

Utilization of propranolol hydrochloride mucoadhesive invasomes as a locally acting contraceptive: *in-vitro*, *ex-vivo*, and *in-vivo* evaluation

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ABSTRACT

It was found that propranolol hydrochloride (PNL), which is a beta-blocker used for hypertension treatment, has a potent spermicidal activity through local anesthetic activity or beta-blocking effect on sperm cells subsequently it could be used as a contraceptive remedy. This study aimed to entrap PNL into invasomes (INVs) and then formulate it as a locally acting contraceptive gel. PNL-loaded mucoadhesive INVs were prepared via the thin-film hydration technique. The D-optimal design was utilized to fabricate INVs employing lipid concentration (X_1), terpenes concentration (X_2), terpenes type (X_3), and chitosan concentration (X_4) as independent variables, while their impact was observed for entrapment efficiency percent (Y_1 ; EE%), particle size (Y_2 ; PS), zeta potential (Y_3 ; ZP), and amount of drug released after 6 h (Y_4 ; Q6h). Design Expert[®] was bestowed to nominate the desired formula. The selected INV was subjected to further studies and formulated into a mucoadhesive gel for *ex-vivo* and *in-vivo* investigations. The optimum INV showed a spherical shape with EE% of $65.01 \pm 1.24\%$, PS of 243.75 ± 8.13 nm, PDI of 0.203 ± 0.01 , ZP of 49.80 ± 0.42 mV, and Q6h of $53.16 \pm 0.73\%$. Differential scanning calorimetry study asserted the capability of INVs to entrap PNL. Permeation studies confirmed the desired sustained effect of PNL-loaded INVs-gel compared to PNL-gel, INVs, and PNL solution. Sperm motility assay proved the potency of INVs-gel to inhibit sperm motility. Besides, the histopathological investigation verified the tolerability of the prepared INVs-gel. Taken together, the gained data justified the efficacy of PNL-loaded INVs-gel as a potential locally acting contraceptive.

ARTICLE HISTORY

Received 30 May 2022
Revised 30 June 2022
Accepted 4 July 2022

KEYWORDS

Contraception;
histopathological study;
mucoadhesive invasomes;
propranolol hydrochloride;
vaginal drug delivery;
spermicide

Introduction

Contraception is considered an important part of unremitting efforts to decrease pregnancy. Several effective contraceptive approaches are available, such as oral contraceptives, abstinence, barrier techniques, injections, and implants. Various research outcomes have displayed a diversity of oral contraceptives with moderate measures of hormones subsequently decreasing their undesirable effects (Greydanus et al., 2001). Spermicides are regarded as non-hormonal locally acting contraceptives. When they are present in vaginal tissues throughout intercourse, they immobilize or kill sperm without causing any systemic actions (Iyer & Poddar, 2008). The merits of spermicides include ease of administration, safety, and acceptability. Furthermore, they are suitable for short time utilization or as adjuvants with other methods. There are four classes of spermicidal agents: surface-active agents, enzyme inhibitors, membrane stabilizing agents, and sulfhydryl binding agents (Jalalvandi et al., 2021).

Nonoxynol-9 is a common surface-active agent spermicide. It is commercially available as vaginal pessaries, acts by destroying the lipids in the sperm, and causes excessive loss of sperm motion. On the other side, it causes ulcers to genital tissues and inflammation, especially if utilized daily. As a

result, spermicides without detergent-type membrane toxicity might provide significant merit over detergent-type spermicides (Tasdighi et al., 2012). Membrane-stabilizing drugs such as quinine, propranolol hydrochloride (PNL), chlorpromazine, and phenoxybenzamine were reported to restrain the motility of spermatozoa *in-vitro* (Moudgil et al., 2002). Hong et al. (1981) studies and clinical studies by Zipper et al. (1983), supported that PNL could be used as a spermicide when administered vaginally.

PNL is known as a beta-blocker that is used mainly for hypertension, angina, myocardial infarction, arrhythmias, and sinus tachycardia (Abruzzo et al., 2012). Several mechanisms have been suggested for the justification of PNL spermicidal action. As previously mentioned, PNL has localized anesthetic or membrane-stabilizing action with long duration and short latency. The potency of locally acting anesthetics is based on their lipid solubility, where the sperm plasma membrane consists of lipoprotein, and thus the immobilizing action on the sperm might occur (Srivastava & Coutinho, 2010). PNL is composed of a hydrophilic molecule with a hydrophobic aliphatic chain that might provide amphiphilic character to PNL (Ubrich et al., 2004). We speculate that PNL amphiphilic nature might aid in PNL adsorption onto sperm cells. On the other hand, both alpha and beta-adrenergic receptors have

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been located on human sperm cells. It has been reported that drugs able to block adrenergic receptors might affect the sperm's ability to move (Adeoya-Osiguwa et al., 2006). Additionally, PNL might cause vesicle formation in sperm cells that might significantly damage their integrity (Nicotra & Senatori, 1992).

It is worth mentioning that PNL has been fabricated as spermicidal vaginal gel and vaginal film (Tasdighi et al., 2012; Borumand et al., 2014). According to our understanding, no paper has been published yet regarding loading PNL in nano-system that might sustain PNL spermicidal activity. In the present study invasomes (INVs) (terpenes enriched vesicles) have been utilized as carriers for PNL. INVs are elastic nano-vesicular systems containing phospholipids, terpenes, and/or ethanol (Qadri et al., 2017; Tawfik et al., 2020). Terpenes were previously proved synergetic effects as antifungal and anti-inflammatory with several drugs loaded into INVs (Albash et al., 2021a; 2021b). Moreover, it was previously reported that the addition of terpenes in extended semen had an immediate spermicidal effect (Cavalleri et al., 2018).

The human vagina appears as an S-shaped fibromuscular collapsible tube between 6 and 10 cm long. The vaginal wall consists of three layers: the epithelial layer, the muscular coat, and the tunica adventitia. The vaginal route can be used for both local and systemic administration (Daoud et al., 2017). In a topical technique of contraception, the bio-actives are transported through the vaginal wall into fluids and mucus present in the vagina resulting in the maintenance of a concentration sufficient to immobilize or kill sperms (Iyer & Poddar, 2008). Vaginal products must be designed for women's convenience and must have the following criteria: no side effects during intercourse; colorless and odorless; might be topically applied before coitus; no leakage, no irritation, burning, or swelling; and suitable to be inserted easily (Vermani & Garg, 2000).

