Development of a Vaginal Film for Delivering a Sperm-Deactivating Non-Hormonal Contraceptive Candidate, Lupeol

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To date, there are only limited nonhormonal contraceptive options available. Therefore, development of nonhormonal contraceptive products represents a gap in female reproductive healthcare needs. Lupeol, a triterpenoid found in a number of fruits and vegetables, is currently being evaluated by YourChoice Therapeutics (CA, USA) as a potential novel nonhormonal contraceptive agent. Sperm hyperactivation is required for fertilization to occur as it facilitates the sperm cells' ability to migrate in the high viscosity fluids within the female genital tract and allows for penetration of the cumulus cells surrounding the egg. Abhydrolase domain-containing protein 2 (ABHD2) is a serine hydrolase enzyme expressed in human spermatozoa which is essential for sperm hyperactivation. Lupeol modulates ABHD2 i.e. it blocks progesterone from binding to ABHD2, which is essential for sperm hyperactivation to occur. Therefore, it can deactivate sperm mobility and avoid egg fertilization, thereby preventing pregnancy. To facilitate its use as a contraceptive product, lupeol must be formulated into a vaginal dosage form which women can utilize in the context of sexual intercourse. Vaginal films have been identified as an acceptable vaginal dosing option. Given its extreme hydrophobicity, formulation of lupeol is challenging. This thesis will describe the pre-formulation studies of lupeol, including the development of a critical analytical method and cytotoxicity study of lupeol in VK2 cell line, and the formulation

development based on a polymeric thin film platform. The prototype film product developed was designed to rapidly release lupeol in the vaginal compartment using a hydroxyethyl cellulose based polymeric thin film. The physicochemical properties of the lupeol immediate-release vaginal film were characterized and were followed by a short-term stability to evaluate drug content and physical properties, such as the water content, disintegration time, puncture strength and contact angle. Additionally, optimization of the formulation was proposed to improve the physical properties of the film prototype. Development of the novel nonhormonal contraceptive drug candidate, lupeol, into a coitally-dependent product is essential for its advancement to future evaluation in women.

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Preface

The work described in this thesis is a collaborative work under grant R43HD097944, funded by the United States NICHD. The purpose of this thesis is to introduce and demonstrate the development of an immediate-release polymeric thin film containing lupeol, a novel highly potent contraceptive agent but possessing unfavorable hydrophobic properties that present as a challenge during pharmaceutical product development. The approaches and findings in this work should be of interest to scientists who are investigating non hormonal contraceptive product development.

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1.0 Introduction

1.1 Contraceptives

1.1.1 The importance of birth control

Nearly half of the pregnancies were unintended worldwide (44% between 2010 and 2014) [1]. Moreover, unintended pregnancies often result in abortions, which contributes to serious and long-term negative health effects in women, especially in developing countries where people are in poverty, malnourished, and lack sanitation [2]. The long-term negative consequences include infertility and maternal death. In 2017, 862,320 abortions occurred with an abortion rate of 13.5 abortions per 1,000 women aged from 15 to 44 years old [3]. The unintended pregnancies and resulting abortions lead to high social, personal, and economic burden. The annual U.S. government's expense in births, abortions, and miscarriages resulting from unintended pregnancies has been estimated to be $5.5 billion in 2018 [4]. Birth control is essential for cost saving in public funding and women's reproductive health. Different forms of birth control are available on the market and can be broadly divided into three categories, hormonal contraceptives, nonhormonal contraceptives, and sterilization. Of these, condoms, vaginal caps, copper IUD etc. are nonhormonal contraceptives, and birth control pills, implants, patches etc. are hormonal contranceptives. The effectiveness of contraceptives is affected by the efficacy of the contraceptive drug, possible contraindications, side effects and level of user compliance. Therefore, it is essential to evaluate the contraceptive effectiveness, possible contraindications, and side effects as well as

formulate the contraceptive drug into a dosage form that fits the lifestyle and meets the needs of women in reproductive age.

1.1.2 Hormonal Contraceptives

1.1.2.1 Menstrual cycle and mechanism of action for hormonal contraceptives

Before introducing the hormonal contraceptives agents, it is important to understand how hormones regulate menstrual cycle and how inhibition of ovulation can provide contraception. The four phases of menstrual cycles are menstruation, the follicular phase, ovulation, and the luteal phase. Menstruation (period) is the elimination of the thickened uterine lining from the body through the vagina. The eliminated menstrual fluid contains endometrial cells, mucus and blood. At this phase, the levels of estrogen and progesterone are low. In the menstrual cycle, menstruation is the end of the cycle and the follicular phase is the first stage of the cycle as the beginning of egg formation occurs. Follicular phase is induced by the pituitary gland, located at hypothalamus, which releases follicle stimulating hormone (FSH). During this phase, FSH stimulates 10 to 20 follicles to develop and only one dominant follicle will mature into an egg cell. The maturation of egg happens as a high-level threshold of FSH is reached. The mature egg moves to the surface of the ovary and then, the menstrual cycle proceeds to next phase, ovulation. Ovulation is the release of a mature egg from the ovary. The rising level of estrogen resulted from the follicular phase triggers the production of gonadotrophin-releasing hormone (GnRH) by the hypothalamus. GnRH prompts the production of luteinizing hormone (LH) and FSH from pituitary gland which results in LH surge. The high levels of LH then initiates ovulation and releases the egg from its follicle. The egg is then funneled into fallopian tube towards the uterus. The remnant follicle transforms into the corpus luteum which releases progesterone and small amount of estrogen. This hormone

combination maintains the thickened uterus lining and prepares uterus for the implantation of a fertilized egg. If fertilization does not occur, the corpus luteum withers and progesterone level decreases. The reduction in progesterone levels results in the shedding of uterus lining which is known as menstruation, and the cycle repeats. Fertilization occurs when a sperm cell successfully penetrates through the protective vestment surrounding the mature egg. The lifespan of sperms is longer than egg (1 day) as they remain motile in cervical mucus for at least seven days *in vivo* and reserve the ability to fertilize ova *in vitro* after five days at room temperature [5] [6]. Therefore, controlling ovulation is a vital step for contraception.

FSH and LH are the two sex hormones responsible for ovulation and preparing the uterus for pregnancy. During the menstrual cycle, the LH surge correlates with the day of ovulation [7]. Without the LH surge, the follicular development and ovulation are inhibited. Moreover, the increased amount of estrogen signals the hypothalamus via negative feedback leading to decreased secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH). The decreased levels of FSH and LH further result in the inhibition of follicular development, ovulation, and corpus luteum development. Furthermore, the elevated level of progesterone not only inhibits the ovulation but also thickens the cervical mucus and reduces the endometrial proliferation. As a result, the increased level of estrogen and progesterone inhibits the fertilization. These biological effects demonstrate the contraception effects when women are administered with hormonal contraceptives containing progestin and estrogen (i.e. ethinyl-estradiol).

The combined use of both estrogen (i.e. ethinyl-estradiol) and progestin is widely implemented in birth control product due to the antigonadotropic effect and the inhibitory effect in ovulation. Estrogen component suppresses the FSH production by pituitary gland via the negative feedback at the hypothalamus [8]. It also suppresses the dominant follicle development

and increases the amount of sex hormone-binding globulin (SHBG) [9]. SHBG inhibits the function of hormones by binding and preventing them from entering the cell membrane and interacting with the receptors located in cells [10]. Moreover, through same negative feedback at hypothalamus similar to estrogen, progestin component decreases the GnRH secretion, suppresses the LH production, and inhibits LH surge [11]. It also thickens the cervical mucus and creates an unfavorable uterine environment for sperm. On the other hand, progestin-only contraceptive is also developed for women who cannot tolerate estrogen. The majority of users who select progestin-only contraceptives over combined contraceptives are women who are breastfeeding or with contraindications to estrogen use, including estrogen-positive breast cancer and potential to develop deep vein thrombosis [12].

1.1.2.2 Types of hormonal contraceptive agents

Hormonal contraceptives contain either estrogen (i.e. ethinyl estradiol) or progestin as active agents. Progestins are synthetic progestogens. They can interact with progesterone and other receptor families, including glucocorticoid and androgen receptors [13]. The interactions between individual progestins and individual receptors can be either weaker or stronger than that of progesterone depending on whether the progestin activates or blocks the receptor. These interactions determine contraception effectiveness and may contribute to many side effects associated with synthetic progestogens.

The biological effects of progestins can be classified as progestational, androgenic and estrogenic effects. Briefly, progestational effects prevent ovulation, androgenic effects are often considered as unwanted side effects such as acne and hirsutism in women. Estrogenic effects counteract the androgenic effects and mainly rely on the amount of ethinyl estradiol in the contraceptives [14]. The combinational birth control pills lessen the androgenic effects due to the

presence of ethinyl estradiol. As described previously, ethinyl estradiol suppresses the androgen production from ovaries and increases the amount of SHBG [9]. SHBG inhibits the function of hormones by inhibiting them from interacting with the receptors located in cells [10]. Therefore, a low level of SHBG is associated with hyperandrogenism and endometrial cancer because of the increased exposure to androgens and estrogens.

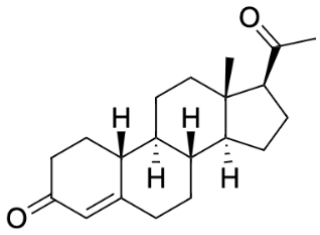
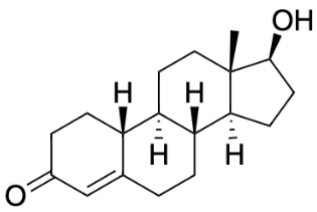
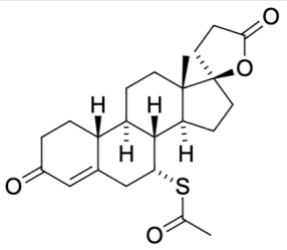
In general, progestins can be classified into four different generations (Table 1) [15]. A Cochrane 2004 review revealed that 2nd and 3rd generation progestins performed better than 1st generation progestins [16]. Few discontinuations and better cycle control were observed in participants using 2nd and 3rd generations. As a result, women preferred using contraceptives containing 2nd and 3rd generation progestins over those with 1st generation progestins such as ethynodiol diacetate, northindrone acetate (NETA) and northindrone (NET).

Table 1. Progestin generations [15]

Generation	Kind of Progestin
1st	Northindrone (NET)
	Northindrone acetate (NETA)
	Ethynodiol Diacetate
2nd	Levonogestrel (LNG)
	Norgestrel
3rd	Desogestrel
	Norgestimate
4th	Drospirenone

The more scientific approach for classifications is based on the hormones they were created from such as testosterone, progesterone and spironolactone (Table 2) [17, 18]. Based on the hormones they are derived from, progestins can be classified into three families, 19-nortestosterone, C-21 progesterone, and 17α-spironolactone.

Table 2. Progestin families [17]

Families	Progestins derived from C-21 progesterone	Progestins derived from 19-nortestosterone	Progestins derived from α-spironolactone
Chemical structure			
Progestins	Medroxyprogesterone acetate (MPA)	Northindrone (NET)	Drospirenone
	Chlormadinone acetate (CMA)	Northindrone acetate (NETA)	
	Cyproterone acetate (CPA)	Levonorgestrel (LNG)	
		Ethynodiol diacetate	
		Desogestrel	
		Norgestrel	

Progestins generated from 19-nortestosterone include NET, NETA, levonorgestrel (LNG), ethynodiol diacetate, desogestrel, and norgestrel [18]. The structures of NET and NETA are both related to testosterone: NET has an ethinyl group at carbon-17 whereas NETA lacks a methyl group at carbon-10 [19]. NET and NETA have progestational and estrogenic activities. They also have lesser androgenic effects compared with LNG and norgestrel. The physiological effects of

estrogen include decreasing LDL-C (low-density lipoprotein cholesterol) and increasing HDL-C (high-density lipoprotein cholesterol) levels [20]. The risk of cardiovascular disease decreases as the level of HDL-C increases in women [21]. NET has been reported to raise the level of HDL and reduce the level of LDL cholesterol in plasma and thus, improves lipid profiles and reduces the risk of cardiovascular disease [22]. Studies have reported that NETA alone did not exhibit effects on lipoproteins but reduces LDL-C level when administrated with estradiol [23]. NETA is available in both combination with estrogen and alone in progestogen-only pills for birth control. Moreover, they both have other medical uses. NETA is a component of hormonal therapy for the treatment of menopausal symptoms [24]. NET alleviates the painful symptoms caused by endometriosis due to induced endometrial proliferation during secretory phase which can relieve the pain.

LNG is a potent second-generation progestin that creates an unfavorable uterine environment for sperm by thickening the cervical mucus to prevent the sperm from reaching the fallopian tubes [25]. FDA has approved LNG as emergency contraception (ECP) although studies have shown that other alternative regimen consisting of ethinyl estradiol and NET may also be used for emergency contraception [26, 27]. LNG is the most widely used contraceptive and has high progestational and weak androgenic effects. Moreover, LNG has multiple potential medical uses other than contraception, including relieving the pain symptoms related to endometriosis and the bleeding symptoms associated with adenomyosis [28].

Progestins that are derived from C-21 progesterone, also known as pregnanes and 17α-hydroxyprogesterone, include medroxyprogesterone acetate (MPA), chlormadinone acetate (CMA) and cyproterone acetate (CPA) [18]. These progestins are considered as antiandrogen medication. Briefly, they block androgens from activating the androgen receptor via attachment to

the binding site. Thus, they have not only been used for contraception but also used as a treatment for prostate cancer in men, hirsutism and hormone replacement therapy in women [29-31]. During hormone replacement therapy, MPA is protective to postmenopausal women as MPA reduces estrogen-induced endometrial proliferation [32].

The only progestin derivative from 17α-spironolactone is Drospirenone [18]. Drospirenone is an aldosterone receptor antagonist (PARA) as it binds the mineralocorticoid or glucocorticoid receptors. Due to the binding with the aldosterone receptor, Drospirenone can prevent excessive loss of sodium and regulate blood pressure by modulating the renin-angiotensin-aldosterone system (RAAS) [33]. When used as a hormone replacement therapy, RAAS stimulation due to estrogen is absent and thus, results in increased blood pressure [34]. Therefore, Drospirenone can induce sodium excretion, reduce water retention, and lower blood pressure. At the same time, Drospirenone may cause bloating and higher potassium level, thus women with chronic kidney, liver, or adrenal disease should be more cautious [33]. Moreover, studies have reported that Drospirenone is associated with an increased risk of blood clots. The risk of venous thromboembolism is higher with the use of Drospirenone in comparison with the use of LNG [35].

1.1.2.3 Dosage forms of hormonal contraceptives

Oral contraceptive pills are the most commonly used product, but other drug dosage forms are also available, including injections, implants, hormonal vaginal rings, and patches. Contraceptive effectiveness is not solely dependent on the mechanism of action of the product but also relies on the correct application in users. Methods such as hormonal contraceptive pills that require frequent administration have a higher failure rate with typical use (7%) compared with perfect use (0.3%) [36, 37]. Due to the requirement of frequent user compliance, hormonal contraceptive pills are less effective than contraceptive implants and intrauterine contraceptive

device (IUD), which require a surgical or nonsurgical procedure involving a healthcare provider. The hormonal contraceptives that have less than 1% failure rate with typical use are the progestin-containing implants and hormonal IUDs [37]. The long-acting birth control implant is surgically inserted into the subcutaneous layer of the arm, where it continuously releases progestin over a period of 5 years. LNG IUD is a contraceptive that incorporates hormonal component, LNG in the IUD delivery system. It shows reduced menstrual bleeding compared to copper IUDs. Because LNG is released locally by the IUDs, the systemic level of progestin is lower than the low dosage of progestogen-only oral contraceptives. The implant and the LNG IUD are considered as long-acting reversible contraceptives (LARCs) which provide protection for an extended period without multiple dosing. LARC is more convenient and cost-effective compared to contraceptive pills due to their contraceptive effectiveness, high continuation rate, and additional medical benefits such as treating endometriosis and endometrial hyperplasia ; however, only 14% of women using contraceptives relied on LARC [38]. This could be due to women's misperceptions and misinformation about these methods, higher initial cost as compared to other contraceptives and the requirements for specific clinical professionals and facilities [39]. An additional dosage form that is administered to the female reproductive tract is a vaginal ring. Vaginal ring is a small, flexible and ring-shape plastic that can be inserted into the vagina to provide contraception by user themselves. It releases hormonal agents for up to 3 weeks and prevents ovulation via same mechanisms as COCs. The failure rate of vaginal ring is 7 % with typical use [37].

