacy even after 4-fold dilution. This covers the margin of dilution with physiological secretions inside the vaginal cavity.

Pre-clinical toxicity study reveals the absolute safe profile of the gel, with no macroscopic signs of toxicity. Biopsy and histopathology confirmed no sign of alteration when control, placebo and treated groups were compared. Systemic safety conformation was made from haematogram results from animal of each group. CBC and DLC data showed no significant elevation compare to untreated control group, reflecting high systemic safety. Safety of gel was further conformed in rabbit vaginal irritation test. Minor signs of toxicity were observed on both lactobacillus strains but this effect had no existence after dilution, confirming the insignificance of the toxicity observations.

## 5. Conclusion

This unique approach of presenting a metal–herbal chelate with synergized spermicidal profile worked very well. β-CD inclusion not only provided an pharmaceutical acceptability but also increased the safety margins for vaginal use. Spermicidal assays revealed the direct effect of (MC-40) on the sperm membrane structure and function. Formulated 1.5% (MC-40) gel showed significant sperm motility inhibition even after fold dilutions. Toxicity and safety studies concluded an effective potential of the product to be used as vaginal contraceptive.

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