The vaginal route seems to be suitable for mucoadhesive drug delivery systems for managing mainly local conditions, or for utilization in contraception (Das Neves & Bahia, 2006). The major merit of mucoadhesive systems is the ability to augment the residence time, thus decreasing the frequency of applications. The most utilized mucoadhesive vaginal drug delivery systems are semi-solid gels. Gels are polymeric matrices consisting of little quantities of solids, scattered in great quantities of liquid, yet having a more solid-like character. Among vaginal dosage forms, gels are characterized by their easy manufacture and the capability to extend and adhere to vaginal walls (Woodley, 2001). Furthermore, due to their high-water composition, they provide the additional advantage of a hydrating and lubricating effect. The strength of lubrication supplied by a product will crucially determine its acceptance and usage (Das Neves & Bahia, 2006). A bioadhesive vaginal dosage form could not be importantly composed of a medicinal substance but may be utilized as a moisturizer for dry vagina syndrome (Ahuja et al., 1997). Either polycarbophil or Carbopol has been utilized as the bioadhesive polymer in several vaginal dosage forms. It is essential to assess the bioadhesion aspects of polymers and

formulations in a simulated vaginal environment to choose the suitable polymers in terms of bioadhesion to vaginal walls.

The success of PNL as a vaginal spermicide has already been described in the literature. Hence, in this research trials were designed to develop PNL-loaded mucoadhesive INVs gel that not only has lubricating action but also prolongs adhesion to the vaginal tissues for an expanded time, that might maintain longer contraceptive action.

Materials

Propranolol hydrochloride (PNL) was supplied by Kahira Pharmaceutical Co. (Cairo, Egypt). Phosphatidylcholine from soya bean (PC), chitosan (Mw 260,000 Da), and mucin were obtained from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). Cineole and limonene were bought from Alfa Aesar (Germany). Methanol and chloroform were gained from El-Nasr Pharmaceutical Co. (Cairo, Egypt). Carbopol 934 P was acquired from Goodrich Chemical Co. (USA).

Methods

Preparation of PNL-INVs

INVs were prepared by using two types of terpenes (cineole, and limonene) at various concentrations through the thin-film hydration method (Table 1) (El-Nabarawi et al., 2018). Firstly, terpenes and PC were weighted in a round bottom flask and dissolved in 10 mL methanol: chloroform (2:1) v/v. By keeping pressure under vacuum for 30 min, the organic phase was evaporated at 40 °C utilizing a rotatory evaporator (Heidolph VV 2000, Germany) at 150 rpm hence a thin film of INVs was formed. The film was hydrated using 10 mL phosphate buffer pH4.6 containing (50 mg PNL) at 40 °C. To maintain complete hydration of invasomal film, glass beads were used for 45 min. The INVs were stored at 4 °C for maturation. To improve the mucoadhesive properties of INVs chitosan-coated INVs were prepared. Briefly, chitosan (0.3 and 0.6%) w/v was dissolved into 1% glacial acetic acid and 10 ml water then added to equivalent volume from the previously prepared INVs then stirred for 15 min.

Table 1. D-optimal design for optimization of PNL loaded INVs.

Factors (independent variables)	Levels	
	Low (-1)	High (+1)
X₁: Lipid concentration (mg)	100	200
X₂: Terpene concentration (%)	0.5	1.5
X₃: Terpene type	Cineole	Limonene
X₄: Chitosan concentration (%)	0	0.6
Responses (dependent variables)	Constraints	
Y₁: EE (%)	Maximize	
Y₂: PS (nm)	Maximize	
Y₃: ZP (mV)	Maximize	
Y₄: Q6h (%)	Minimize	

Abbreviations: **EE%**; Entrapment Efficiency Percent, **PS**; Particle Size, **ZP**; Zeta Potential; **Q6h**; Amount of drug released after 6 hours, **PNL**; Propranolol Hydrochloride and **INVs**; Invasomes.

Characterization of PNL-INV_s

Determination of entrapment efficiency percentage (EE%)

One ml from the prepared INV_s was centrifuged at 20,000 rpm for 1 h at 4 °C employing a cooling-centrifuge (Sigma 3 K 30, Germany). The clear supernatant was subsequently diluted and PNL concentration was determined at λ_{max} 289 nm (Kraisit et al., 2018), using a UV-VIS spectrophotometer (Shimadzu UV1650, Japan). EE% was calculated by employing the sequent equation (Mosallam et al., 2021):

$$\text{EE\%} = \left(\frac{\text{Total amount of PNL} - \text{Untrapped PNL}}{\text{Total PNL concentration}} \right) \times 100 \text{ (Eq. 1)}$$

All evaluations were completed in triplicate \pm SD.

Determination of particle size (PS), polydispersity index (PDI), and zeta potential (ZP)

The PS and PDI of INV_s were measured for the fabricated INV_s employing a Malvern Zetasizer 2000 (Malvern Instruments Ltd., Malvern, UK). The ZP assessment was done by observing the electrophoretic motion of the particles. The evaluations were performed after dilution (Imam et al., 2017; Hassan et al., 2018). All measurements were performed in triplicate \pm SD.

Determination of amount of drug released after 6 hrs (Q6h)

The *in-vitro* release was performed employing a locally fabricated Franz's diffusion cell with an area of 0.785 cm². The cellulose membrane was subjected in the middle between the donor and receptor compartments. 1 mL of INV_s dispersions (5 mg PNL) was placed in the donor cells. The receptor compartment was filled with 50 mL of phosphate buffer (pH 4.6) at 37 \pm 1 °C. At a predetermined time, 1 mL of permeation media was withdrawn, and an equal volume of fresh media was added into the receiver compartment (Borumand et al., 2014). Samples were removed at 1, 2, 3, 4, 5 and 6 hrs then tested through a UV spectrophotometer at λ_{max} 289 nm. Trials were completed in triplicates as mean \pm SD.

D-optimal experimental design

A D-optimal experiment was performed to estimate the effect of several factors during INV_s formation adopting Design expert[®] (Stat Ease, Inc., Minneapolis, USA). The design demanded making 20 trials. Four factors were inspected: lipid concentration (mg) (X_1), terpenes concentration (%) (X_2), terpenes type (X_3), and chitosan concentration (%) (X_4) that opted as independent variables, whilst EE% (Y_1), PS (Y_2), ZP (Y_3) and Q6h (Y_4) opted as dependent variables (Table 1).

Selection of the optimum PNL -INV_s

The selection of the optimum INV resulted from the desirability function that allowed the inspection of all responses

simultaneously. The selection was decided to obtain a suggestion with sustained Q6h (%), highest ZP, and reasonable PS and EE%. The solution with the highest desirability was selected (Table 1).

Transmission electron microscopy (TEM)

The shape of the optimum INV was examined by employing TEM (Joel JEM 1230, Japan). The vesicular dispersion of INV_s was placed as a thin film on a grid, stained by phosphotungstic acid 1.5% then inspected and photographed (Abdellatif et al., 2017).

Differential scanning calorimetry (DSC)

The thermal assessment of PNL, PC, and the lyophilized optimum INV was carried out by DSC (Shimadzu Corp., Japan) standardized with indium. Five mg of each specimen was placed in a standard aluminum pan and heated in a temperature range of 10-250 °C at a scanning rate of 5 °C/min under nitrogen flow (Abd-Elsalam et al., 2018).