1.1.2.4 Side effects of hormonal contraceptives

As the popularity of hormonal contraception increased, the awareness of the side effects related to hormonal contraceptives also raised. Since they inhibit ovulation via negative feedback at the hypothalamus, hormonal contraceptives influence the responses that are mediated by the

self-produced progesterone and estrogen. Thus, they affect the human hormonal system, having a potential adverse effect on women's psychological health and increasing the risk of cardiovascular disease in women. Hormonal contraceptives containing estrogen are contraindicated for women who cannot tolerate estrogen. This includes women with estrogen-positive breast cancer [40] [41] and women with a history of cardiac issues such as thromboembolic disorders, hyperlipidemia, and coronary artery disease [42] [43]. Combined oral contraceptive pills (COCs) containing estrogen and progestin were reported to influence the left insular lobe in the cortex region, which is linked to emotional responses including anxiety and aggression [44]. The amygdala habituation slows and thus, COCs might reduce the reactivity of insula toward external stimuli and lead to mood deterioration. Moreover, since estrogen is a lipophilic hormone that can cross through cell membranes and bind to nuclear receptors, it influences the coagulation system by inducing the gene transcription and influence intracellular signaling pathways [45]. Although the decreased level of clotting factor V reduces the risk of thrombosis, the elevated levels of clotting factors II, VII, X, XII, XIII, fibrinogen, and thrombin activatable fibrinolysis inhibitor (TAFI) favor the thrombus formation in blood vessels and the prevention of fibrinolysis to breakdown blood clots [46]. In studies evaluating the risk of venous thromboembolism in women taking hormonal contraceptives, a 4- to 7-fold increased risk dependent on the type of hormonal contraceptives used was observed compared with the non-users [47] [48]. Women using hormonal IUDs were reported to have side effects including irregular menstrual bleeding and unpredictable menstrual cycles. For progestin-only contraceptives, women are more prone to develop ectopic pregnancy and experience bleeding between period [49-51]. Therefore, there is a rising trend among researchers to develop nonhormonal contraceptives to provide women with alternative contraceptives in order to cover all types of contraceptive needs [52]. Despite these side effects observed in some women,

hormonal contraceptives make up the major portion of currently used birth control strategies. It is worth noting that most of the side effects described previously are not common, as only 12.4% of women using hormonal contraceptive experienced nervous system disorders and 6.1% of women experienced metrorrhagia (abnormal bleeding from the uterus) [53].

1.1.3 Nonhormonal Contraceptives

Nonhormonal contraceptives are being developed for both men and women. The mechanism of actions of these nonhormonal contraceptives are distinct compared to hormonal contraceptives. These include acting as a physical barrier between the sperms and the egg cell, inhibiting the fertilization process either by suppressing the sperm motility or by acting as a spermicidal, and suppressing the spermatogenesis by decreasing gonadotropins (LH and FSH) levels [54].

1.1.3.1 Conventional barrier methods

Barrier methods include the diaphragm and male and female condom. The diaphragm blocks sperm cells by covering the cervix in the vagina. Condoms are the most common type of barrier methods and are the only method that can prevent both pregnancy and HIV as well as other STDs (sexually transmitted diseases) [55]. Latex, polyurethane, and polyisoprene are the three major types of condom materials. Latex composes the majority of the condoms in the market because of its stretch properties. However, the antigenic proteins presented in latex will trigger Type I allergic reactions in some users who are allergic to latex [56]. Also, latex condoms should not be used with oil-based lubricants since the mineral oil will deteriorate the integrity of the condom and diminish the ability of condoms to prevent pregnancy and STDs [57]. Therefore, other

materials such as polyurethane and polyisoprene were developed as alternative options for people who are allergic to latex. Polyurethane is a non-latex condom that is made from a plastic material. It is thinner than the latex and can transfer body heat better than the latex condom: therefore, polyurethane is not as noticeable as latex condom. As for polyisoprene, it is a second non-latex option that has a similar structure to latex but does not contain the antigenic proteins that will cause allergic reactions in humans. The main disadvantage of barrier methods is their high failure rate (13% accidental pregnancies) under typical use, which is due to the high dependence on user compliance and product knowledge [37].

1.1.3.2 Spermicides

Spermicides interrupt fertility by killing sperm cells. Currently available spermicides are available over the counter and are reversible method of contraception. Spermicides are marketed in various product forms, such as foams, gels, creams, suppositories, and films. Currently, Nonoxynol-9 (N-9) is the active ingredient of all the currently available spermicide products on the market [58]. N-9 is a nonionic surfactant and is also widely used in various cleaning and cosmetic products. It prevents pregnancy by targeting the acrosomal membranes of the sperm, which leads to immobilized sperm cells. One of the major marketed N-9 products is a vaginal polymeric film, VCF®. VCF® is an on-demand and unnoticeable contraceptive as it dissolves rapidly upon contacting with fluids after inserting into the vagina. The users do not need to worry about the removal of films as they dissolve and get washed away with the cervicovaginal fluid. Also, VCF® is acceptable among women because of its small size, portability and can be used privately [59].

However, the failure rate of spermicides is 21% with typical use which is higher when compared to other contraceptives [37]. Moreover, in a study by Van Damme et al, N-9 was found

to disrupt the epithelial integrity in the vaginal area. Studies showed that N-9 could be cytotoxic and increases the risk of HIV and other STDs as the repeated use of N-9 is associated with vaginal ulceration and the disturbance of vaginal microbial flora [60]. This finding is consistent with the study that has shown an inverse relationship between the lactobacilli and vaginal microbial flora against HIV-1 infection, gonorrhea and trichomoniasis [61]. Moreover, the increased risk of infection is reported to be associated with the frequency of use [62]. The drawback of N-9 in increasing the risk of STDs reinforces the necessity to develop safer alternative spermicides to protect women from STDs and prevent pregnancy [63].

1.1.3.3 Copper intrauterine device

The copper IUD is a functional spermicide that disrupts sperm mobility by the copper ion. An *in vitro* study showed that the concentration of copper ions, found within the uterus with CuT-380A IUD, inhibited the motility and viability of sperm cells by blocking the activation of acrosomal enzymes; and thus, sperms failed to penetrate the zona pellucida and fertilize the egg [64]. The device also induces inflammation within the uterus, preventing the implantation of the blastocyst. In a study investigating the recovery of sperm cells from fallopian tubes in IUD users, no sperm cell was recovered, whereas the control group had 14 sperm cells. Also, the number of leukocytes in the group with the IUD was significantly higher than the control group without the IUD [65]. Therefore, it is concluded that the phagocytic activity of leukocytes resulting from inflammation exhibited the spermicide activity. According to FDA, a copper releasing IUD provides 10 years of pregnancy protection, which is 5 years more than LNG-IUD. The failure rate of copper IUD is 0.8% whereas LNG-IUD is 0.2% [37] [66]. However, there are several complications following the implantation of Cu- and LNG-IUD, which include cramping coupled with heavy menstrual bleeding, perforation, expulsion, and infections [67]. Perforation implies

that the IUD is pushed into or through the muscle of the uterus, which can lead to penetration of uterus wall and migration of IUD to the pelvis, abdominal cavity, and bladder. Expulsion is the most common complication, which refers to the displacement of IUD into the vaginal cavity. The rarest case of IUD complication is perforation and if such a case occurs, surgical removal of IUD is required. An improper position of the device will affect the effectiveness of the contraception. The most serious complication is an infection, which occurs due to the killing of normal bacteria inhabiting the vaginal area as a result of antibiotics taken before the IUD insertion to reduce the infection of upper genital tract infection, and thus, leading to the outgrowth of vaginal yeast [67].

1.1.4 Sterilization

Sterilization is an irreversible birth control method and an alternative option for people with a contraindication for hormonal contraceptives. Vasectomy and laparoscopic tubal ligation are permanent sterilization methods that involve a surgical procedure. Laparoscopic tubal ligation is a female sterilization procedure performed by sealing the fallopian tube and thus, preventing the contact between sperm cells and egg cell [68]. On the other hand, hysteroscopic tubal occlusion , which is also a female sterilization method, involves non-surgical placement of permanent micro-inserts into fallopian tubes through a vaginal approach [69]. Further, for hysteroscopic tubal occlusion, only local anesthesia is needed whereas general anesthesia is required for laparoscopic tubal ligation surgery. Therefore, hysteroscopic tubal occlusion is less invasive as compared to laparoscopic tubal ligation. Vasectomy is male sterilization achieved by sealing or cutting off the vas deferens tubes that transport the sperm cells out of testes. The failure rate of vasectomy is 0.15%, whereas the failure rate of tubal occlusion is 0.5% [37].

However, the reversal of tubal ligation increases the risk of ectopic pregnancy [70]. After sterilization, there is a rare chance that the tubes will heal and the fetus will implant in the fallopian tube instead of the uterus, leading to an ectopic pregnancy. Other potential risks following the tubal ligation surgery includes infections, bleedings, and scarring of the fallopian tubes [71, 72]. On the other hand, the reversal of vasectomy increases the risk of bleeding within the scrotum and infection at the surgical site.

1.1.5 Novel non-hormonal contraceptive products and potential non-hormonal contraceptive agents

Currently, many novel non-hormonal contraceptive products have been developed and are in either pre-clinical or clinical studies. Ovaprene®, a hormone-free monthly vaginal ring contraceptive that has successfully completed the postcoital test clinical study, is currently in clinical development and is potentially the first monthly non-hormonal contraceptive product [73]. It acts as a physical barrier due to the mesh and impedes the motility of sperms for preventing sperms to enter the cervical canal. Amphora® , also a hormone-free on-demand contraceptive, is a vaginal noncytotoxic spermicide gel tested in phase III clinical trial for its safety, efficacy, and tolerability as a contraceptive [74]. The product is an investigational multipurpose vaginal pH regulator (MVP-R) designed to regulate vaginal pH within the range of 3.5 to 4.5 even in the presence of semen, and thus, immobilize and kill sperm cells. SMART (System Mute until Activation by a Remote Trigger) is an on-demand non-hormonal contraception that maintain a protective and inhospitable vaginal environment for sperm by using polymer fibers [75]. These novel bio-responsive polymer compositions can maintain a physiologically acidic pH environment and instantly increase the viscosity of fluids which decreases the sperm motility.

Several compounds have been identified as novel spermicidal agents and have the potential to be developed into nonhormonal contraceptives. Desgalactotigonin (DGT), found in the seed of *Chenopodium album*, was reported to exhibit spermicidal effects at minimum effect concentration (MEC) 58.03 μM when tested in human spermatozoa [63]. DGT was biocompatible with *Lactobacillus acidophilus* and showed low cytotoxicity in HeLa cells. The EC50 of DGT (29.8 μM) was lower than N-9 (78.34 μM) but the IC50 of DGT (135.54 μM) was higher than N-9 (0.675 μM). These findings suggested that DGT has better therapeutic window, specificity, and selectivity compared with N-9. Therefore, DGT is a potential active agent for a contraceptive that is compatible with vaginal microflora and safe for vaginal cells. Other studies have shown that CD52g, an antigen secreted by epithelial cells in the male genital tract, was found to insert into the sperm membrane and the seminal leukocytes which transmit HIV-1. Therefore, the anti-CD52g monoclonal antibodies were hypothesized to be a multipurpose prevention technology that prevents HIV transmission and provides contraception [76].

Several novel contraception targets of male fertility have been reported and are in different status of development, including sperm calcium channel (CatSper), Na,K-ATPase (NKA) and SLO3 K channels[77-79]. The mechanisms of action for each candidate differ but all are associated with the disruption of sperm cells or the reduced production of sperm cells, including targeting sperm motility, impairing spermatogenesis, and inhibiting sperm passage through vas deferens [80]. One of the novel sperm-deactivating agents that have been identified to reduce sperm motility via inhibiting the activation of CatSper through binding to ABHD2 (Abhydrolase domain-containing protein2) is called lupeol, which is a pentacyclic triterpenoid molecule.

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1.2 Triterpenoids

Triterpenoids are phytosterols that are commonly present in plants, where they form a critical structural component of the plant cell membrane [81]. Phytosterols have a similar structure to cholesterol, where the former stabilizes cell membranes in animals but it is absent in plants. Therefore, phytosterols serve the same function as cholesterol but in plants, which is to stabilize the phospholipid bilayers and increase the membrane rigidity [81]. Triterpenoids consist of six isoprene units and are synthesized from the 30-carbon squalene via cyclization (Figure 1) [82], and include protostanes, lanostanes, holostanes, cycloartanes, dammaranes, euphanes, tirucallanes, tetranortriterpenoids, quassinoids, lupanes (Figure 2), oleananes, friedelanes, ursanes, hopanes, isomalabaricanes, and saponins [83].

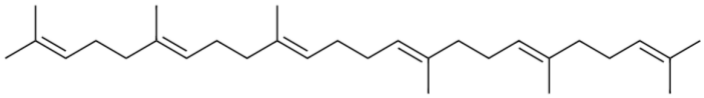

Figure 1. Chemical structure of squalene

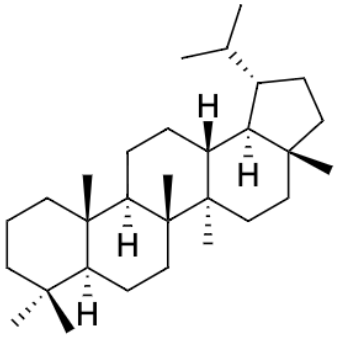

Figure 2. Chemical structure of lupane

Triterpenoids can be broadly categorized based on the number of rings in the structure. Pentacyclic triterpenoids, such as oleanolic acid (OA) and lupeol, form the dominant group. They can be found in a variety of edible vegetables as well as traditional medicinal herbs. Triterpenoids can also be found in the general food supply, including olive oil. For countries where diets are mainly olive oil-based, the average intake of triterpenes could reach 400 mg/kg for a person in a day [84]. Also, triterpenoids can be found in the wax-coating of a variety of plants and fruits such as seaweed and apple peels [85]. Researchers have identified thirteen triterpenoid compounds from apple peels that have anti-cancer pharmacological activities [85]. These isolated compounds were found to possess antiproliferative activity toward human HepG2 liver cancer cells, MCF-7 cancer cells and Caco-2 cancer cells [85]. Moreover, it was discovered that triterpenoids also possessed antioxidant, antibacterial, and anti-inflammatory activities [86-88]. Studies regarding the antioxidant activity of triterpenoids have demonstrated that for diseases involving oxidative stress, such as liver disease, tumor, and inflammatory conditions, OA could reduce the concentration of serum alanine transaminase and the liver centrilobular necrosis [89]. In leukemia cell studies, OA and ursolic acid demonstrated a protective effect toward H_2O_2-induced DNA damage. In other studies, triterpenoids were shown to possess antibacterial and antivirus activities [90]. With this wide spectrum of pharmacological activities, there is an unprecedented escalation of interest in triterpenes in the past few decades. Lupeol is a promising compound that has drawn scientists' attention. A substantial amount of published literature suggests the utility of lupeol in a wide variety of conditions and it has been extensively investigated to develop treatments for clinical use [91-98]. These clinical trials studied triterpenes in various conditions, including cardiovascular diseases, breast cancer, and diabetes. Furthermore, triterpene-based products are being sold commercially in the market.

1.2.1 Lupeol

Lupeol, betulin, and betulinic acid are pentacyclic triterpenes of the lupane-type saponins. Lupeol can be found in a wide variety of vegetables and fruits, including white cabbages, strawberries, grapes and green peppers; as well as in various medicinal herbs, including *Tamarindus indica, Allanblackia monticola, Himatanthus sucuuba*, *Celastrus paniculatus, Zanthoxylum riedelianum, Leptadenia hastata, Crataeva nurvala, Bombax ceiba,* and *Sebastiania adenophora,* which are used by people in different parts of the world [84]. The chemical structure of lupeol is as presented in Figure 3, and the chemical formula is $C_{30}H_{50}O$. The molecular weight of lupeol is 426.7174 g/mol, the topological polar surface area is 20.2 $Å^2$, and the heavy atom count is 31. Lupeol has 1 H-Bond donor, 1 H-Bond receptor, and 1 rotatable bond (PubChem, NIH library, Compound ID 259846). The cytotoxicity of lupeol in various cell lines has been reported. The IC_{50} of lupeol in a human breast cancer cell line MCF-7 was found to be 80 μM after 24 hours [99]. In a study investigating the cytotoxicity of lupeol in cancer cells, lupeol was tested for up to 72 hours. This study showed that lupeol exhibited various levels of cytotoxicity in lymphoma LCL, human foreskin fibroblast (HFF), HeLa and Burkitt's lymphoma (BL41) cells. Within 24 hours after the treatment, lupeol showed little to no adverse effect to HFF and HeLa cells. The IC_{50} value of lupeol at 72 hours in LCL, HFF, HeLa and BL41 was 51.8, 79.7, 63.3 and 56.9 μM, respectively [100]. Lupeol has LD50 of 2g/kg in mouse and rat through oral administration [101]. Lupeol has been shown to exhibit various pharmacological activities *in vitro* and *in vivo*. These include the activity to decrease inflammation and cancer, inhibit microbial growth and prevent pregnancy.

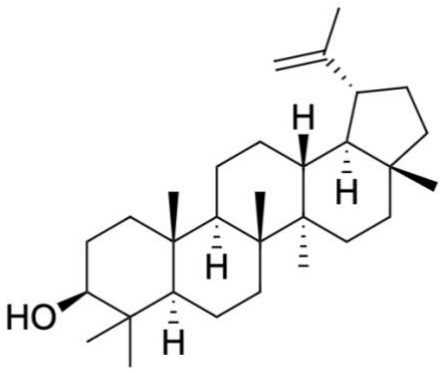

Figure 3. Chemical structure of lupeol

1.2.1.1 Anti-inflammatory activity of lupeol

The anti-inflammatory activity of lupeol was investigated via different routes of administration as well as the mechanism of pathways. For topical application, studies showed that lupeol suppressed the production of pro-inflammatory mediators [102]. The application of 0.5 and 1 mg/ear of lupeol could diminish 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation in the mouse ear model. The results demonstrated that the level of neutrophil specific marker myeloperoxidase decreased after applying lupeol; thus, leading to a reduction of cell infiltration into inflamed tissues. The mechanism of action is that lupeol decreases the production of prostaglandin E2 and inhibit tumor necrosis factor- α and interleukin-1β cytokine production in macrophages [102]. Moreover, lupeol was reported to manifest high wound healing potential in a dead space wound mouse model. It showed that the wound healing activity was improved significantly when topically applied (8 mg/mL lupeol) in 0.2% sodium alginate gel [102]. The result was even better than the commonly used wound healing skin ointment Nitrofurazone. Chronic inflammation is known to prolong the healing process of damaged tissues. The proven wound healing property of lupeol suggest that it reduces inflammation, which has the potential to be further investigated.