Stability study

The stability of the optimum INV was monitored to estimate vesicles' growth, drug leakage, or any change. The optimum INV was conserved at the refrigerator for 3 months and its stability was inspected by comparing the PS, PDI, EE%, ZP, and Q6h of the conserved INV_s with the freshly prepared INV_s. Further, the system was visually assessed for any sedimentation (Albash et al., 2021c). Statistical significance was performed by Student's *t*-test utilizing SPSS[®] software 22.0.

Evaluation of mucoadhesion aspects of INV_s

The mucoadhesion aspects of optimum INV were assessed where 1% w/v of mucin solution was admixed with an equivalent quantity of INV_s dispersion through mixing via stirrer. The stirring was maintained for five minutes, and the mixture was subjected to equate overnight. The surface charge of mucin alone and INV_s with mucin was assessed by ZP evaluation as previously mentioned (Abdellatif et al., 2020).

Preparation of PNL loaded mucoadhesive INV_s gel

To enhance the vaginal adhesion of PNL, the optimum INV was loaded into a gel matrix. In brief, 0.5% (w/v) Carbopol 934P was dispersed in 10 ml water by mixing at 500 rpm employing a magnetic stirrer for 1 h to produce a Carbopol 943 P solution. Then, PNL-loaded INV_s equivalent to 50 mg PNL was mixed with the equivalent quantity of Carbopol 934 P solution. Finally, triethanolamine was added to augment the pH of the Carbopol 934 P mixture, and the formation of gel took place (Jana et al., 2014). A control gel containing the same amount of PNL was fabricated as previously described for comparative assessment.

Gel characterization

Visual inspection

The appearance, homogeneity, and precipitation of freshly fabricated gels (PNL-gel, and INVs loaded gel) were inspected by visual examination under black and white surface (Abdelmonem et al., 2018).

Determination of pH

The pH of PNL- gel and the optimum INVs loaded gel was evaluated, in triplicate, using a calibrated pH meter (Hanna, type 211, Romania). The average reading was recorded (Abdellatif et al., 2021).

Viscosity

The viscosity of fabricated gels (PNL-gel, and INVs loaded gel) was evaluated with no dilution employing Brookfield DV III ultra V6.0 RV cone and plate rheometer (Brookfield Engineering Laboratories, Inc., Middleboro, MA) using spindle # CPE40 at $25 \pm 0.5^\circ\text{C}$. All the experiments were made in three times. The spindle was maintained at 30 r.p.m and 60 s^{-1} as a shear rate (Abdellatif et al., 2021).

Ex-vivo permeation studies

Permeation experiments were implemented by Franz's diffusion cell with a diffusion area of 0.785 cm^2 . Rat vaginal tissue was mounted between the donor and receptor compartments (Albash et al., 2020). 1 mL of PNL solution, INVs, PNL-gel, and INVs loaded gel equivalent to 5 mg was placed in the donor compartment. The receptor compartment was filled with 50 mL of phosphate buffer (pH 4.6) at $37 \pm 1^\circ\text{C}$. At a predetermined time, 1 mL of permeation media was withdrawn, and an equal volume of fresh media was added to the receiver cell (Abdellatif et al., 2020). The samples were evaluated utilizing a validated HPLC method. Statistical significance was inspected by one-way ANOVA employing SPSS[®] software. Post hoc analysis proceeded via Tukey's honestly significant difference (HSD) test.

In-vitro spermicidal activity assessment

Sperm motility assay

10 μl of the optimum INV loaded into gel-plus spermatozoa were added to the slide, and the sperm motility was evaluated by light microscope using the computer-assisted sperm analysis (CASA) method (Wakimoto et al., 2018). The percent of sperm motility was determined by monitoring, and

recording progressive, non-progressive, and immotile sperm, and the results were the average of six determinations (Ghafarizadeh et al., 2021).

In-vivo studies

Animals

Eighteen female Sprague Dawley rats with an average weight of (150-200 gm) were housed at $25 \pm 2^\circ\text{C}$, with the 12:12 hours light-dark cycle. Rats were furnished with food and water ad libitum. Animal procedures were accepted by the Research Ethics Committee at the Faculty of Pharmacy, Cairo University, Egypt in compliance with the national regulatory standards set via the animal care committee at Cairo University (PI2816).

Histopathologic evaluation

Animals were categorized into three groups 6 rats per group. Group one behaved as a negative control, group two received PNL gel, and group three received INVs-loaded gel, respectively. The treatments were supplied per group once daily. Each group was subdivided into two groups of three animals in which the first group was subjected to a short-term application while the other received a long-term application of treatments. After mercy killing, vaginal tissues were dissected out and preserved in 10% formalin. After fixing, samples were handled through the passage in alcohol and xylene and then added into paraffin. Sections were divided, stained, then visualized by a light microscope (Abdellatif et al., 2020).

Results and discussion

Optimization of INVs using D-optimal design

Several trials were performed to evaluate the impact of the independent variables. Response surface methodology is based on the experimental design that aims to determine the best variables for a specific target of the response, utilizing minimal experiments (Aboelazayem et al., 2018). The prepared INVs were optimized via applying D-optimal design using Design-expert[®] which produced 20 experimental trials. The model selected was quadratic for EE%, and Q6h (%) while it was linear and 2FI for PS and ZP, respectively. For adequate- precision a ratio greater than 4 is preferred which was observed for all dependent variables as illustrated in Table 2. The predicted R^2 values were in correspondence with the adjusted R^2 in all dependent variables (Table 2).

Table 2. Output data of the D-optimal analysis of INVs formulations and predicted and observed values for the selected INV (INV14).

Responses	EE%	PS (nm)	ZP (mV)	Q6h (%)
Adequate precision	24.856	13.221	49.352	29.326
Adjusted R^2	0.9811	0.7982	0.9932	0.9841
Predicted R^2	0.8693	0.6327	0.9897	0.9070
Significant factors	(X_1 , X_2 and X_4)	(X_1 and X_4)	(X_4)	(X_2 , X_3 and X_4)
Predicted value of selected formula (INV14)	63.41	208.45	47.00	54.55
Observed value of selected formula (INV14)	65.01	243.75	49.80	53.16

Abbreviations: **EE%**; Entrapment Efficiency Percentage, **PS**; Particle Size, **ZP**; Zeta Potential, **Q6h**; Amount of drug released after 6 hours and **INVs**; Invasomes.

Table 3. Experimental runs, independent variables, and measured response of the D-optimal design of PNL loaded INVs.