For oral administration, reports demonstrated that 60 mg/kg of lupeol was observed to alleviate the mucus production and the inflammation by significantly reducing the level of type II cytokines interleukin (IL)-4, IL-5, and IL-13 in the bronchial asthma mouse model [103]. Mice treated with lupeol also had reduced cellularity and eosinophils in the broncho-alveolar fluid. The result was comparable to the mice that were treated with dexamethasone (30 mg/kg). Dexamethasone is a corticosteroid used for treating various lung and bronchial-related diseases, including asthma and bronchospasm [104]. However, the side effects of chronic corticosteroids include hyperglycemia and osteopenia [104]. Therefore, the discovery of an agent that possesses the ability to restrict the allergic airway inflammation but potentially has lower toxicity is a great breakthrough. Furthermore, many studies have compared the anti-inflammatory activity of lupeol with other anti-inflammatory agents. For example, indomethacin, a selective cyclo-oxygenase inhibitor and a commonly used non-steroid anti-inflammatory drug, was compared with the herbal medicine used by Caribbean-region doctors for inflammation, *pimento racemose*. The extracts from the *pimento racemose* plant manifested high anti-inflammatory activity in animal models that are comparable to indomethacin. The extracts were later identified to be enriched with lupeol [105].

1.2.1.2 Anti-cancer activity of lupeol

Studies have reported that phytochemical compounds can reduce the risk of cancer, and that triterpenoids have shown to manifest anti-proliferative activity in various cancer cell lines. Several studies demonstrated that lupeol acts as an antineoplastic agent by various pathways related to mutagenesis and tumorigenesis.

Mutations which occur through DNA strand breaks can be the precursors of the development of cancer, and cells that harbor mutations with excessive cell growth are at risk of

forming into malignant tumors. In several studies, lupeol was demonstrated to exhibit anti-mutagenic activity *in vitro* and *in vivo*. A study performed in the mouse skin model indicated that the topical application of 200 μg/mouse could prevent 7, 12-dimethylbenz[a]anthracene (DMBA)-induced DNA alkylation damage [106]. At the 96-hour time point, the pre-treatment of lupeol showed 56.05% prevention against DMBA-induced DNA strand breaks. In the other study conducted in the mouse model, lupeol was reported to suppress the B(a)P-induced genotoxicity. The mice were administrated with one dose of B(a)P (100 mg/kg) after the pre-treatment of 1 mg/animal lupeol for 7 consecutive days. The results indicated the clastogenicity induced by B(a)P was inhibited by lupeol and led to an increase in mitotic index, suggesting lupeol increased the cell population that underwent mitosis [107]. In the *in vivo* study, the protective effects of lupeol against Mancozeb-induced genotoxicity were investigated in cultured human lymphocytes [83]. The results showed a decreased expression of Mancozeb-induced DNA-damaged genes and an increased expression of DNA repair genes in the lymphocytes [83]. These findings suggested lupeol attenuates the oxidative stress caused by different external factors that can potentially lead to mutagenesis. Therefore, the anticancer effect of lupeol is associated with its antioxidant properties. Furthermore, the anticancer effects of lupeol can be achieved through targeting multiple signaling pathways, including the inhibition of phosphatidylinositol-3-kinase (PI3K) pathway, nuclear factor kappa B (NFκB) pathway [108], Wnt/β-catenin signaling and Fas-apoptotic machinery [109].

1.2.1.3 Antifertility and contraceptive activity of lupeol

Compared to other biological activities studied for lupeol, the research associated with the its antifertility activity of lupeol has been minimal. Previously, lupeol was reported to significantly decrease the weight of the reproductive organs in male rats when orally administrated at the dose

level of 10 mg/rat/day [110]. The number and motility of the sperm cells were significantly reduced, and the size of the seminiferous tubules was diminished by 24.62%. As for Leydig cells which are responsible for producing testosterone, the nuclear area and the number of mature Leydig cells were reduced by 27.65% and 35.47% [110].

Sperm hyperactivation is essential for fertilization and is a part of sperm capacitation, as it increases sperm motility and enables the penetration of sperm through the protective vestment surrounding the egg cell (Figure 4) [111]. The calcium channel of sperm cells (CatSper), which is activated by ABHD2 receptor via progesterone binding is essential for sperm hyperactivation to occur (Figure 5) [111]. Lupeol was reported to block the binding of progesterone to ABHD2 and inhibit sperm hyperactivation (Figure 6) [77]. Mannowetz et al. evaluated the changes of the current in CatSper and the curvilinear velocity in sperm cells after incubation with lupeol. The results demonstrated that lupeol significantly diminished the hyperactivation of spermatozoa. The IC_{50} of lupeol for CatSper inhibition in sperm cells was 109 nM [77]. The mechanism of action of the lupeol's inhibitory effect in sperm motility is by blocking the regulation of progesterone in sperm cells. As a result, sperm cells fail to penetrate the protective vestment and enter the egg cell when they are exposed to lupeol (Figure 7). These findings indicate that lupeol has the potential to act as a contraceptive compound by preventing sperm hyperactivation and averting fertilization. Moreover, these studies also suggested that progesterone serves a vital role in mediating the sperm hyperactivation which is responsible for the cascade of actions leading to egg cell fertilization.

1.2.1.3.1 Regulations of progesterone in sperm cells through CatSper

In women, progesterone is not only known to regulate ovulation and prepare the uterus for pregnancy by thickening the endometrium to receive an embryo, it also regulates human sperm cell function upon entering the female reproductive tract [112, 113]. Progesterone released by the egg cell potentiates CatSper, a pH-dependent Ca2+ channel, with nanomolar concentration. When CatSper is activated, it triggers a sudden influx of calcium ions into the sperm tail, resulting in asymmetrical flagellar motion and initiating sperm hyperactivation (Figure 5). Sperm hyperactivation is important because it enables the sperm cells to efficiently pass through viscous luminal fluids of the female vaginal tract and penetrate through the protective vestment surrounding the egg cell (Figure 4). Moreover, CatSper is also responsible for the sperm chemotaxis and the acrosome reaction. Since CatSper is activated by progesterone, it can guide the sperm cells to turn toward the egg cell by sensing the concentration gradient of progesterone. The increasing calcium ion level in the flagellum eventually leads to an increase in the head of the sperm cells, resulting in the Ca2+-dependent acrosome reaction. The acrosome reaction is the fusion of the acrosomal membrane with the sperm cell membrane, contributing to the exposure of the acrosomal contents, which include proteolytic enzymes. The purpose of this process is to help the sperm cells penetrate across the protective vestment surrounding the egg cell by releasing the proteolytic enzymes within the sperm cells. The proteolytic enzymes degrade the egg's vestment, which includes the zona pellucida layer and the vitelline membrane, thus creating a path for sperm cells to get in contact with the egg cell. The sperm cell fuses with the egg cell and results in fertilization.

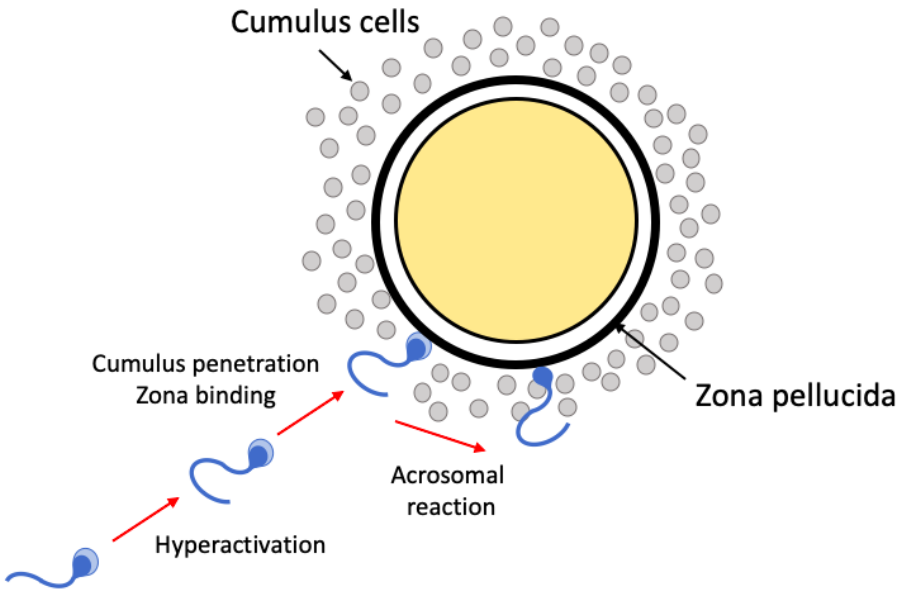

Figure 4. Sperm capacitation schematic

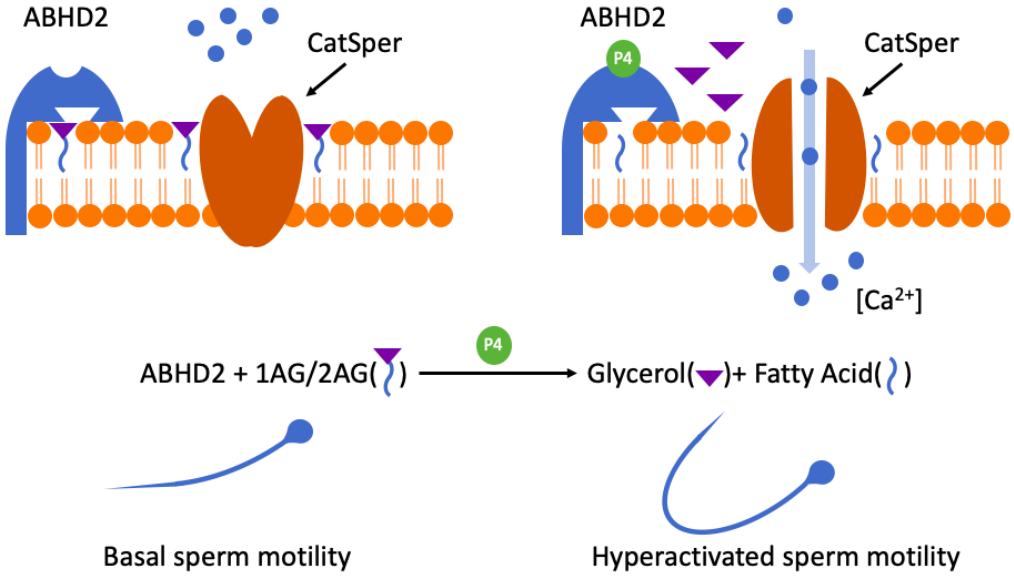

Figure 5. Activation of CatSper via binding of progesterone (P4) to ABHD2.

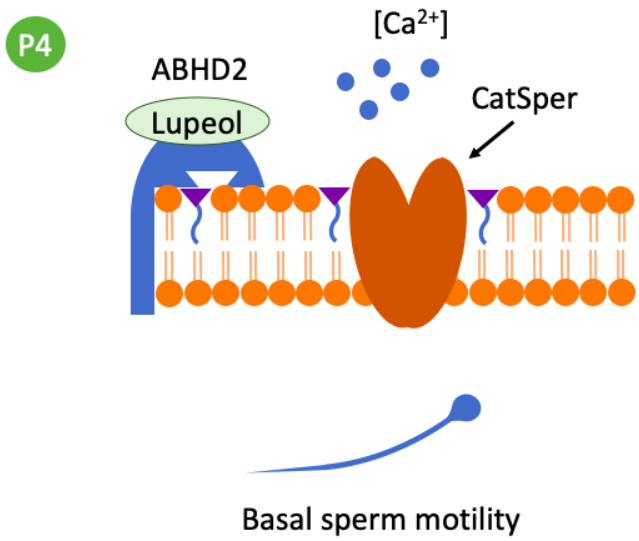

Figure 6. The mechanism of action of lupeol in inhibiting sperm hyperactivation. Lupeol arrests sperm hyperactivtion by inhibition of CatSper via ABHD2 binding.

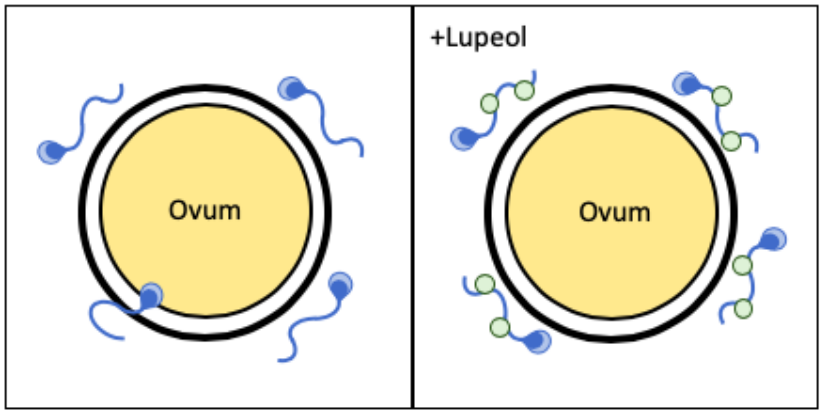

Figure 7. The inhibitory effect of lupeol blocks the entry of sperm cells into ovum.

1.2.1.3.2 The location of CatSper and related receptor

To locate CatSper on the sperm cells, Lishko et al. separated the human spermatozoa into two segments, the head and the flagellum [113]. By conducting the patch-clamp technique, the amplitude of inward current density at CatSper channel was recorded. The amplitude of currents recorded from the integrated human spermatozoa before and after being potentiated by progesterone is close to the one recorded on the flagellum [113]. This indicates that CatSper is located on the flagellum of the sperm cells.

The receptor associated with CatSper activation is ABHD2, an abhydrolase serine protein 2, to which progesterone binds [114]. Since CatSper is essential for sperm motility and fertility, inhibition of CatSper could arrest the sperm hyperactivation and prevent women from pregnancy. Lupeol has been proven to harbor contraceptive activity by blocking the binding of progesterone to the ABHD2, and thus inhibiting the sperm hyperactivation [114]. Triterpenes are the precursor of all steroids, which gives lupeol a similar structure compared with progesterone [115]. This characteristic may be the reason why lupeol can compete with progesterone for the binding to ABHD2.

Since the target site of lupeol is sperm-specific receptor that is expressed predominantly on sperm flagellum, topical delivery is highly recommended (i.e. vaginal delivery) to maximize lupeol's exposure to sperm cells and not be limited by its low availability [116].

1.3 Dosage form and drug delivery system

A pharmaceutical dosage form is defined as the physical design structure of a dose for an active pharmaceutical ingredient (API) that is used as a medication for administration into the body. There are different types of dosage forms, such as tablets, capsules, sachets, controlled release dosage forms, parenteral dosage forms, transdermal dosage forms, emulsions, and suspension inhalants. The choice of dosage form depends on various factors, including the route of drug administration, the drug release rate, and the physicochemical properties of the drug. The ideal drug delivery system should be able to deliver sufficient amount of active agent and channel active agent to the target site. Pharmaceutical excipients are used as inactive ingredients that have little or no therapeutic value but useful in structuring and manufacturing the dosage form and preserving the active agent. The ideal properties of excipients include non-toxic, commercially available, economical, stable, and no undesired interaction with the API.

For topical delivery, the drug dosage forms include solutions, gels, creams, foams, suppositories, and films. The topical delivery system is used for restricting the effects at the site of the application and avoiding the first-pass metabolism, the gastric conditions, and the risks and inconveniences of the parental drug delivery systems [117]. The additives for topical products, such as suppositories and films, include emulsifiers, biodegradable polymers, and plasticizers. Suppositories are designed for the insertion into orifices such as the vagina or the rectum. It may be the preferable dosage form when the patient has nausea or intense vomiting reaction [118]. Some patients avoid the use of the suppositories because it is uncomfortable. Whereas for vaginal films, users can self-administer digitally (i.e. using finger) with minimal discomfort. Vaginal drug delivery has several advantages besides bypassing first-pass metabolism, including large surface area, rich blood supply, and relatively high permeability to some drugs [119]. These biological

advantages of the vagina makes it an ideal route for bioadhesive drug delivery systems to retain drugs for localized treatment and contraception [120]. In order to extend the drug retention time within the vaginal vault, bioadhesive polymeric film delivery system has been developed as a solid dosage form to incorporate the API [120]. Due to the rich blood supply in the rectal and vaginal area, it provides exceptional absorption of the drug and allows both for local and systemic action.

Vaginal polymeric films have lesser leakage, do not require applicators to insert the product, are more convenient and portable as compared to gels and foams [121, 122]. Thus, vaginal films have greater user acceptability compared to other vaginal dosage forms [123]. Moreover, vaginal films are thin, lightweight, and flexible. These physical properties of films make them more resistible to physical forces as compared to tablets and can be stored in individual sealed flat packages which are easy to carry.

2.0 The objective

Project hypothesis: It is hypothesized that by delivering lupeol using vaginal films high local concentrations in the cervicovaginal environment can be achieved. As a result, lupeol can efficiently interact with CatSper located on the flagellum and immobilize sperm leading to prevention of unintended pregnancy.