F	Lipid Concentration (mg) (X_1)	Terpene Concentration (%) (X_2)	Terpene Type (X_3)	Chitosan Concentration (%) (X_4)	EE%	PS (nm)	PDI	ZP (mV)	Q6h (%)
F1	100	1	Cineole	0.6	54.03 ± 1.11	167.20 ± 2.26	0.212 ± 0.01	53.45 ± 1.06	53.16 ± 1.24
F2	100	0.5	Cineole	0.3	53.23 ± 1.69	228.10 ± 4.10	0.262 ± 0.09	38.60 ± 0.14	73.16 ± 2.23
F3	100	1.5	Cineole	0	78.02 ± 0.48	60.20 ± 0.84	0.137 ± 0.02	-22.45 ± 1.06	40.26 ± 0.62
F4	100	1	Cineole	0.6	55.85 ± 2.20	174.80 ± 2.82	0.231 ± 0.01	47.35 ± 1.34	52.63 ± 0.99
F5	100	0.5	Limonene	0	77.45 ± 0.62	169.70 ± 1.97	0.607 ± 0.03	-29.75 ± 0.49	45.17 ± 0.37
F6	100	1.5	Limonene	0	76.92 ± 0.86	263.20 ± 0.84	0.254 ± 0.04	-26.70 ± 0.42	43.59 ± 1.12
F7	100	1.5	Limonene	0.6	56.15 ± 1.47	170.75 ± 0.91	0.235 ± 0.01	41.45 ± 0.21	55.61 ± 0.74
F8	100	0.5	Limonene	0.6	52.05 ± 1.56	193.05 ± 4.03	0.206 ± 0.01	40.90 ± 1.27	62.98 ± 1.24
F9	150	0.5	Cineole	0	77.10 ± 0.41	153.55 ± 2.05	0.408 ± 0.10	-31.80 ± 0.28	43.24 ± 0.62
F10	150	1.5	Cineole	0.3	55.04 ± 2.26	177.50 ± 4.10	0.158 ± 0.01	36.00 ± 0.28	55.08 ± 0.98
F11	150	1	Limonene	0.3	57.44 ± 1.04	205.95 ± 1.76	0.141 ± 0.01	40.10 ± 0.56	55.96 ± 0.26
F12	150	1.5	Limonene	0	77.72 ± 0.84	183.10 ± 2.40	0.476 ± 0.02	-30.40 ± 0.70	44.12 ± 2.11
F13	200	1	Cineole	0.3	58.87 ± 2.24	193.20 ± 5.37	0.282 ± 0.06	36.80 ± 0.14	56.66 ± 2.23
F14	200	1.5	Cineole	0.6	65.01 ± 1.24	243.75 ± 8.13	0.203 ± 0.01	49.80 ± 0.42	53.16 ± 0.73
F15	200	0.5	Cineole	0.6	52.70 ± 1.15	199.75 ± 0.07	0.178 ± 0.06	42.70 ± 1.55	64.73 ± 1.74
F16	200	1.5	Cineole	0	85.17 ± 0.19	145.90 ± 0.42	0.480 ± 0.01	-27.10 ± 2.12	41.31 ± 0.62
F17	200	0.5	Limonene	0	77.29 ± 1.13	166.40 ± 0.98	0.502 ± 0.03	-33.25 ± 2.19	42.72 ± 0.62
F18	200	1.5	Limonene	0.6	61.82 ± 0.79	240.10 ± 4.24	0.156 ± 0.01	42.60 ± 1.13	70.17 ± 1.97
F19	200	1.5	Limonene	0.6	61.01 ± 0.65	240.10 ± 2.26	0.136 ± 0.01	44.90 ± 0.56	70.35 ± 2.73
F20	200	0.5	Limonene	0	77.59 ± 1.15	128.20 ± 4.80	0.493 ± 0.02	-33.20 ± 0.70	39.74 ± 1.37

Note: Data represented as mean ± SD ($n=3$). Abbreviations: **EE%**; Entrapment Efficiency Percentage, **PS**; Particle Size, **ZP**; Zeta Potential, **PDI**; Polydispersity Index, **Q6h**; Amount of drug released after 6 hours, **PNL**; Propranolol Hydrochloride and **INVs**; Invasomes.

Effect of formulation variables on the EE%

Entrapment of drugs via PC containing formulae provides the required delivery, good stability, protection, and permeability based on lipid constituents and aspects. EE% of PNL-loaded INVs ranged from 52.05 ± 1.56 to 85.17 ± 0.19%. The reasonable EE% of PNL inside INVs might be related to PC are ampholytic surfactants capable to augment the lipo-solubility of drugs, hence enhancing PNL EE% inside INVs (Abd-Elsalam et al., 2018). The effect of the independent variables, lipid concentration (X_1), terpenes concentration (X_2), terpenes type (X_3), and chitosan concentration (X_4) on the EE% of PNL in INVs illustrated in Table 3 and is graphically explained as three-dimensional (3-D) surface plots in Figure 1A-B. Lipid concentration (X_1) had a significant effect on EE% ($p=0.0053$). It was obvious that EE% increased by augmenting the concentration of lipid from 100 to 200 mg our results complied with Singh et al. (2005) who stated that EE% declined as lipid amount diminished which might be related to the reduction in the space available for PNL loading inside INVs. Moreover, PC may form shell-like structures embracing the drug this might enhance the stability of the formulated INVs and inhibit the leakage of the drug to the external media (Radwan et al., 2020). Terpenes concentration (X_2) had a significant impact on EE% ($p=0.0021$) as EE% increased by augmenting the concentration of terpenes from 0.5 to 1.5%. It was previously reported that the addition of terpenes to PC bilayers induces structural changes increasing lamellarity. As the lamellarity of INVs increases, it provides more area for drug entrapment inside INVs (Subongkot et al., 2012). Considering terpenes type (X_3) ($p=0.4589$) the factor was not significant in the response. On the other hand, chitosan concentration (X_4) ($p<0.0001$) had a significant negative impact on EE% of PNL. This could be related to the surface charge of both PNL and chitosan. PNL is positively charged bioactive (Abruzzo et al., 2012). Additionally, chitosan bears a positive

charge, it was expected that both PNL and chitosan might compete for PC, based on their affinity, EE% of PNL decreased. The previous findings were observed when leuprolide (positively charged) was incorporated inside chitosan-coated liposomes (Guo et al., 2003).

Effect of formulation variables on PS

PS of the prepared INVs ranged from 60.20 ± 0.84 to 263.20 ± 0.84 nm. The impact of lipid concentration (X_1), terpenes concentration (X_2), terpenes type (X_3) and chitosan concentration (X_4) on the PS of PNL loaded INVs is shown in Table 3 and is graphically explained as three-dimensional (3-D) surface plots in Figure 1C-D. Lipid concentration (X_1) affected PS of PNL loaded INVs significantly ($p=0.0028$). It was reported by augmenting PC concentration the PS increased this might be related to PC tending to form thick bilayers around INVs that subsequently increased the PS of the formed INVs (Shreya et al., 2016). For both terpenes concentration (X_2) and terpenes type (X_3), the factors were not significant in the response with p values of 0.6591 and 0.1914, respectively. Concerning chitosan concentration (X_4) ($p=0.0006$) by increasing chitosan concentration there was a linear increase in the PS of the formed INVs. It was reported that by augmenting the concentration of chitosan, the dispersion viscosity might increase and result in larger vesicle formation (Patil & Sawant, 2011).