Project goal: The overarching goal of the project is to develop a vaginal drug delivery system for the novel sperm-inhibiting triterpenoid, lupeol. To this end, an immediate-release vaginal contraceptive film containing lupeol was developed to administer precoitally. To achieve this goal, following specific aims were proposed:

Specific Aim 1: To develop and validate a reliable analytical method using HPLC and perform pre-formulation studies of lupeol. A HPLC method that can reliably quantify lupeol under varying conditions was developed. Pre-formulation studies included lupeol solubility studies, forced degradation studies, and cytotoxicity studies.

Specific Aim 2: To develop a lupeol immediate-release vaginal film using solvent casting method. A polymeric vaginal film platform which has immediate-release profile was selected as a base for formulation development efforts. An optimized formulation with improved dissolution profiles was developed.

Specific Aim 3: To evaluate film physicochemical properties, including drug content, content uniformity, dissolution profile and physical properties. A solid-phase extraction method with sufficient drug extraction efficiency

was established for evaluating drug content and content uniformity. Dissolution profile was assessed using USP I apparatus (basket) and physicochemical properties of the films were analyzed using Karl Fischer titrator, tensiometer and TA.XT texture analyzer.

3.0 Pre-formulation studies of lupeol

3.1 Introduction

Pre-formulation studies include the development and validation of an analytical assay and the characterization of physical and chemical properties of a drug candidate. Pre-formulation studies provide important information about the factors that could affect drug performance and formulation design. They also support the need for formulation modification.

3.1.1 HPLC method development

A reliable analytical method could detect and separate any process impurities, intermediates, degradation products, and excipients which might interfere with quantification of the analyte of interest. Reversed-phase high-performance liquid chromatography (RP-HPLC) was identified as the analytical method for lupeol. RP-HPLC has a non-polar stationary phase and a moderately-polar mobile phase. The stationary phase is silica, which is surface-modified with a long chain (C_{18} or C_8) of hydrocarbons. The interaction between the alkyl group and the non-polar molecules is formed through Van der Waals forces and hydrophobic interactions. Also, the non-polar molecules are less soluble in the aqueous mobile phase which facilitates their interactions with the stationary phase. Therefore, the retention time is longer for less polar analytes and polar molecules will be eluted earlier. RP-HPLC is preferred because it allows the use of water-based solvents making the analysis more cost-effective. Moreover, the silica in Normal Phase-HPLC tends to absorb water and distort the retention time of the analytes.

3.1.2 Solubility study

Studies have shown that lupeol exhibits significant biological activity, including anticancer, antiviral, antibacterial, and antioxidant properties [124]. However, its pharmaceutical development is greatly impeded due to its physicochemical properties. It is reported that lupeol is sparingly soluble in aqueous solutions and is soluble in organic solvents, such as acetone, methanol (MeOH), and ethanol (EtOH). The solubility of lupeol in water is 195 ng/mL [125]. The log P is 7.45 which makes it a highly hydrophobic compound. The molecular weight of lupeol is 426.729 g/mol (PubChem, NIH library, Compound ID 259846). In the process of developing an active agent into pharmaceutical products, the bioavailability of the API is an important factor in determining its therapeutic efficacy. Lupeol is a BCS (biopharmaceutical classification system) class II drug which has low solubility but high permeability. When administered via the oral route, due to the predominately hydrophilic environment in the gastrointestinal tract, the BCS class II drugs will have low solubility leading to inadequate bioavailability. Therefore, the bioavailability of lupeol is limited by its poor solubility. To select the potential solvent matrix for developing an analytical assay and the media for dissolution study, we screened lupeol with several solvents of interest.

3.1.3 Forced degradation study

Forced degradation studies are important evaluations to determine the stability of drug candidates. These studies elucidate the major degradation factors associated with the drug which are not evident from the standard stability studies. To identify potential degradation pathways for lupeol in these studies, accelerated conditions were applied, including elevated temperature,

exposure of the drug to intensive visible light, or addition of other reactants such as peroxides, strong base and strong acid. Forced degradation studies require analytical methods that are capable of differentiating drug candidate and degradants. Understanding mechanisms of potential drug instability assists the development process to identify potential issues and develop strategies to overcome them.

3.1.4 Cytotoxicity study

In addition to identification of degradation pathways, it is also important in pre-formulation studies to evaluate potential for toxicity. Typically, relevant cell lines are used to assess toxicity of new compounds. Cytotoxicity assays determine the toxicity of an agent in cells by measuring the number of live cells and cell proliferation; in other words, the cell viability. Cytotoxicity assays are used for drug screening and to eliminate compounds that exhibit cytotoxic effects. There are a variety of assays based on different cell functions, such as the activity of cellular enzymes (ex. ATP, LDH), cell membrane integrity, cell population, or nucleotide uptake activity.

Although cytotoxicity of lupeol has been investigated in several cancer cell lines [99, 100], to date no vaginal-related cell lines have been evaluated with respect to lupeol exposure. Therefore, to better understand the therapeutic window of lupeol for vaginal administration, we conducted a cytotoxicity test using a vaginal epithelial cell model, the VK2/E6E7 cell line that is commonly used to study the agents intended for intravaginal applications.

3.2 Materials

Lupeol was purchased from BOC Sciences (Shirley, NY). HPLC grade methanol (MeOH), HPLC grade acetonitrile (ACN), acetic acid, 37% hydrochloric acid (HCl), 5 N sodium hydroxide (NaOH), and 30% hydrogen peroxide were purchased from Fisher Scientific (Fair Lawn, NJ). Ethanol (EtOH) was purchased from Pharmco (Brookfield, CT). SDS (sodium dodecyl sulfate), CTAB (Cetyltrimethylammonium bromide) and Tween80 were purchased from Spectrum Chemical (Gardena, CA). Keratinocyte serum-free medium (KSFM) was purchased from Gibco (Carlsbad, CA). CellTiter-Glo® assay kit was purchased from Promega (Madison, WI). VK2/E6E7 (ATCC® CRL-2616™) was purchased from ATCC (Manassas, VA). 0.22 μm polytetrafluoroethylene (PTFE) filter (0.22μm pore diameter, 13mm in diameter) was purchased from RESTEK (Bellefonte, PA). Luna C8 column (Luna 3μm C8(2) 100Å 50 x 4.6 mm) was purchased from Phenomenex (Torrance, CA). Ultrapure water was obtained from an in-house Milli-Q® water purification system (Millipore Sigma Advantage A10).

3.3 Methods

3.3.1 RP-HPLC analysis

3.3.1.1 Instrument method

A high-performance liquid chromatography method was developed to quantify lupeol within the film. Quantification was achieved using RP-HPLC (Waters Alliance 2695), with UV detection at 210 nm. The mobile phase composition was a mixture of 100% ACN and 0.1% acetic

acid in water at a ratio 88:12. The ACN and the aqueous solution were mixed and sonicated for 5 minutes to remove excessive air bubbles. A Luna C8 column (Luna 3μm C8(2) 100Å 50 x 4.6 mm) was employed to obtain the chromatographic separation under isocratic elution at a flow rate of 1 mL/min. The temperature of the column and the sample rack were maintained at 25°C. The run time for each sample was set at 10 minutes. The injection volume was 10 μL. The calibration curve range of lupeol investigated for linearity was between 25 to 200 μg/mL. The method was validated for linearity, repeatability, precision, specificity, the limit of quantification (LOQ), and the limit of detection (LOD).

3.3.1.2 Sample preparation method

To prepare a lupeol stock solution with a concentration of 200 μg/mL in 90% MeOH, lupeol 4.0 mg was accurately weighed and transferred into a 20 mL volumetric flask. Lupeol was dissolved by the addition of 15 mL of MeOH and 2 mL of Milli-Q water. The volumetric flask was then sonicated for 5 minutes. After sonication, volume was adjusted by adding additional MeOH and vortexed afterward. To construct a standard calibration curve, lupeol stock solution was diluted with 90% MeOH to a range of concentrations (25 to 200 μg/mL). The concentrations of the quality controls were 85, 125, and 175 μg/mL. The linearity of the calibration curve was obtained by plotting the concentration of lupeol versus analyte area (AUC). The amount of lupeol in each sample was then back calculated based on this calibration curve.

3.3.2 Lupeol forced degradation study

In a 100 mL volumetric flask, 20.8 mg of lupeol was accurately weighed to prepare a stock solution with a concentration of 208 μg/mL in 90% MeOH. Lupeol was dissolved by adding 80 mL of MeOH and 10 mL of milli-Q water. The volumetric flask was then sonicated for 5 minutes. After the sonication, the volume was made up with MeOH.

3.3.2.1 Unstressed control samples

5 mL of lupeol stock solution (208 μg/mL) was placed in glass vials in triplicate. Samples were sealed with parafilm, covered with aluminum foil and placed at room temperature for 7 days. At predetermined time points, aliquots were withdrawn from the samples and diluted with 90% (v/v) MeOH to a concentration of 130 μg/mL. Each aliquot was then filtered with a 0.22 μm PTFE filter. The filtrate was analyzed using the developed HPLC method.

3.3.2.2 Thermal stability

5 mL of lupeol stock solution (208 μg/mL) was placed in glass vials in triplicate. Samples were sealed with parafilm, covered with aluminum foil and incubated at 65°C for 7 days. At predetermined time points, aliquots were withdrawn from the samples and diluted with 90% (v/v) MeOH to a concentration of 130 μg/mL. Each aliquot was then filtered with a 0.22 μm PTFE filter. The filtrate was analyzed using the developed HPLC method.

3.3.2.3 Photolysis

5 mL of lupeol stock solution (208 μg/mL) was placed in glass vials in triplicate. Samples were sealed with parafilm and exposed to an intensive visible light at room temperature for 7 days.

At predetermined time points, aliquots were withdrawn from the samples and diluted with 90% (v/v) MeOH to a concentration of 130 μg/mL. Each aliquot was then filtered with a 0.22 μm PTFE filter. The filtrate was analyzed using the developed HPLC method.

3.3.2.4 Oxidation

Lupeol stock solution (208 μg/mL) was diluted with 30% (v/v) H_2O_2 to achieve a H_2O_2 concentration of 3% (v/v) and lupeol concentration of 187.2 μg/mL. Samples were placed in glass vials in triplicate. Vials were sealed with parafilm, covered with aluminum foil and placed in room temperature for 7 days. At predetermined time points, aliquots were withdrawn from the samples and diluted with 90% (v/v) MeOH to achieve a lupeol concentration of 130 μg/mL. The aliquots were then filtered with a 0.22 μm PTFE filter, and the filtrate was analyzed using the developed HPLC method.

3.3.2.5 Acidic hydrolysis

Lupeol stock solution (208 μg/mL) was diluted with 37% HCl to achieve a HCl concentration of 0.5 N and lupeol concentration of 199.41 μg/mL. Samples were prepared in vials in triplicate. Vials were sealed with parafilm, covered with aluminum foil and placed in room temperature for 7 days. At predetermined time points, aliquots were withdrawn from the samples, neutralized with 5 N NaOH and diluted with 90% (v/v) MeOH to 130 μg/mL. The aliquots were then filtered with a 0.22 μm PTFE filter, and the filtrate was analyzed the developed HPLC method.

3.3.2.6 Basic hydrolysis

Lupeol stock solution (208 μg/mL) was diluted with 5 N NaOH to achieve a NaOH concentration of 0.5 N and lupeol concentration of 187.2 μg/mL. Samples were prepared in vials in triplicate. Vials were sealed with parafilm, covered with aluminum foil and placed in room temperature for 7 days. At predetermined time points, aliquots were withdrawn from the samples, neutralized with 37% HCl and diluted with 90% (v/v) MeOH to 130 μg/mL. The aliquots were then filtered with a 0.22 μm PTFE filter, and the filtrate was analyzed using the developed HPLC method.

3.3.3 Lupeol solubility study

The solubility of lupeol in various solvents was determined including 70% MeOH, 80% MeOH, 90% MeOH, 90% EtOH, 90% ACN, 0.5% SDS, 1% SDS, 1% CTAB, 2% CTAB and 5% CTAB. An excess amount of lupeol was added to a known amount of solvent in a glass vial. The mixtures were then sealed tightly using parafilm, covered in aluminum foil and placed on a rotator overnight at room temperature. Then, the mixtures were filtered with 0.22 μm PTFE filter and the filtrate of each sample was quantified using the HPLC method previously described.

3.3.4 Lupeol cytotoxicity study

VK2/E6E7 cells were seeded into 96-well plates at a density of 10^4 cells per well. A stock of the drug solution was prepared by dissolving 3 mg of lupeol into 234 μL of 37°C EtOH to achieve 30 mM in 100% EtOH. The lupeol stock solution was then diluted with KSFM (Keratinocyte serum-free medium) to achieve a sample stock of 60 μM lupeol in 0.2% EtOH. The

sample stock was then diluted with KSFM to a predetermined testing concentration in the range of 0.47 – 60 μM. The testing concentrations were prepared by serial dilution. After incubating cells at 37°C and 5% CO_2 for 10 hours to allow cells to adhere to the culture plate, they were treated with the testing solutions by replacing the media with 200 μL of solutions that contained different lupeol concentrations. The cell viability was assessed using CellTiter-Glo® assay at 24 hours, 48 hours, and 72 hours after the treatment. The result was obtained by measuring the luminescence signal using a microplate reader. Because all living microorganisms utilize ATP for storing metabolic energy, the detection and quantification of ATP can be used to quantitate living microorganisms, including bacteria and cells. The CellTiter-Glo® assay used in these studies relies on the ATP-dependence of the luciferase reaction to detect and quantitate live cells. Therefore, the detected luminescence is proportional to viable cells. By calculating the relative cell viability of test group to control group, biocompatible drug concentrations are determined if the relative viability is greater than 80%.

3.4 Results

3.4.1 RP-HPLC validation

The developed method was qualified for linearity, accuracy, precision, repeatability, LOD and LOQ. Linearity represents the ability of the method to measure test results that are proportional to the concentration of the analytes within a given range. It is evaluated by monitoring the regression coefficient (R_2) of the calibration curve. Linearity tested on three different days showed R_2 greater than 0.999 (Table 3). The accuracy of an analytical method is the degree of closeness

between the theoretical value of analytes in the samples and the values determined by the method using the linear regression equation of the calibration curve. The accuracy of three different levels on three different days were within the range of 95% to 105% (Table 4). The precision of the method is the closeness of a series of measurements of an analytes when analytical procedure is applied repeatedly to multiple aliquots. The precision of the method was determined based on the %RSD, and the acceptable criteria is less than 2% (Table 5). The %RSD of three different levels in the method were lower than 2% on three different days. The repeatability of the method is measured by preparing six samples at the designated concentrations and the %RSD for these samples should be less than 2% (Table 6). The average recovery of 100 µg/mL is 100.44% with RSD 0.71%. LOD and LOQ are determined based on the signal-to-noise ratio (s/n). The s/n of LOD should be greater than 3.3 and the s/n of LOQ should be greater than 10 (Table 7). LOD is the lowest concentration level of an analyte that can be statistically differentiated from a blank matrix. LOQ is the concentration level of the analyte that can be obtained with specified degree of confidence.

Table 3. Linearity.

The linearity was determined by calculating the correlation coefficient (R_2) (n=3).

Linearity	Day 1	Day 2	Day 3
R_2	0.9999	0.9996	0.9997

Table 4. Accuracy.

Intra-day accuracy was performed on 3 different days with the same sample preparation method and instrument method. %Accuracy for different levels of control should fall between 95%- 105%.

Values are represented as mean± SD with n=3.

Quality controls	(µg/mL)	%Accuracy		
		Day 1	Day 2	Day 3
Low conc.	85	97.8 ± 0.5	100.5 ± 0.5	98.1 ± 1.0
Mid conc.	125	101.0 ± 1.0	102.2 ± 0.4	99.0 ± 0.2

High conc.	175	100.6 ± 0.3	101.0 ± 0.5	99.3 ± 0.2

Table 5. Precision.

Intra-day precision was performed on 3 different days with the same sample preparation method and instrument method. The %RSD for standards should be less than 2.0% (n=3).

Quality controls	(µg/mL)	%RSD		
		Day 1	Day 2	Day 3
Low conc.	85	0.54	0.47	1.01
Mid conc.	125	0.96	0.35	0.25
High conc.	175	0.28	0.53	0.24

Table 6. Repeatability.

The % RSD is calculated based on the peak areas of 3 injections of each sample.

The %RSD should be less than 2.0%. Samples were prepared at the level of 100 µg/mL lupeol (n=6).

Theoretical Concentration (µg/mL)	Actual Concentration (µg/mL)	%RSD	Recovery%	Recovery %RSD
100	101.05	0.36	100.44	0.71
100	100.58	0.18		
100	101.11	0.98		
100	100.36	0.75		
100	99.13	0.91		
100	100.43	1.09		

Table 7. LOQ and LOD.