Evaluation of PDI

A zero value of PDI manifests as a monodisperse nanosystem. Conversely, a PDI value of 1 resembles polydisperse vesicles (Al-Mahallawi et al., 2015). PDI values ranged from 0.136 ± 0.01 to 0.607 ± 0.03 (Table 3) this viewed that the fabricated INVs were polydisperse but within the acceptable range (Stetefeld et al., 2016).

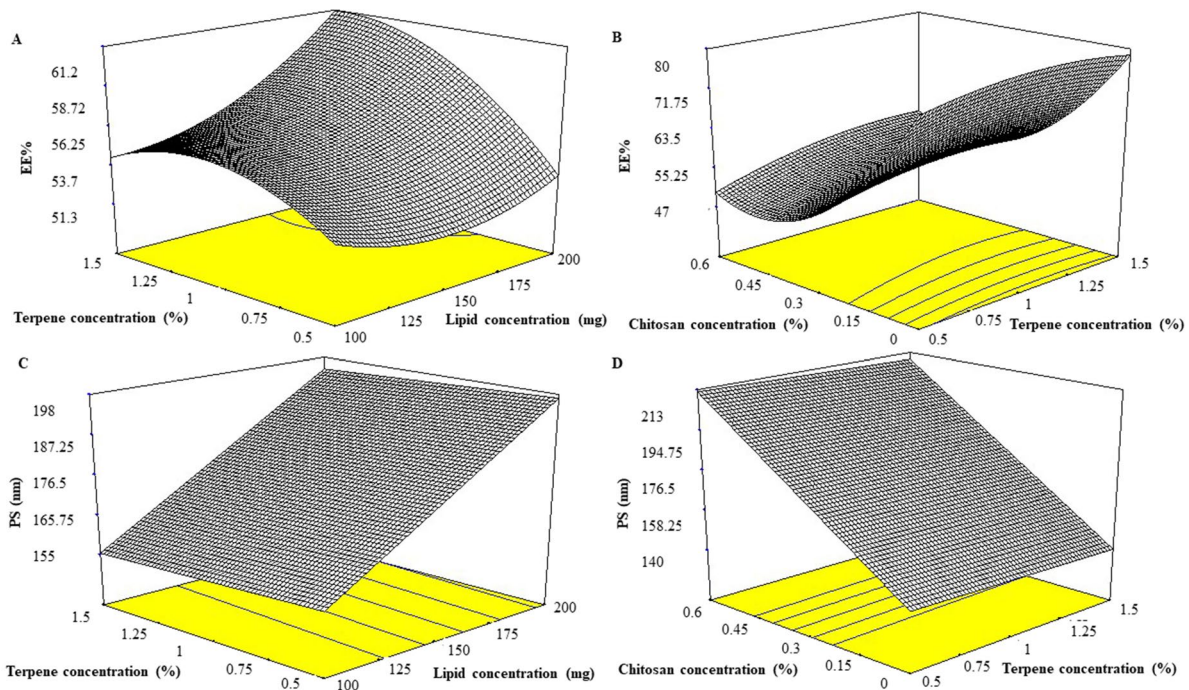


Figure 1. Effect of formulation variables on EE% of PNL-INV_s (a-b) and PS (c-d). **EE%:** Entrapment Efficiency Percentage, **PS:** Particle Size, **PNL:** Propranolol Hydrochloride, **INV_s:** Invasomes.

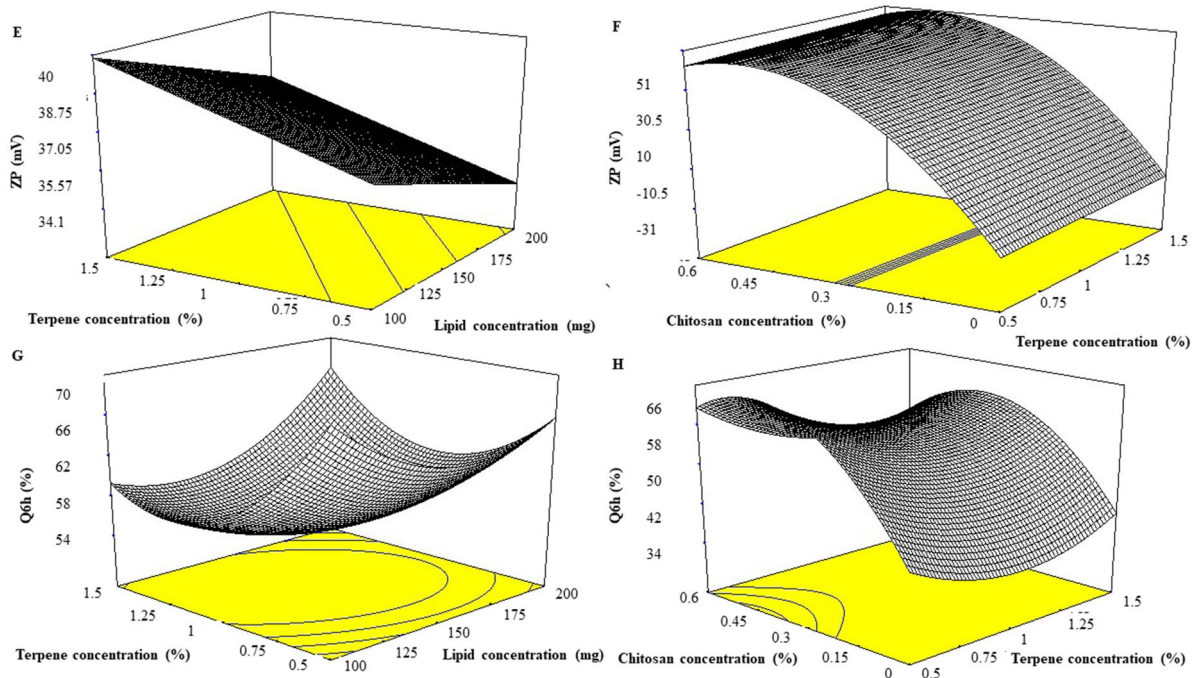


Figure 2. Effect of formulation variables on ZP of PNL-INV_s (e-f) and Q_{6h} (g-h). **ZP:** Zeta Potential, **Q_{6h}:** Amount of drug released after six hours, **PNL:** Propranolol Hydrochloride, **INV_s:** Invasomes.