	S/N ratio	Average		S/N ratio	Average
LOQ (6 µg/mL)	13.73	12.47	LOD (2 µg/mL)	3.08	3.9
	13.02			4.27	
	10.30			4.05	
	12.55			4.54	
	11.90			3.58	
	13.31			3.87	

3.4.2 Lupeol forced degradation study

For lupeol forced degradation studies, lupeol was exposed to different accelerated conditions. The degradation results were analyzed based on the chromatograms, recovery rate and peak purity of the samples. After exposing to thermal (65°C) and photolysis conditions, the concentration of lupeol did not decrease significantly (less than 6%, Table 8) and little degradant peaks were observed in the chromatograms (Figure 10, 11). Therefore, lupeol was determined to be stable under thermal and photolysis conditions. In the oxidative and acidic conditions, degradant peaks were fully resolved from the lupeol peak, suggesting that our method was stability indicating and able to distinguish lupeol peak from any degradants or impurities (Figure 12, 13). Lupeol recovery decreased by less than 10% in 0.5 N HCl and approximately 15% in 3% H_2O_2 (Table 8). Upon 0.5 N NaOH exposure, sample precipitation occurred at 24-hour time point. Thus, the concentration of lupeol dropped significantly after precipitation was observed in the samples (Figure 8). The recovery of lupeol reduced by almost 40% at the 168-hour time point (Table 8). It might indicate that lupeol was degraded by 0.5 N NaOH, however, the degradants were not soluble in the solvent and were filtered out by the 0.22 μm PTFE filter. Therefore, no obvious changes were observed in the chromatogram (Figure 14). To rule out the suspicion of impurities in the peaks, purity angle and threshold angle for each sample was evaluated. The purity angle represents the spectral heterogeneity of a peak based on the comparison of the spectrum over all of the peaks. The threshold angle is the sum of the noise angle and solvent angle. The purity flag indicates the spectral homogeneity based on the comparison of purity angle and threshold angle. If the purity angle is smaller than the threshold angle, there is no purity flag suggesting that there is no impurity co-eluting with the main API peak in HPLC. It is essential to evaluate the purity flag as not all the degradation peaks appear well-resolved in the chromatogram. Moreover, underlying peaks might

affect the integration of the peak and affect the result. For lupeol degradation studies, only one out of eighteen samples had an impurity flag (Table 8). In conclusion, our forced degradation studies provide confidence that the developed HPLC analysis method has high specificity and can distinguish degradants from the lupeol peak.

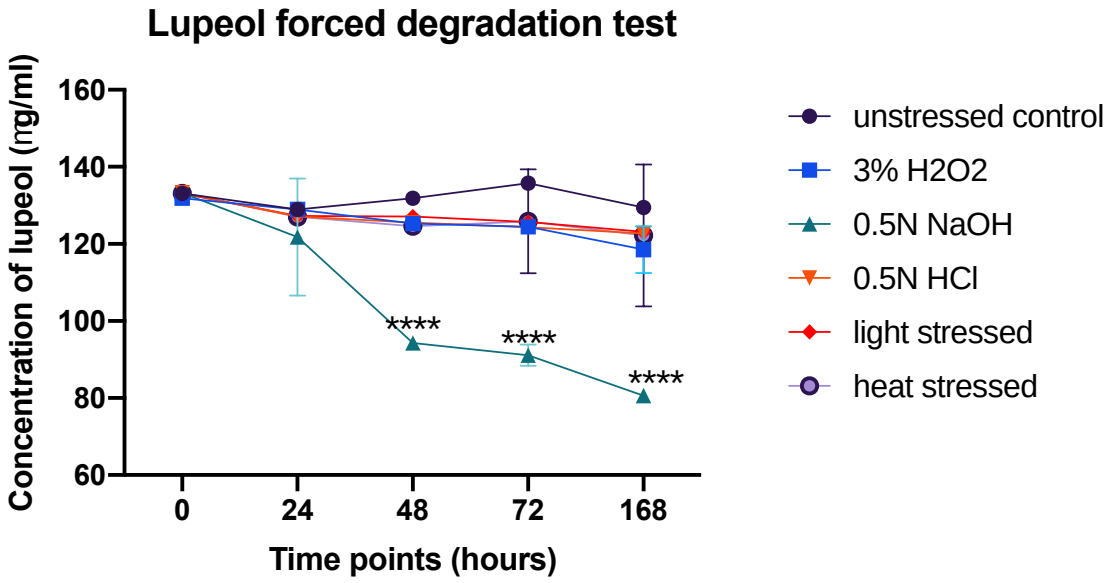

Figure 8. Lupeol concentration in accelerated conditions over 168-hour time point. Results are presented as the mean±standard deviation, where bars represent standard deviations of 3 different samples. At each time point, results were compared with time 0 result. Statistical significant (two-way ANOVA) is indicated by ****p<0.0001.

Table 8. Recovery and Peak purity of forced degradation samples at 168-hour time point. *Sample evaporation resulted in drastically increased concentration.

Conditions	Sample No.	Recovery (%)	Purity flag
Control	1	99.5	No
	2	99.5	No
	3	99.7	No
3% H_2O_2	1	85.8	No
	2	94.6	No
	3	93.2	No
0.5N NaOH	1	61.4	No
	2	60.9	No
	3	63.7	No
0.5N HCl	1	95.2	No
	2	94.5	No
	3	93.4	No
Photolysis	1	94.6	Yes
	2	94.6	No
	3	95	No
65ºC	1	93.9	No
	2	118.6*	No
	3	94.1	No

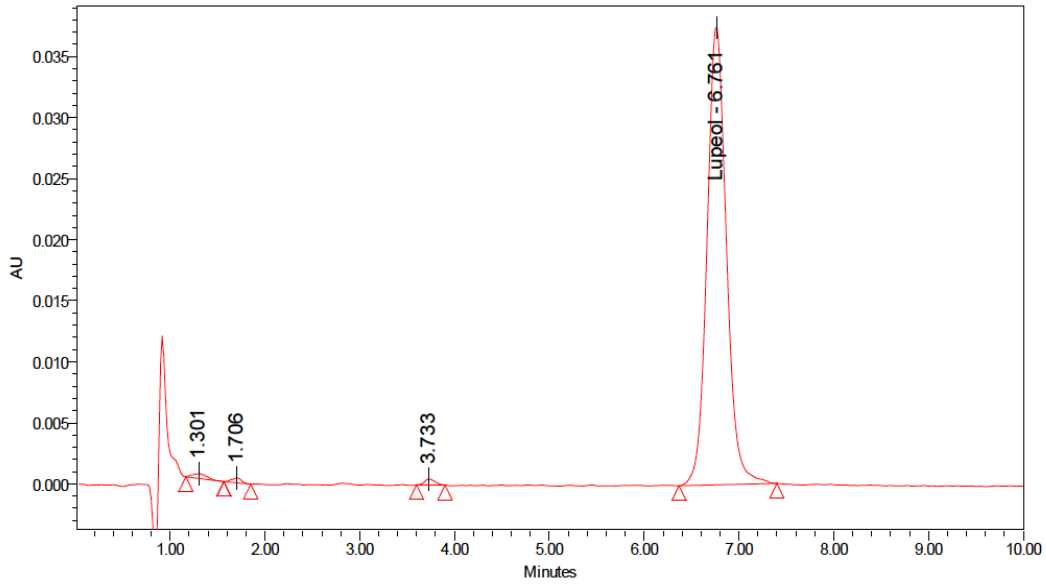

Figure 9. Chromatogram of the control samples in forced degradation studies

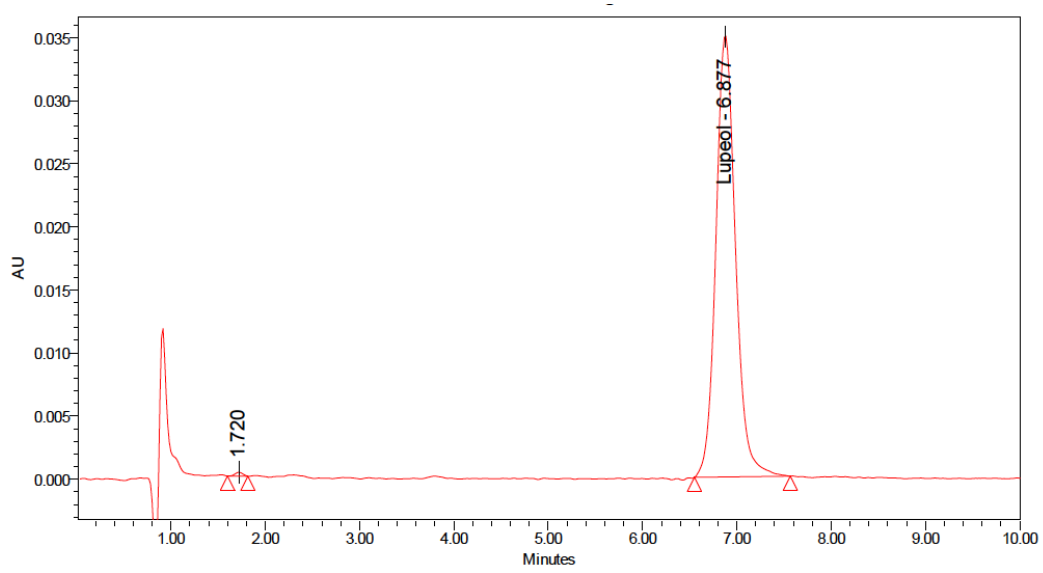

Figure 10. Chromatogram of the samples in light-exposed condition in forced degradation studies

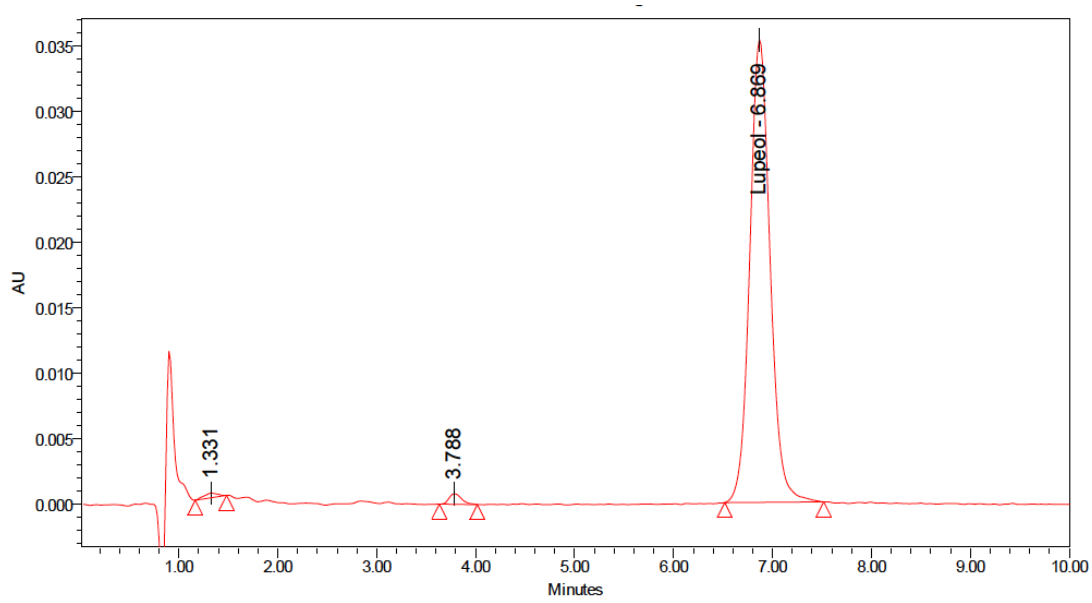

Figure 11. Chromatogram of the samples in heat-stressed condition in forced degradation studies

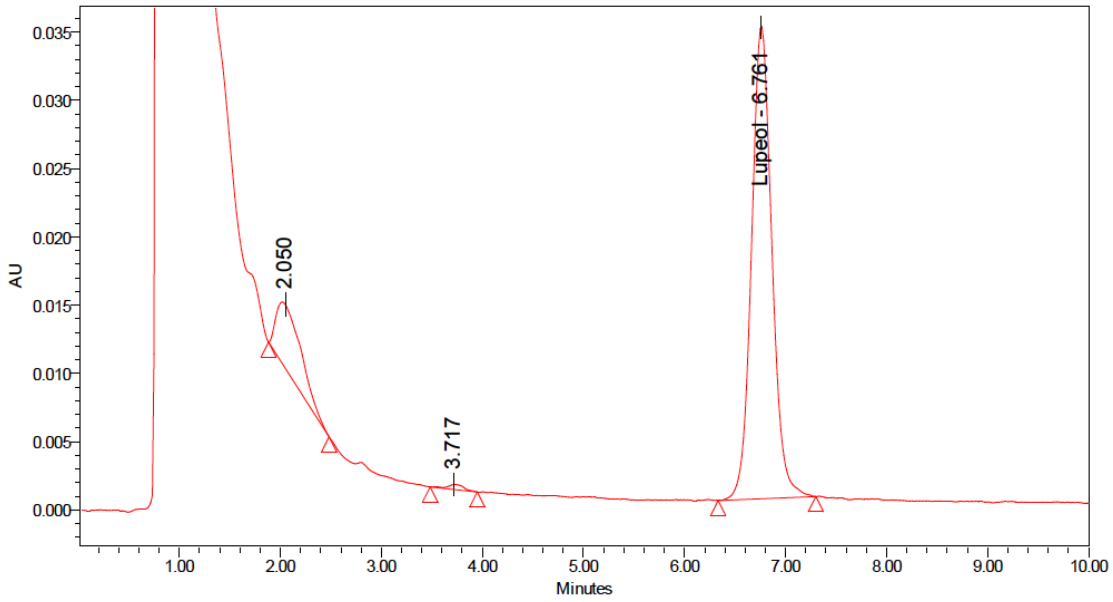

Figure 12. Chromatogram of the samples in 3% H_2O_2 stressed condition in forced degradation studies

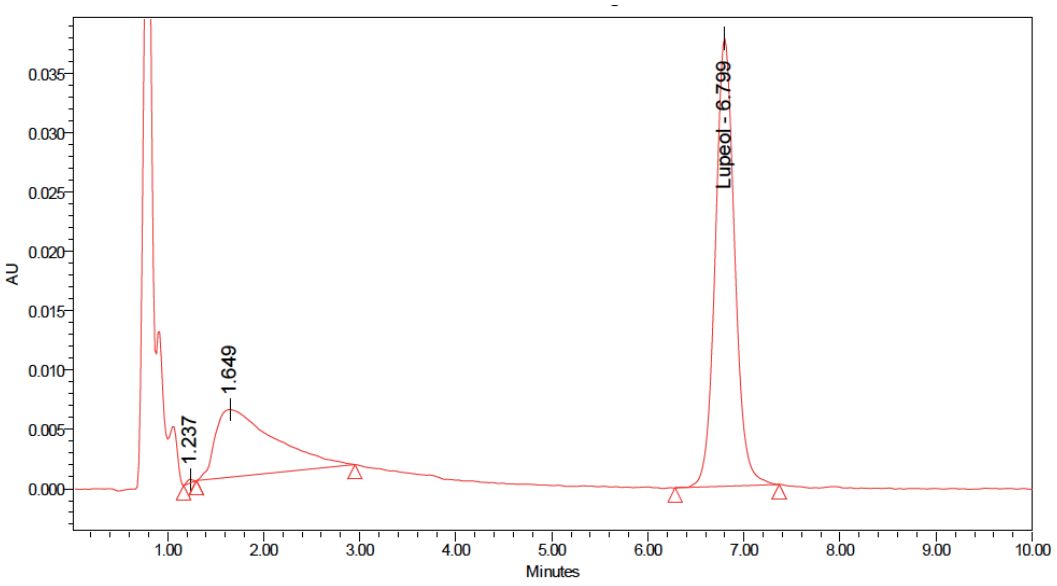

Figure 13. Chromatogram of the samples in 0.5N HCl stressed condition in forced degradation studies

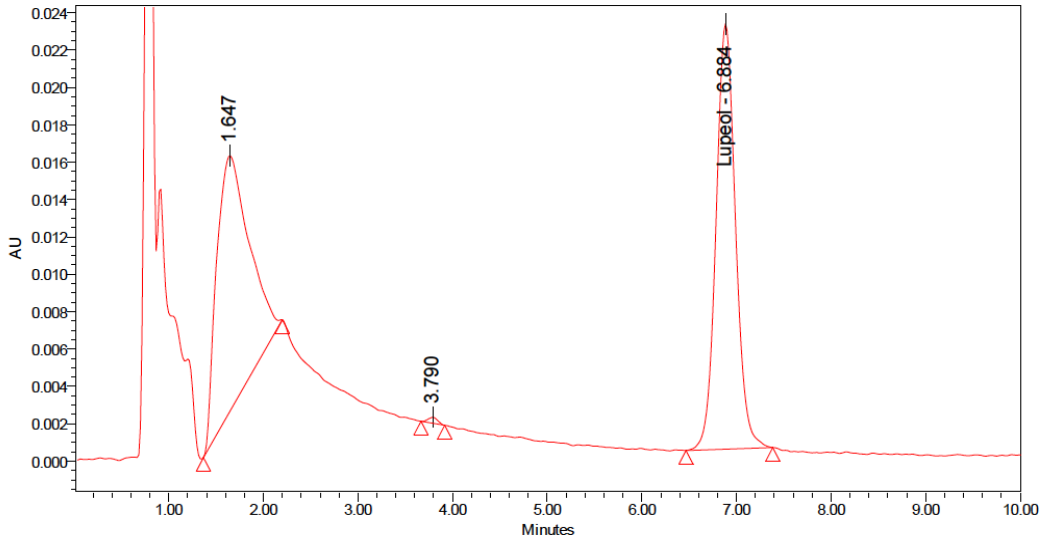

Figure 14. Chromatogram of the samples in 0.5N NaOH stressed condition in forced degradation studies

3.4.3 Lupeol solubility study

Lupeol is very slightly soluble in aqueous solutions and is soluble in organic solvents. The solubility of lupeol in various solvents is shown in Table 9. Solubility studies were used to direct the design of *in vitro* release test methods. Specifically, to determine the appropriate media for the dissolution testing, three different types of surfactants were evaluated. Tween 80 is a nonionic surfactant, which is mostly used for neutral compounds [126]. CTAB is a cationic surfactant and SDS is an anionic surfactant. These are the surfactants that are commonly used in dissolution methods for hydrophobic drugs. However, none of the surfactants provided sufficient solubility of lupeol for use in the dissolution media. Among the three concentrations of MeOH tested, 80% and 90% MeOH had solubility of 150 µg/mL and 1165 µg/mL respectively, which were considered as the candidates for the dissolution media. The 90% MeOH was chosen as the sample matrix for HPLC due to its greatest solubilization potential for lupeol.

Table 9. The solubility of lupeol in various solvents (n=1).

Solvent	Solubility (µg/mL)
70% MeOH	20
80% MeOH	150
90% MeOH	1165
90% EtOH	1400
90% ACN	398
0.5% SDS	20
1% SDS	29

1% CTAB	75
2% CTAB	123
5% CTAB	380

3.4.4 Lupeol cytotoxicity study

By calculating the relative viability of treatment groups compared with the control group, the concentration that has relative viability below 80% is considered as toxic. The testing concentrations were limited by the lupeol solubility in 100% EtOH (30 mM) [127]. The lupeol stock solution (30 mM) was later diluted using KFSM to maintain EtOH concentration at or below 0.2% and achieve lupeol concentration between 0.47 - 60 μM. Based on the results, only 60 μM lupeol group showed that the relative viability decreased to 79.0% after 72 hours exposure, which was lower than the target 80% relative viability (Figure 15). Based on the VK2 cell morphology observed under the microscope, a noticeable change of morphology was observed in groups treated with lupeol for 72 hours at concentrations above 15 μM. Cells were formed into clumps and detached from the plates. Therefore, 7.5 μM is considered the biocompatible lupeol concentration in vaginal epithelium cells. The IC50 of lupeol in human sperm cells was reported to be 109 nM [77]. Based on the toxicity data, the therapeutic window of lupeol can be suggested to be from 109 nM to 7.5 μM. However, it should be noted that *in vivo* studies are required to confirm this therapeutic range. The drug amount in lupeol film is determined based on the equation in figure 16 calculated using the IC50 of 109 nM and 2mL vaginal fluid volume. Since this dose will be small for reproducibly manufacturing films, the loading dose in films was increased to 1 mg. Thus,

the label claim of each 2"x2" film was 1 mg. Moreover, the theoretical lupeol concentration after vaginal application was 1.2 μM. In conclusion, the loading dose of lupeol in our immediate-release vaginal film is biocompatible with the vaginal epithelium cells.

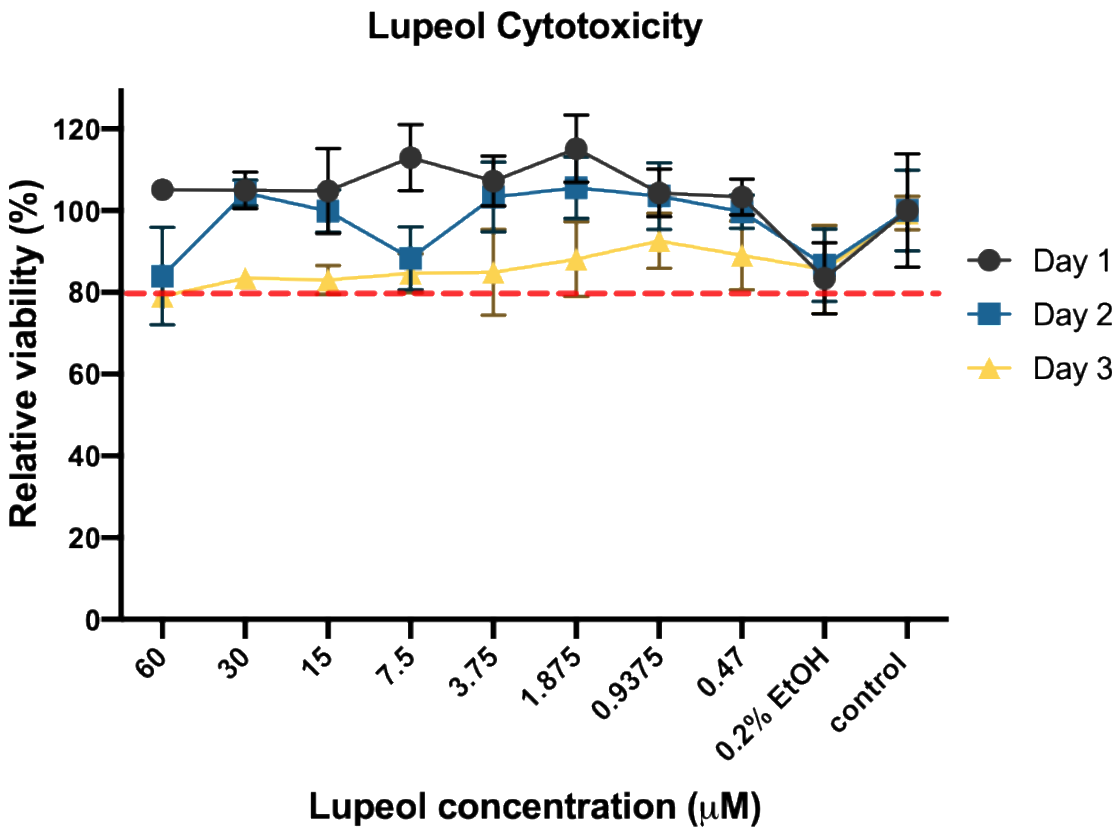

Figure 15. Lupeol cytotoxicity. Dash line: The threshold (80% relative viability) to determine the biocompatible drug concentration in VK2 cell line. The range of testing concentration was 0.47 – 60 μM. Control group was only incubated with KFSM media which did not contain 0.2%EtOH or lupeol.

*Therapeutic dosage in human (g) =conc. (M) * fluid volume (L) * 1000 * the molecular weight (g/mol) of the compound.*

Figure 16. The conversion of drug concentration to therapeutic dosage

3.5 Summary

The pre-formulation studies evaluated the physical and chemical properties of lupeol. We have determined the sample matrix for HPLC method and the media for dissolution studies based on the solubility studies. An HPLC method was successfully developed and validated despite the high hydrophobicity of lupeol posing problems with sample preparation. The forced degradation studies have generated the stability profile of lupeol under the accelerated conditions and identified 0.5 N NaOH as the major degradation condition of lupeol that should be avoided during the development process.

The cytotoxicity profile of lupeol was evaluated in the vaginal epithelial cell line VK2/E5E6. The epithelial cells in the female reproductive tract are the primary site of sexually transmitted infections, including HIV. They serve as a physical barrier against initial HIV and STD acquisition [128]. It is essential to examine the epithelial integrity and the epithelial cell viability when developing vaginal delivery products as any damage to the epithelial cells can increase the risk of infection. Therefore, the cytotoxicity study of the drug in epithelial cells is important as it determines the non-toxic concentration based on the effect on cell viability. Our studies demonstrated that 7.5 μM was the biocompatible concentration in VK2 cell line, which is greater than the theoretical lupeol concentration expected after the application of films into the vagina (~1.2 μM). In conclusion, the drug dosing of lupeol films (1mg) determined based on the IC50 manufacturing feasibility, and cytotoxicity of lupeol in sperm cells is biocompatible with the vaginal epithelium cells.

4.0 Formulation development and assessments

4.1 Introduction

To deliver drugs vaginally, polymeric thin films are advantageous because of their high efficiency in releasing the drug, negligible vaginal leakage compared with conventional vaginal gel products and individual privacy for each user. Gel and cream vaginal dosage forms have been associated with messiness and leakage leading to decreased user compliance and loss of therapeutic efficacy [129]. Bio-adhesive vaginal films can overcome these limitations because films dissolve rapidly in the vagina forming a viscous and bio-adhesive gel with minimal contribution to the vaginal fluid content, thus minimizing leakage [120]. Also, vaginal films are an economically feasible dosage form and are stable during storage and transportation [130]. Many studies have been conducted to investigate the potential of combining anti-infective drugs and contraceptives to deliver drugs locally and prevent STDs and pregnancy [131, 132]. Films provide a female-controlled option that puts the discretion into women's hands [133].

4.1.1 Immediate-release vaginal film development

Excipients play an important role in determining the properties of the film. A typical film formulation includes film-forming polymers, plasticizers, disintegrants, dispersing aids, and solvents [129]. All of the ingredients should be non-toxic and non-irritating to the vaginal cells. The type and amount of excipients can be modified to achieve different film properties, such as the drug release rate, disintegration, drug loading capacity and film elasticity. The film-forming

polymers are an essential component of the formulation. They affect the loading capacity of the drug and the toughness of the film. The plasticizers are used to enhance the softness and flexibility of the film; they also reduce the brittleness by improving the mobility of the polymer chains, decreasing the intermolecular forces, and reduced glass transition temperature of the system [134]. Further, they reduce the viscosity of the polymer solution and allow uniform dispersion of the drug.

Two methods have been used to manufacture film dosage form, solvent casting and hot-melt extrusion. The solvent casting technique is most widely used. It is an easy, inexpensive and convenient setup that can be applied at the laboratory scale [135]. Moreover, solvent casting is ideal for manufacturing films that contain heat-sensitive API since a relatively low temperature is needed for the removal of the solvent, whereas hot-melt extrusion (HME) normally requires much higher temperatures. Solvent casting involves multiple steps which includes preparation of a homogenous excipient solution or dispersion, casting the solution onto a substrate, drying the film sheet, and cutting the film sheet into unit doses. On the other hand, HME avoids the use of a solvent. Since HME manufactures the films through extrusion processes using high temperature, pressure and sheer, it is suitable for a wide variety of APIs regardless of BCS class as the uniformity of matrix does not rely on solubilization or dispersion [136, 137].

4.1.2 Short-term stability studies of lupeol immediate-release vaginal films

Drug product testing includes characterization for physicochemical properties, evaluation of dissolution profile and stability studies for the films. Stability studies are essential studies for the drug development process because they not only determine the integrity and safety of the drug but also the shelf life and storage conditions of the products. Stability studies assess the influence

of environmental factors, such as temperature and relative humidity (RH) on the quality (physical, chemical, and biological properties) of a drug product under. According to ICH guidelines, in climatic zone II (i.e. US), stability studies for a drug product should be evaluated at 25°C/60% RH and at an accelerated ambient condition, 40°C/75% RH, for its thermal stability and sensitivity to moisture [138].

4.1.3 Dissolution studies of lupeol immediate-release vaginal films

In the process of developing a pharmaceutical product, *in vitro* drug dissolution testing is used to evaluate how an API is released from the formulation. It can be used for both quality control purposes to evaluate batch-to-batch consistency and the stability of the product, and for the purpose of determining the *in vitro-in vivo* correlation (IVIVC) to predict drug release profile *in vivo*. Dissolution studies are performed at 37°C to simulate the physiological temperature in the human body. To develop a dissolution test, there are several factors that need to be examined to achieve a robust and biorelevant method. Components such as the apparatus type, sink condition, media composition and properties (e.g. pH, osmolality), and the agitation rate should be evaluated to ensure they are appropriate for the test product. A commonly used apparatus for testing *in vitro* drug release for film products is a USP I (basket) [139]. A second apparatus which has been applied is a USP 4 (flow-through-system). It is important to maintain sink condition throughout release testing. Sink condition is the ability of the dissolution media to dissolve at least 3 times the amount of the API in the dosage form [140]. Based on the Noyes-Whitney equation (Figure 17), the dissolution rate is proportional to the (Cs-Ct), where Cs is the concentration at saturation and Ct is the concentration at a given time [141]. Therefore, if the concentration gets close to the saturation, the dissolution rate would be slowed. If the sink condition is achieved, the dissolution

rate will be solely dependent on the drug release rate from the product but not the solubility of the drug. Under sink conditions, the drug concentration (Ct) will be extremely small and considered negligible in the bulk medium. In other words, the concentration gradient (Cs-Ct) tends toward Cs, and the impact of drug solubility on dissolution becomes negligible.

$$\frac{dX}{dt} = Rate = \frac{DA}{h}(Cs - Ct)$$

Figure 17. Noyes-Whitney equation. M: mass (mg), t: time (s), D: diffusion coefficient (cm^2/s), A: surface area of the drug (cm^2), h: thickness of the diffusion layer (cm), Cs: saturation solubility of the drug (mg/cm^3), Ct: concentration of the drug dissolve at a particular time (mg/cm^3) [114].

4.1.4 Formulation optimization to develop a lupeol optimized prototype film

Initial dissolution testing showed that the developed lupeol film did not achieve the desired release profile. This issue could be due to decreased disintegration of hydrophilic matrix in 90% MeOH which was selected as dissolution media based on lupeol solubility. Therefore, increasing the lupeol solubility in formulation will allow the usage of aqueous-based solution as dissolution media. As a result, drug release will be improved in dissolution studies. For this reason, optimization of formulation was conducted and investigated to improve the drug solubility of lupeol in the formulation and drug release from lupeol films. Several solubilizers such as the Labrasol®, Labrafil M 1944 CS®, Capryol 90®, Lauroglycol 90® and Transcutol P® were selected as candidates to improve lupeol solubility in aqueous solution [142]. Labrafil® (oleoyl polyoxyl-6 glycerides) is generally used as a soluble surfactant and oral bioavailability enhancer. Capryol 90® (propylene glycol monocaprylate) is a water insoluble cosurfactant as well as a topical penetration

enhancer. Labrasol® (caprylocaproyl macrogol-9 glycerides) is a self-emulsifying surfactant and Transcutol P® (diethylene glycol monoethyl ether) is a strong solubilizer. Lauroglycol 90® (propylene glycol monolaurate type II) is a solubilizer for poorly-soluble drugs and bioavailability enhancer. Additional solubility studies of lupeol using these solubilizers suggested that Transcutol P® and Capryol 90® were the most effective at solubilizing lupeol. These solubilizers are widely used in topical cream formulations which have been approved by the FDA.

Solubilizers can be categorized into oil, surfactant, and co-surfactants [143]. Co-surfactants are used with surfactants and oil to increase the solubility and enhance the dispersibility of drugs in aqueous phase. These oil, surfactant and co-surfactant mixtures are commonly used in self-emulsifying drug delivery systems (SEDDSs) to improve the oral absorption of highly lipophilic drug compounds [144]. When introduced into an aqueous solution with gentle mixing, SEDDSs emulsify spontaneously into fine oil-in-water emulsions. Therefore, by introducing this system into our formulation design, we are aiming to develop a lupeol film that has increased total drug release and enhanced solubility of lupeol. The goal of this strategy is to improve the product effectiveness.

4.2 Materials

Labrasol®, Labrafil M 1944 CS®, Capryol 90®, Lauroglycol 90® and Transcutol P® were obtained from Gattefosse (Paramus, NJ). HPMC (Methocel™ E5) was obtained from the Dow Company (Midland, MI). CMC 7LF PH (Sodium carboxymethylcellulose), PEG 400 and glycerin were obtained from Spectrum Chemicals and Laboratory Products (Gardena, CA). HEC (Natrosol™ 250L) was obtained from Ashland Global Chemical Company (Wilming, DE). Oasis®

Hydrophilic-Lipophilic-Balanced (HLB) cartridges were purchased from Waters (Milford, MA). HYDRANAL™ Water Standard KF-Oven (140-160 °C) and HYDRANAL™ - Composite 2 were purchased from Honeywell Fluka™ (Muskegon, MI).

4.3 Methods

4.3.1 Lupeol immediate-release vaginal film development (original prototype films)

Lupeol films were manufactured using a solvent casting technique. The film components are listed in Table 10. The preparation of polymer solution was initiated by pre-mixing the dry ingredients, including 6 g hydroxyethyl cellulose (HEC), 6 g hydroxypropyl methylcellulose (HPMC), and 2 g sodium carboxymethylcellulose (CMC). The dry mix was then gradually added into 100 mL milli-Q water at room temperature in a beaker. The solution was stirred overnight using an overhead stirrer. 55.2 mg of lupeol was dispersed in 2 mL of EtOH using probe sonicator (Vibra Cell Sonics, probe diameter: 2 mm) and then added to 2 g of glycerin. The lupeol dispersion was then added into the polymer solution. To allow for adequate dispersion and polymer hydration, lupeol polymer solution was stirred overnight. The mixture was then cast onto the heated (71°C) surface of the film applicator (Elcometer 4340 automatic Film Applicator), which was lined with a PET (polyethylene terephthalae) sheet. Thickness of the polymer cast was controlled using a doctor blade set to thickness of 0.11mm. After 12 minutes, the dried film sheets were then cut into 2”x 2” units and stored in aluminum foil pouches. The targeted drug loading in a 2”x 2” film was 1 mg. The drug loading was selected to provide vaginal concentrations that are several logs above the IC50 of lupeol (Equation 1).

Table 10. Components of the lupeol immediate-release film formulation.