Effect of formulation variables on ZP

Vesicle agglomeration might not happen for nano-system that have a ZP of $|30|\text{mV}$ or more due to electrostatic repulsion (Albash et al., 2019). ANOVA results demonstrated that chitosan concentration (X_4), displayed a significant impact on ZP ($P < 0.0001$) as depicted in Table 3 and graphically explained as response 3-D plots in Figure 2E-F. ZP values

ranged from -22.45 ± 1.06 to $+53.45 \pm 1.06$ mV. As observed, ZP values fluctuated between negative and positive values. The negative values of uncoated INV_s could be explained due to the presence of PC as it was reported that the head of PC is directed in a manner that the phosphatidyl group is outside but the choline group is inside when they are present in a low ionic strength medium that might impart

a negative surface charge (Makino et al., 1991). It is worth mentioning that Tawfik et al. (2020) prepared Agomelatine-loaded INVs by using limonene and cineole as terpenes and their ZP ranged from -33.3 ± 2.8 to -73 ± 3.6 supporting our finding for negative surface charge subsequently good colloidal stability of INVs. On the other side, the addition of chitosan resulted in positive ZP values INVs. The positive charge of chitosan is due to the protonation of the amine group available on the surface of chitosan that forms the coating surrounding the vesicles (Shukla et al., 2020). Further, positive ZP increased as the chitosan concentration increased our findings were following Bashiri et al., (2020). as they found that positive ZP values increased by increasing chitosan concentration from 0.1 to 0.15% upon preparing nanostructured lipid carriers coated with chitosan due to an increment in the number of amine groups that reside on the surface of particles.

Effect of formulation variables on Q6h

The quantity of PNL emitted after 6 hrs ranged from 39.74 ± 1.37 to $73.16 \pm 2.23\%$ (Table 3). The effect of lipid concentration (X_1), terpenes concentration (X_2), terpenes type (X_3), and chitosan concentration (X_4) is graphically explained in 3-D surface plots in Figure 2G-H. Lipid concentration (X_1) displayed no significant influence on Q6h ($p=0.0519$). Terpenes concentration (X_2) ($p=0.0008$) showed that by augmenting terpenes concentration from 0.5 to 1.5% the amount of PNL released decreased. For terpenes type (X_3) ($p=0.0042$), limonene resulted in a higher amount of PNL released compared to cineole. The higher release profiles of INVs containing limonene might be ascribed to its low boiling point. Limonene has a lower boiling point (176°C), followed by cineole (177°C). The lower the boiling points of the terpene, the weaker the coherence of the molecules or self-association (El-Nabarawi et al., 2018). Regarding chitosan concentration (X_4) ($p<0.0001$), as observed increasing chitosan concentration resulted in a high amount of PNL released from INVs.

The previous phenomenon was observed previously upon preparation of Indomethacin-loaded chitosan microspheres. As the authors reported upon increasing chitosan the release of Indomethacin was enhanced due to chitosan swelling that resulted in a lower crosslinking degree of chitosan and subsequently aided in high PNL release (Oriente et al, 1996). In addition, chitosan possesses a hydrophilic character that might aid in enhancing PNL releases upon increasing its concentration to INVs (Meng et al., 2011).

Determination of the selected INV

INVs were optimized by the constraints in Table 1 with the relegation of the non-significant response (PDI). The best values of the variables were gained by numerical optimization relied on the desirability function employing the Design-Expert® software. A suggested optimum formula (INV14) containing 200 mg PC, 1.5% cineole, and chitosan at 0.6%. The predicted and observed values of INVs (INV14) are depicted in Table 2. The high resemblance between the observed and predicted responses of INV might validate the design to anticipate the responses.

Transmission electron microscopy (TEM)

Figure 3 proved that the optimum INV was a spherical invasome with narrow size distribution and no INVs with irregular morphology were shown. INVs PS determined by Zetasizer was in an accordance with that visualized employing TEM. This also represented that the coating process did not produce agglomeration or aggregation of INVs (Dung et al., 2009).

Differential scanning calorimetry (DSC)

PNL, PC, and optimum INV DSC thermograms are illustrated in Figure 4. PNL thermogram viewed an endothermic peak at 166.91°C , proving the crystalline nature of PNL (Chaturvedi et al., 2010). In addition, PC showed two

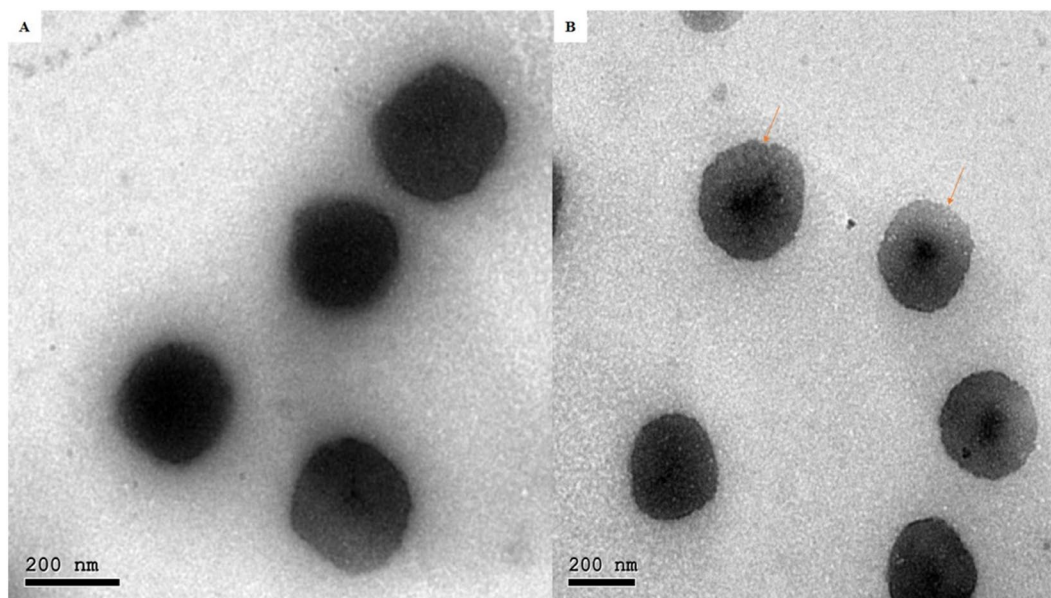


Figure 3. Transmission electron micrographs of INV14. INVs: Invasomes.

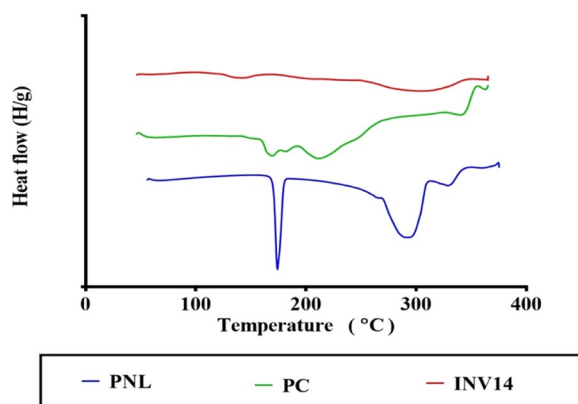


Figure 4. DSC thermograms of PNL, PC and the optimum INV. **PC:** Phospholipid, **PNL:** Propranolol Hydrochloride, **INV:** Invasome.

endothermic peaks. The first peak (159.9°C) was described as mild, showing hot movements of polarity parts of PC. The second peak manifested at 234.6°C, that might be attributed to the transition from gel to liquid crystal state and the melting of the carbon-hydrogen chain in PC, and changes in isomers or the crystal (Ruan et al., 2010). However, the lyophilized optimum INV did not have a sharp peak. This might reflect that PNL, when formulated as INV was entrapped inside the INVs in an amorphous state (Elsenosy et al., 2020).

Stability study

The visual checking of the stocked INVs did not view any INVs sedimentation through the storage time. Moreover, PS, PDI, ZP, EE%, and Q6h measurements of the stocked optimum INV were 220.00 ± 12.00 nm, 0.347 ± 0.034 , 47.87 ± 1.23 mV, $64.00 \pm 1.00\%$, and $56.00 \pm 2.00\%$ that illustrated insignificant difference from the freshly prepared INVs ($p > 0.05$). These consequences might reveal the excellent stability of INVs which might be correlated to the steric effect generated via chitosan coating (Goyal et al., 2016).

Evaluation of mucoadhesion aspects of INVs

Mucin surface charge was -9.17 ± 0.02 mV shifted by combing with INVs (INV14). A positive value was gained from the complexes created between mucin and INV14, whereas the negative ZP charge of the mucin might be neutralized by the positive charge of the INV14 adhered to mucin. It was previously found that if the component has a mucoadhesive character it might change the surface aspects of the mucin (Albash et al., 2021b). The change from negative mucin ZP to a positive mucin ZP value of 17.86 ± 0.30 mV demonstrated the mucoadhesive property of INV14.

Gel characterization

Visual inspection

All fabricated gels revealed homogenous consistency with no precipitation or aggregation.

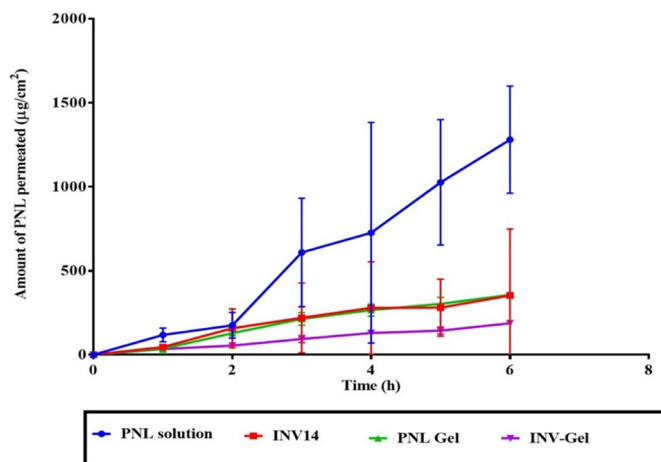


Figure 5. Ex-vivo permeation profile of PNL from INV-gel, PNL-gel, INV14 compared to PNL solution. **PNL:** Propranolol Hydrochloride, **INV:** Invasome.

Determination of pH

The observed pH value for all formulae ranged 5.2 ± 0.01 to 5.4 ± 0.20 , which is considered suitable for vaginal application.

Viscosity

PNL-gel, and INVs-gel formulations showed high viscosity values of 2063.50 ± 211.50 cp, and 3188.50 ± 39.50 cp, respectively. In general, mucoadhesive gel preparations are characterized by high viscosity. Previous studies reported that for obtaining a gel with a desirable viscosity for vaginal route it must be ranged from 200 to 5000cp supporting that both gel formulae with good viscosity (Mourtas et al., 2010). In addition, we speculate the higher viscosity values of INVs-gel compared to PNL-gel due to the presence of both phospholipids, and terpenes our results were in accordance with the previously reported literature (Ensign et al., 2014).

Ex-vivo permeation

The efficacy of drug transport to mucus membranes is based on the retention and distribution of drugs on the mucosal membranes and their infiltration via the mucus membranes (Abdellatif et al., 2020). Cervical mucus acts as an excellent site for the retention and distribution of spermicides and, at the same time, as the main barrier to sperm movement (Han et al., 2017). From Figure 5, it is distinct that the quantity of PNL permeated from optimum INV loaded gel was significantly ($p < 0.5$) the lowest related to PNL-gel and INV14 and PNL solution. The significant lowest permeability from INVs loaded gel could be explained in the light of two reasons. First, it is well known that cationic drugs (as PNL) form complexes with either anionic surfactants or polymers and that the complexes might have an impact on the emission of the bio-active through the matrix. The complex formation is possibly the cause for the observed reduction in the total permeation of the drug from the Carbopol matrix (Perez-Marcos et al., 1994). Second, Carbopol gel is known to produce a controlled release system due to the increment in viscosity of the fabricated system (Jaiswal et al., 2016). Further, the high-bioadhesive potential of the formulated gel could be

ascribed to the aspects of the anionic polymer; carbopol which has several carboxylic moieties that might form H-bonding with the mucus membranes and hence, resulting in excellent bioadhesion and sustained drug effect (Mahmoud et al., 2020). The previous reasons justified the significant sustained effect from INVs loaded gel compared to PNL-gel, INVs, and PNL solution. It is worth noting, that the amphoteric property of PNL justified its highest permeation profile compared to other formulae.

In-vitro spermicidal activity assessment

Sperm motility assay

Motility is an essential aspect of sperm and determination of this parameter is critical for sperm evaluation. CASA is a technique devised to take the image from a microscope through visualization of sperm on the video to provide quantitative information on the kinematic behavior of sperm, and subsequently permit objective assessment of sperm kinetics (Wakimoto et al., 2018). In this research, it was shown that the motility of sperm was significantly terminated in samples treated with optimum INV-loaded gel after 10 sec of incubation (Table 4). In addition, the sperm cell integrity (without flagella) was damaged as presented in Figure 6. This could be justified as follows; firstly, PNL decreased sperm motion by its membrane stabilizing activity in addition to its ability to block the beta receptors

present in sperm cells as previously mentioned. Secondly, the existence of cineole might induce oxidative damage to spermatozoa by reactive oxygen species (Dörsam et al., 2015). The findings of this research are consistent with the previous results indicating that the essential oils as cineole (at concentrations of 1% and 0.1%) in semen had immediate spermicidal potential that was evaluated by CASA and demonstrated a significant increase of damaged cells (Cavalleri et al., 2018).

In-vivo studies

Histological assessment of samples from the negative control group as illustrated in (Figure 7) displayed no histopathological alteration with the ordinary histological composition of the stratified mucosal epithelium with the underlying lamina propria. For group II treated with PNL-gel mild edema was observed in the lamina propria and sub-mucosal layers after short-term application. On the other hand, there was no histopathological alternation after long-term application. Further, in group III treated with INVs-gel, there was no histopathological alteration in the vaginal tissues with the absence of inflammatory cells in both short and long-term application groups. The previous findings confirmed the safety of INVs gel as a topically administered spermicide. In addition, this investigation validated the possibility of utilization of INVs gel on daily basis without any inflammation.