Ingredients	Role	Percentage %w/w
Hydroxyethyl cellulose (Natrosol 250L)	Film forming	6
Hydroxypropyl methylcellulose (Methocel E5)	Film forming	6
Sodium carboxymethylcellulose (CMC 7LF PH)	Film forming	2
Glycerin	Plasticizer / Dispersing aid	2
Lupeol	API / contraceptive agent	0.0552
Milli-Q water	Solvent	84
EtOH	Dispersing aid	2*

* EtOH evaporated after solvent casting.

4.3.2 Physicochemical characterization of lupeol films

The characterization of the film included weight, thickness, appearance, water content, disintegration time, puncture strength, contact angle, drug content, content uniformity and dissolution. The weight of the film was measured using the analytical balance. The thickness of the film was measured using the digital thickness gauge (Mitutoyo 547-520S). The appearance (texture and color) of the film was visually evaluated.

4.3.2.1 Water content

The water content was assessed using the Karl Fischer method (Metrohm, 890 Titrando). In Karl Fischer method, MeOH and sulfur dioxide reagents were used. They interact to form an intermediate alkylsulfite salt, which could be oxidized by the iodine present in the Karl Fischer reagent. This reaction is an oxidation reaction and it consumes water. Therefore, the water presented in the samples promotes the oxidation reaction and consumes the iodine. When the water present in the sample is depleted, the excess iodine will be detected electrometrically by the titrator's electrode. It will signal the endpoint of the titration. The instrument will calculate the amount of water present in the samples according to the concentration of the iodine in the reagent and the amount of the reagent that is consumed during the titration analysis and provide the results as %w/w water.

4.3.2.2 Puncture strength

Puncture strength was determined using a previously reported method [145]. Briefly, the film was placed on the TA-108S5 fixture with only a flat round surface of the film was exposed. TA8A 1/8 probe was placed on top of the film and punctured the film at a rate of 1.0 mm/sec and a trigger force of 1.0g. The maximum force required to penetrate the film was recorded using TA.XT texture analyzer as the puncture strength. The puncture strength was then calculated with the equation below (Figure 18):

$$\text{Puncture strength} = \frac{Force\ (kg)}{Film\ thickness\ (mm)}$$

Figure 18. Puncture strength

4.3.2.3 Disintegration time

Film disintegration time was measured using a previously developed method that employed TA.XT texture analyzer [146]. The program measured the time difference between the application of 15 µL of water onto the film and the penetration of probe through the film as the force applied equals to zero. The time difference was then normalized by the thickness of the film (Figure 19):

$$\text{Disintegration time} = \frac{Disintegration\ time\ (s)}{Film\ thickness\ (mm)}$$

Figure 19. Disintegration time

4.3.2.4 Contact angle

To examine the surface hydrophilicity property of films, the contact angle of water on the films was measured using the optical tensiometer (Biolin Scientific). Each film was assessed in triplicate by placing water droplets at three different positions and the contact angle for the water formed water droplet was recorded. The volume of the attached water droplets and the recording time frame were kept the same for repeatability. The contact angle (CA°, θ) is defined as the angle created by a liquid with a solid or liquid boundary that it was placed on. The lower value of the contact angle, for instance lesser than 90°, indicates that the surface was wetted by the liquid and has better wettability.

4.3.2.5 Drug content and content uniformity

To determine the drug content, the film was first dissolved in Milli-Q water and MeOH to form a 70% MeOH matrix. Lupeol was extracted by performing solid-phase extraction (SPE) before HPLC analysis. Film drug content uniformity was evaluated by randomly selecting films

from six different locations within the same sheet and then quantifying lupeol content in each sample.

SPE is a purification technique where substances dissolved or suspended in a liquid solvent are separated from the matrix by being adsorbed to the cartridge containing solid adsorbents. Reverse phase SPE can retain non-polar analytes since the hydrophilic groups of the silica are chemically modified with hydrophobic alky or aryl functional groups. The typical extraction procedure of reverse phase SPE involves four basic steps (Figure 20), which are conditioning, sample loading, washing, and eluting. The SPE procedure for lupeol extraction includes conditioning the cartridge with 1 mL of MeOH and 1 mL of Milli-Q water, loading the cartridge with 1 mL of sample solution, washing the cartridge with 1 mL of Milli-Q water and eluting the cartridge with 1.5 mL of MeOH. The eluent was then evaporated and reconstituted with 90% MeOH before HPLC analysis. The vacuum port was applied to the SPE manifold to efficiently elute the solution.

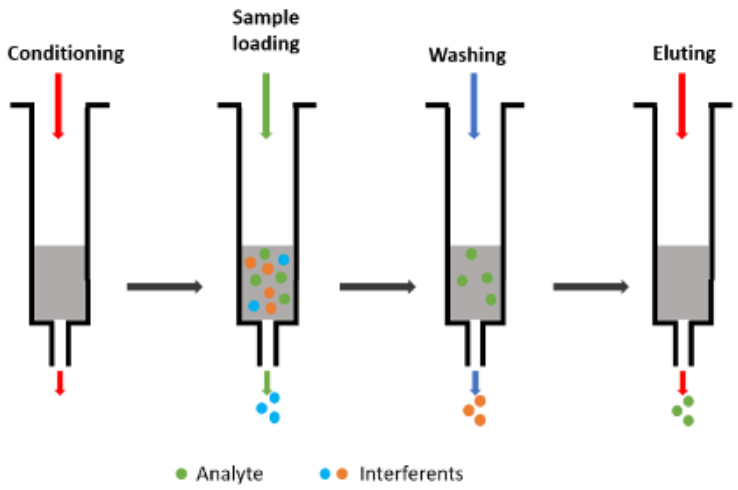

Figure 20. Shematic showing steps involved in solid phase extraction

4.3.3 Short-term stability study of lupeol original prototype films

Lupeol films were individually sealed in aluminum foil pouches and were stored into two different conditions, 25°C/60% RH and 40°C/75% RH, to perform short term film stability studies for 3 months. At specific time points, films were tested for drug content, puncture strength, disintegration time, water content and contact angle using the method described previously.

4.3.4 Dissolution development

The dissolution profile of lupeol films was determined using a DISTEK USP I apparatus. Under 25°C and 37°C, 45mL of 80% and 90% MeOH was used as the dissolution media, and the rotating rate of the 40-mesh basket was 50 rpm. The media were sampled up to 3 hours after the films were placed in the media. At predetermined time points, 3 mL of the media was collected and replaced with 3 mL of fresh media. The samples were then filtered with 0.22 μm PTFE filter, before being analyzed using the HPLC method described in section 3.3.1. Because a large media volume was used, the film polymers were greatly diluted in 45mL media. Thus, the cleanup of film polymers from collected dissolution samples using SPE method was not needed before HPLC analysis. A different calibration curve was used for the HPLC analysis in dissolution development. The calibration curve range of lupeol was between 10 to 100 μg/mL. The concentrations of quality controls were 15, 20, and 35 μg/mL. The standard samples were prepared in 80% MeOH.

4.3.5 Lupeol immediate-release vaginal film optimization (optimized prototype films)

In order to increase drug release from the films, additional solubilizers Capryol 90®, Transcutol P® and PEG 400 were introduced to the original formulation to improve the solubility of lupeol. This optimized prototype film was manufactured by a solvent casting technique. The ingredients are listed in Table 11. The mixture of Transcutol P®, Capryol 90® and PEG 400 (1:1:1) were heated to 40°C to dissolve lupeol. Milli-Q water was added in a dropwise manner while hand mixing. The mixture was then subjected to probe sonication in ice bath for 30 minutes until the solution was distributed uniformly. HPMC, HEC and CMC polymer powder mix were gradually added into the lupeol emulsion using an overhead stirrer and stirred overnight. The mixture was then cast onto a preheated (71°C) film applicator (Elcometer 4340 automatic) that was lined with PET liner secured under vacuum to allow for water evaporation within 12 minutes. The film sheets were then cut into 2"x2" units and stored in aluminum foil pouches.

Table 11. Components of the lupeol optimized film formulation

Ingredients	Role	Percentage %
Hydroxyethyl cellulose (Natrosol 250L)	Film forming	6
Hydroxypropyl methylcellulose (Methocel E5)	Film forming	6
Sodium carboxymethylcellulose (CMC 7LF PH)	Film forming	2
Glycerin	Plasticizer / Dispersing aid	2
Transcutol P	Solubilizer	3.3
Capryol 90	Solubilizer	3.3

PEG 400	Solubilizer	3.3
Lupeol	API / contraceptive agent	0.0791
Milli-Q water	Solvent	74

4.4 Results

4.4.1 Lupeol extraction using solid-phase extraction method

Three experiments were designed for SPE method development. Samples tested for each experiment are listed below:

Table 12. Samples used in experiments for solid-phase extraction method development

Samples	Components		Note
	Lupeol	Film polymers	
Lupeol spiked-control samples	Yes	No	50 µg/mL lupeol solution
Lupeol-loaded films	Yes	Yes	1 mg of lupeol per film
Lupeol spiked-placebo films	Yes	Yes	Placebo films spiked with lupeol (100, 125, 150 µg/mL)

4.4.1.1 The recovery of lupeol spiked-control samples after SPE

To develop an SPE method for extracting lupeol from a polymeric matrix, Oasis HLB cartridge was selected based on the property of the sorbent and the hydrophobic characteristic of lupeol. Sample matrix preparation is important since it determines the behavior and property of

the compound while it interacts with the cartridge and affects the extraction efficiency of the compound. Because the solubility of lupeol is higher in organic solvent than in aqueous solution, different percentages of MeOH were tested for extraction efficiency. In 70%, 80%, and 90% MeOH, the solutions were spiked with lupeol stock solution to achieve a nominal concentration of 50 μg/mL (Table 12). After the extraction, the drug content in the samples was analyzed using HPLC analysis.

The results showed that the recovery rate was 96.17% for samples that were prepared in 90% MeOH (Figure 21A). 80% and 70% MeOH resulted in higher recovery rates (101.82% and 99.8%, respectively). These results imply that lupeol had greater affinity to 90% MeOH solution than the cartridge, and thus, the cartridge was not as effective at capturing lupeol. Therefore, use of 80% and 70% MeOH solution in the solid-phase extraction method was further tested with films containing a known amount of lupeol to determine the extraction efficiency in the presence of film polymers.

4.4.1.2 The recovery of lupeol-loaded films after SPE

Lupeol-loaded films were manufactured with 1mg of lupeol to determine the extraction efficiency in the presence of film components (Table 12). To prepare lupeol-loaded film samples in 70% and 80% MeOH, films were disintegrated with milli-Q water before MeOH was added to dissolve lupeol. Afterward, samples underwent the same SPE procedure as described previously (Figure 20). The recovery rate was 91.60% for samples prepared in 70% MeOH and 93.57% for samples prepared in 80% MeOH (Figure 21B). However, for samples prepared in 80% MeOH, one of them failed to be eluted from the cartridge even with an increased vacuum force. This suggested that 80% MeOH failed to dissolve the film polymers leaving undissolved polymer

particles in the matrix, and thus, disrupting the extraction process. Therefore, 70% MeOH was selected as the sample matrix for solid-phase extraction.

4.4.1.3 Recovery of lupeol spiked-placebo films after SPE

The validation of the SPE method was conducted using lupeol spiked-placebo films at three different levels (80%, 100%, and 120%) of the target concentration (125 μg/mL) (Table 12). The results showed that SPE had around 100% recovery rate in all the levels of target concentration with low standard deviation (Figure 21C). This indicated that the developed SPE method has sufficient drug extraction efficiency for analyzing drug content of lupeol films.

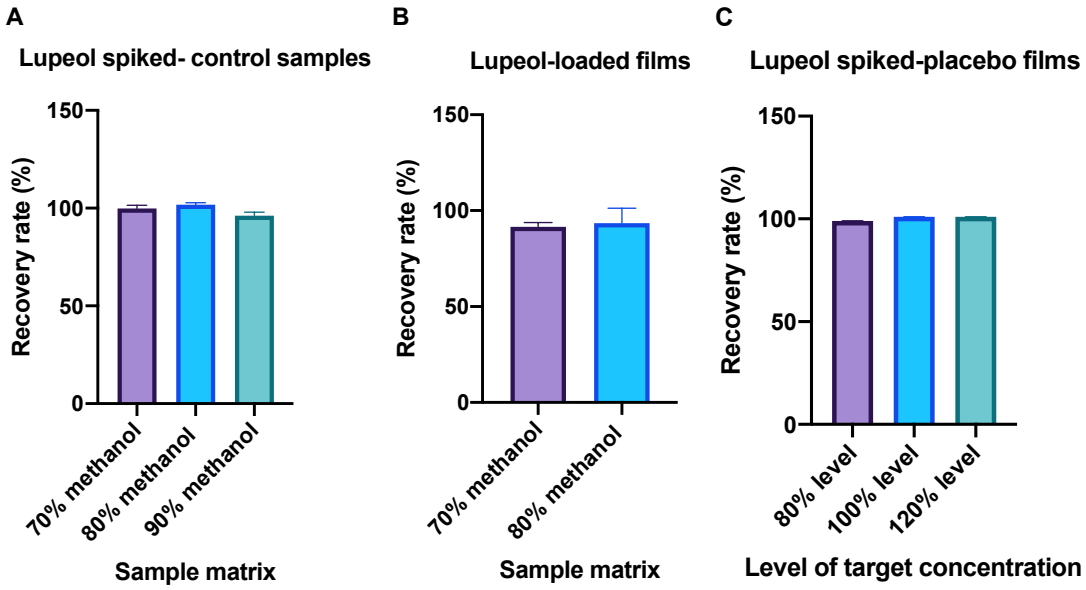

Figure 21. Solid-phase extraction (SPE) method development. A) Recovery of lupeol spiked-control samples in different sample matrices after SPE (n=3). B) Recovery of lupeol-loaded films in different sample matrices after SPE (n=3). C) Recovery of lupeol spiked-placebo films in three levels of target concentration (n=3).

4.4.2 Physicochemical properties of lupeol original prototype films

Lupeol immediate release films consisting of HEC, HPMC, CMC, glycerin, water and lupeol were manufactured using solvent casting technique. The physicochemical properties of the lupeol films were evaluated (Table 13). Drug was extracted from the lupeol films using SPE and then quantified by HPLC analysis. Sufficient drug loading and good content uniformity were achieved for this original prototype film. The drug loading was found to be 1.036 ± 0.064 mg. The drug was uniformly distributed within each film (RSD <5%). The average weight of the films was 267.63 ± 15.5 mg and the average thickness of the films was 93.0 ± 0.7 µm. The average contact angle was 74.5 ± 2.88 CA°. The average water content in the films was 7.12 ± 0.39 % w/w and met target specification (<10% w/w). The puncture strength and disintegration time were measured by the texture analyzer method and were found to be 7.59 ± 1.57 kg/mm and 987.29 ± 155.9 sec/mm, respectively.

Table 13. Physicochemical properties of lupeol original prototype films.

Values are represented as mean± SD with n=6.

Characterizations	Results
Drug content (mg)	1.036 ± 0.064
Recovery (%)	117.79 ± 1.62
RSD (%)	1.38
%w/w	0.377 ± 0.005
Weight (mg)	267.63 ± 15.5
Thickness (µm)	93.0 ± 0.7
Contact angle (CA°)	74.5 ± 2.88

Water content (%)	7.12 ± 0.39
Puncture strength (kg/mm)	7.59 ± 1.57
Disintegration time (sec/mm)	987.29 ± 155.9

4.4.3 Short-term stability study of lupeol original prototype films

Lupeol film stability studies were carried out at 25°C/60% RH and at accelerated condition 40°C/75% RH for a period of 3 months. During this time, the puncture strength of the films remained stable (Figure 22A). Although the mean contact angle increased at the 2 week-time point, it remained stable afterwards (Figure 22B). The disintegration time of the films decreased significantly at 1 month-time point but remained consistent at all later time points (Figure 22C). Change over time was also observed in water content tests (Figure 22D). A significant decrease in water content of the films occurred at the 1 month-time point. Notably, the residual water content of the film products remained within the target specification (< 10% w/w). The changes observed in the physical characterizations may suggest that water evaporation occurred within the samples over time. As for the drug content, the acceptable range is 90%-110% of label claim. The results showed that the drug content remain unchanged during the 3-month time period (Figure 22E). Overall, although it appears that water evaporation may have occurred during storage, the drug content of the films was unaffected and remained stable for the 3-month time period.