Table 4. Comparison of sperm motility before and after addition of optimum INV loaded gel.

Sample no.	Volume (ml)	Sperm count per ejaculate after liquification (10 ⁶)	Motility (%) after 10 sec treatments with INVs loaded gel
1	3.2	81	0
2	4.5	92	0
3	5.1	86	0
4	2.4	78	0
5	3.6	90	0
6	2.2	84	0

Abbreviation: **PNL**; Propranolol Hydrochloride and **INVs**; Invasomes.

Conclusion

Propranolol hydrochloride (PNL) loaded invasomes (INVs) were created by a thin film hydration approach by D-optimal design, after a screening experiment that illustrated the importance of different variables on INVs aspects. The optimum INV (INV14) contained 200 mg phospholipid, 1.5% cineole and 0.6% chitosan. The optimum INV was stable upon refrigeration for 3 months. Transmission electron microscopy

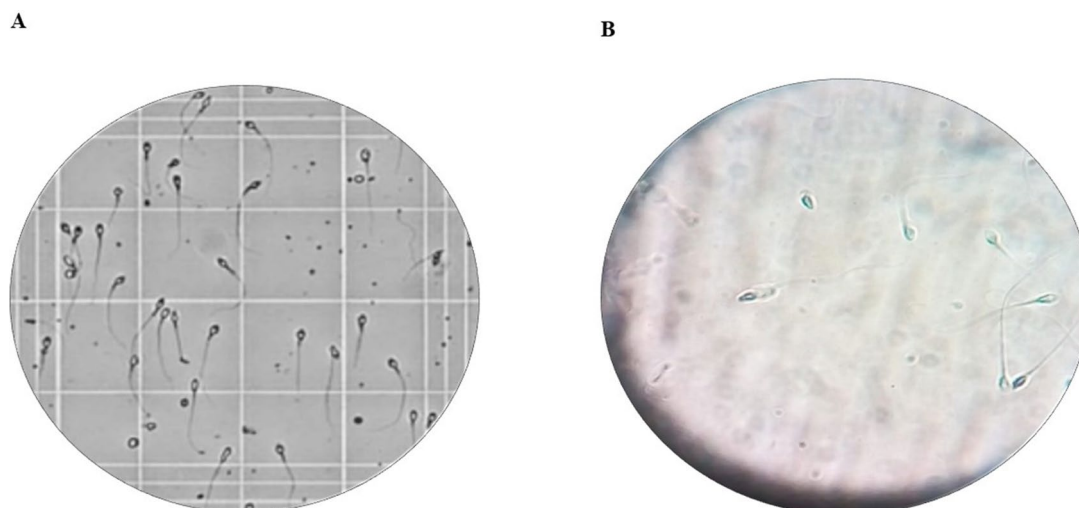


Figure 6. Light microscope photomicrographs showing sperm cells A) before addition of PNL-INV gel B) after addition of PNL-INV gel. **PNL**: Propranolol Hydrochloride, **INV**: Invasome.

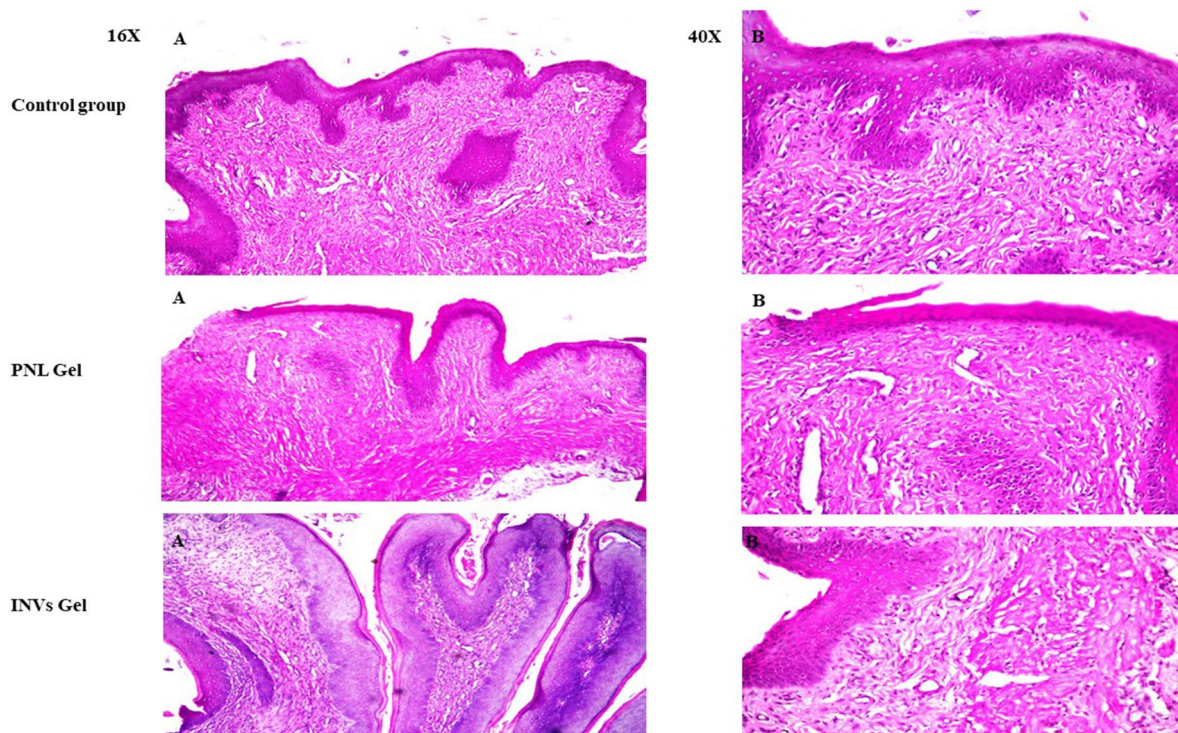


Figure 7. Light microscope photomicrographs showing histopathological sections (hematoxylin and eosin stained) of rat vagina normal control (group I), rat vagina treated with PNL gel (group II) and rat vagina treated with INVs gel (group III) with magnification power of 16X to illustrate all vagina layers (Left side) and magnification power of 40X (Right side). **PNL:** Propranolol Hydrochloride, **INV:** Invasome.

displayed spherical INVs without aggregation. *Ex-vivo* studies concluded sustained effect, good vaginal deposition, and accumulation of PNL-loaded INV gel, compared to PNL gel, INVs, and PNL solution. Further, sperm motility assay proved the powerful inhibitory effect of INVs-gel. Histopathological studies demonstrated the safety of PNL-loaded INV gel for vaginal application to rats. Hence, it could be concluded that PNL-loaded INV gel could be considered a safe and effective formula that can be used to prolong the deposition of PNL within vaginal layers, to control birth, with minimized systemic side effects.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

The author(s) reported there is no funding associated with the work featured in this article.

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