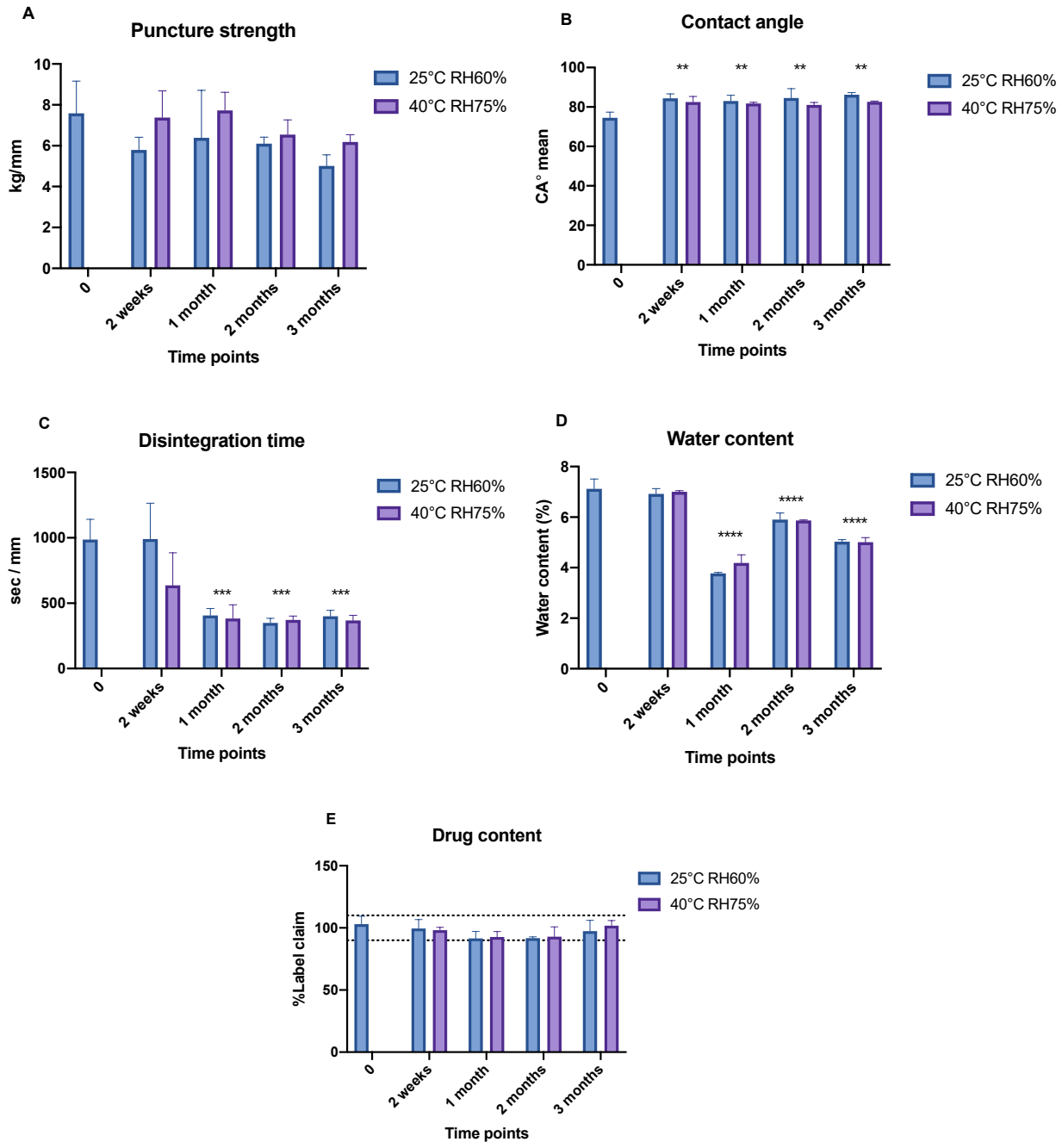

Figure 22. Physicochemical characterizations of lupeol films from the stability study. Lupeol films were stored for 3 months in 25°C/60% RH and 40°C/75% RH. A) Puncture strength, B) contact angle, C) disintegration time, D) water content and E) drug content of lupeol films.The dashed lines in E) represent the acceptable label claim range (90-110%). Results were presented as the mean and bars represented standard deviations from 3 different film samples. At each time point, results were compared with time 0 result. Statistical significant (two-way ANOVA) is indicated by *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

4.4.4 Dissolution study of lupeol original prototype films

Prior to initiation of dissolution testing of the films, studies were conducted to identify the optimal dissolution media. In these studies, two different MeOH water solutions were tested at two different temperatures. Because of the relatively low drug loading in the film, the volume of the media was limited in order to achieve quantifiable concentration of lupeol in HPLC. Given the high solubility of lupeol in organic solvents, 80% and 90% MeOH solutions were tested. It is noteworthy to mention that MeOH will impact the integrity of the film by interacting with polymer components in the formulation. Although HPMC is soluble in MeOH and constitute 6% composition of the total formulation, the majority of polymers in the formulation are composed of HEC (6%) and CMC (2%) which are insoluble in MeOH [147-149]. Therefore, the insolubility of HEC and CMC may limit the disintegration and solubilization of the film in 80% and 90% MeOH solutions.

The dissolution profile demonstrated a burst release of drug from the film in the first 20 minutes (Figure 23). In the experiments conducted at 25°C (Figure 23A, B), the drug release reached plateau at around 60 minutes whereas in the experiments performed at 37°C, the drug release reached plateau at around 40 minutes (Figure 23C, D). This suggested that elevated temperature increased the release of lupeol from the films. The results demonstrated that at 37°C, 87.8% drug release was obtained using 80% MeOH as the dissolution media, whereas in 90% MeOH drug release was slightly decreased (78.9%) (Table 14). Moreover, in 90% MeOH as the media, there was no significant increase in the percentage of drug release as the temperature elevated. The reason may be due to the insolubility of HEC and CMC in MeOH resulting in low disintegration property of polymeric thin film in 90% MeOH. Therefore, 80% MeOH in 37°C was chosen as the final condition for the dissolution test.

Table 14. The average percentage of cumulative drug release. Values are represented as mean± SD with n=3. Results of experiments conducted at 37ºC were compared with their respective experiments conducted at 25ºC. Statistical significant (two-way ANOVA) is indicated by ns (no significance) and * p<0.05.

Avg% Drug Release		Media	
		80% MeOH	90% MeOH
Temperature	25ºC	78.2 ± 3.4	81.6 ± 0.4
	37ºC	87.8 ± 5.2*	78.9 ± 0.5ns

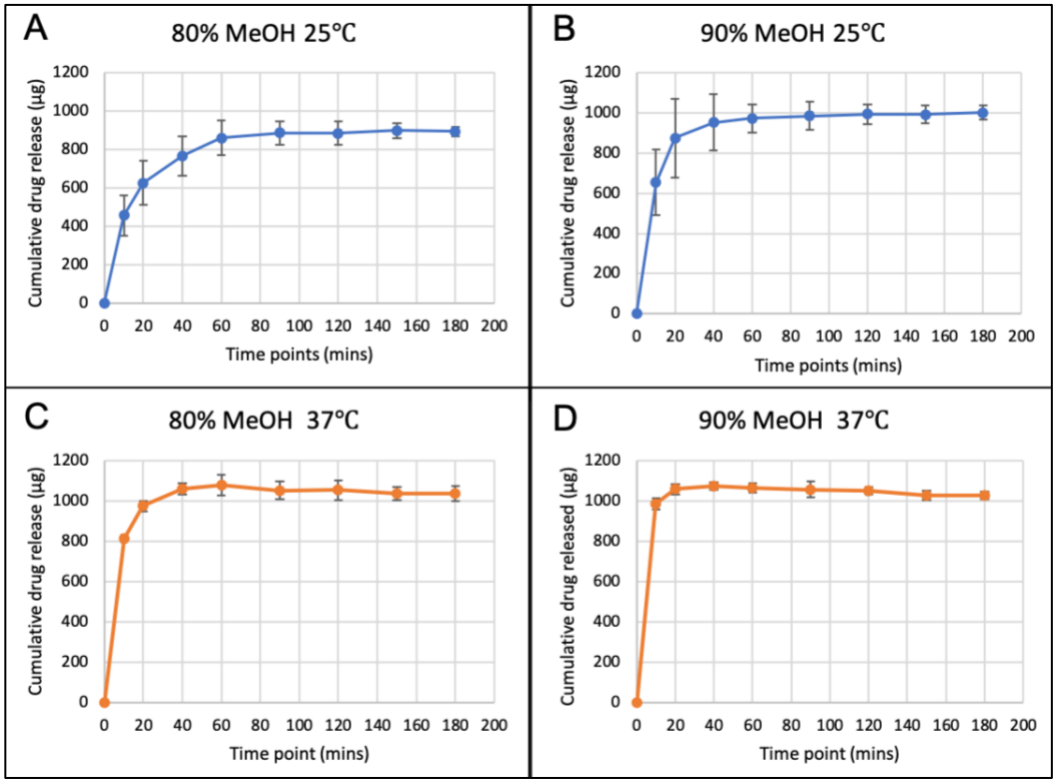

Figure 23. Dissolution profile of lupeol films in various conditions (n=3). A) 80% MeOH as dissolution media at 25°C. B) 90% MeOH as dissolution media at 25°C. C) 80% MeOH as dissolution media at 37°C. D) 90% MeOH as dissolution media at 37°C.

4.4.5 Formulation optimization of lupeol optimized prototype film

4.4.5.1 Physicochemical properties of lupeol optimized prototype films

Under the conditions (37 ºC, 80% MeOH) of dissolution testing, 88% of the loaded lupeol was released from the film. In order to increase the total amount of drug release from the films, additional solubilizers i.e. Capryol 90®, Transcutol P® and PEG 400, were introduced into the formulation to improve the solubility of lupeol. This optimized prototype film was then characterized for its physicochemical properties using the methods described in previous 4.3.2 sections (Table 15). Because a relatively smaller batch of optimized prototype films were manufactured and in order to characterize all of the physicochemical properties, the films were cut into 1"x1" size resulting in 250 µg drug loading for each film. Drug content of the optimized prototype films was found to be 315.53 ± 12.70 µg which exceeded 110% of label claim. However, drug content uniformity within each film was demonstrated to be distributed evenly with 2.03% RSD, which met our target specification (RSD <5%). The average weight of the films was 95.73 ± 5.91 mg and the average thickness of the films was 117 ± 7 µm. The %w/w of the film is 0.322 which is slightly higher than 0.303, the theoretical value of %w/w. The average contact angle was 46.34 ± 4.68 CA°. The average water content in the films was 3.24 ± 0.07 %w/w. The puncture strength and disintegration time were measured using the texture analyzer and found to be 4.57 ± 0.40 kg/mm and 386.24 ± 53.63 sec/mm, respectively, meaning that the films were flexible, soft and rapidly disintegrated.

Table 15. Physicochemical properties of lupeol optimized prototype films.

Values are represented as mean± SD with n=6.

Characterizations	Results
Drug content (µg)	315.53 ± 12.7
Recovery (%)	100.00 ± 2.03
RSD (%)	2.03
Weight (mg)	95.73 ± 5.91
%w/w	0.322 ± 0.006
Thickness (µm)	117 ± 7
Contact angle (CA°)	46.34 ± 4.68
Water content (% w/w)	3.24 ± 0.07
Puncture strength (kg/mm)	4.57 ± 0.40
Disintegration time (sec/mm)	386.24 ± 53.63

4.4.5.2 Comparison of physicochemical properties between lupeol original and optimized prototype films

When comparing the lupeol original prototype film and optimized prototype film, differences in the physical properties were observed (Figure 24). For the original prototype film, the puncture strength and the disintegration time of the films increased significantly after the addition of lupeol into the formulation. However, in the optimized prototype films, the puncture strength and the disintegration time of the films did not change significantly with the addition of lupeol (Figure 24A, B). This could be due to the difference between the dispersion and solubilization systems used in the original and optimized prototype films, respectively. In the original prototype films, lupeol was dispersed by EtOH and glycerin in the films. Thus, lupeol formed into small hard particles in the films. The particles increased puncture strength and the

hydrophobicity of the particles may have increased disintegration time when compared with placebo original prototype films. On the contrary, in the optimized prototype film, lupeol was solubilized in the mixture consisted of oil, surfactant and co-surfactant. The hydrophobicity and hardness of lupeol particles were attenuated; thus, the disintegration time and puncture strength remained unchanged when compared with placebo optimized prototype films. The residual water content in the film products remained below 10% w/w suggesting the films were not prone to promote growth of microorganisms (Figure 24C). The contact angle of original prototype films was significantly higher than optimized prototype films despite the loading of lupeol in the films (Figure 24D). This suggested the optimized prototype films were more hydrophilic compared to the original prototype films.

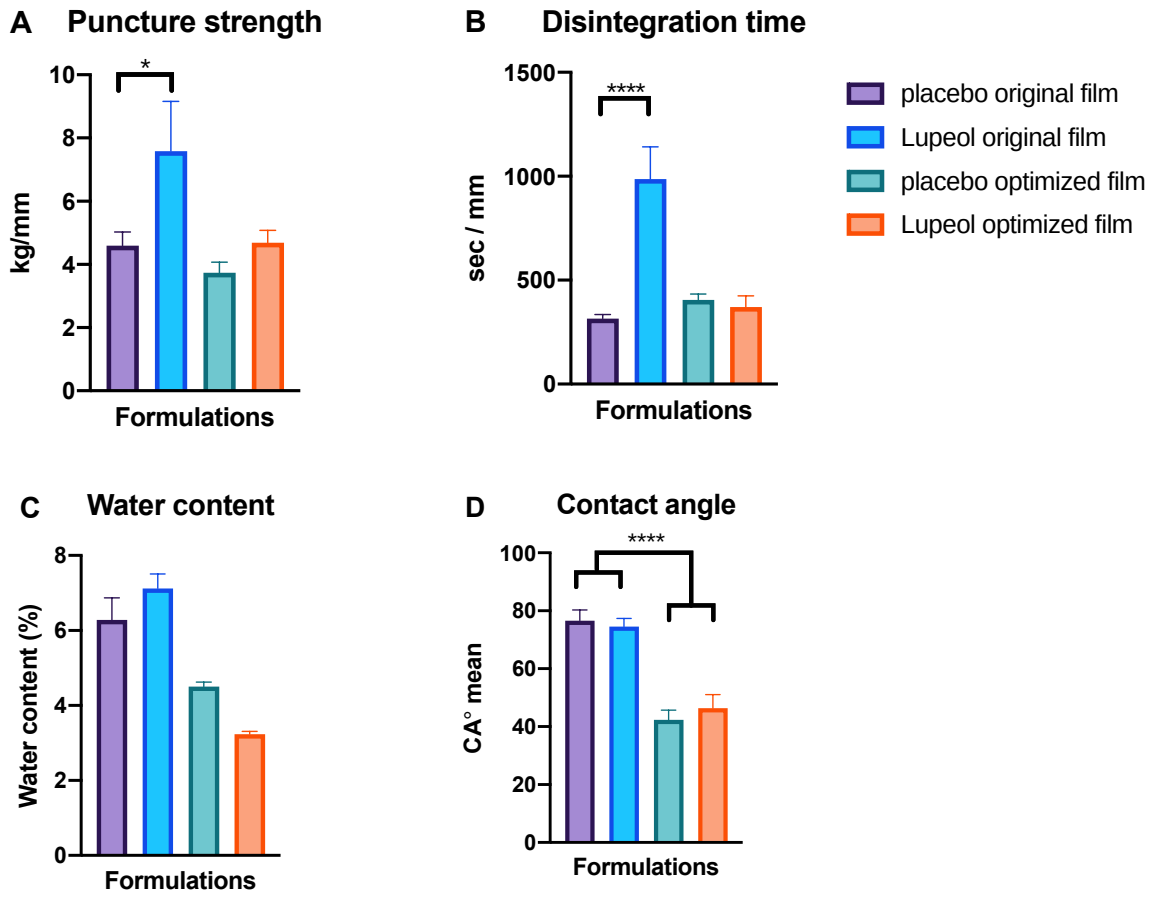

Figure 24. Physical characterizations of different lupeol film formulation. A) Puncture strength and B) Disintegration time were analyzed using TA.XT texture analyzer (n=3). C) Water content was assessed using Karl Fischer titrator (n=3). D) Contact angle was evaluated by tensiometer (n=6). Results were presented as the mean and bars represented standard deviations from 3 different film samples. Statistical significant (one-way ANOVA) is indicated by *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

4.5 Summary

The development of lupeol original prototype films was achieved through modification of a polymeric film platform previously developed and evaluated by our lab in the clinic (NCT02280109) [150]. After the lupeol original prototype films were manufactured, the physical and chemical properties of the film products and their stability at 25°C/60% RH and 40°C/75% RH was investigated. The water content of the films remained below 10% w/w, which could potentially reduce the possibility of bacterial growth while maintaining the stability of film properties to be soft and flexible. The drug content remained stable along the 3-month time period. The dissolution profile demonstrated rapid drug release from the film formulation with over 75% of drug released in the first 10 minutes of the dissolution study. The observed immediate release of lupeol from the film could be associated with the presence of the plasticizer glycerin. Glycerin has been reported to be easily dissolved in water and thus, it accelerates the disintegration and subsequent drug dissolution in oral film formulations [151]. The dissolution studies also assured the batch-to-batch consistency as well as the product uniformity within each batch with only 5% RSD.

Various strategies have been developed in the literature to improve the solubility of hydrophobic drugs, including particle size reduction, solubilized liquid formulations, solid dispersion and self-emulsifying drug delivery systems [152]. Since polymeric films are prepared and manufactured from a semisolid solution, the addition of solubilizers into the original formulation is applicable. The solubilizer mixture consisting of Capryol 90®, Transcutol P® and PEG 400 (1:1:1, *w/w/w*) was introduced to enhance the solubility of lupeol in water. This lupeol optimized prototype film was then characterized for physicochemical properties. The water content of the films remained below 10% w/w and the %RSD for content uniformity was below

5%. The lupeol optimized prototype films resulted in decreased disintegration time and puncture strength. The reduction in disintegration time suggested the optimized prototype films had faster and more drug release then the original prototype films. The reduction in puncture strength suggested the optimized prototype films are more flexible than the original formulation.

In conclusion, lupeol is a highly potent sperm-inhibiting agent which is highly hydrophobic and has a favorable stability profile for pharmaceutical development. Despite the hydrophobicity that posed a challenge to the formulation development of a vaginally administered product, we have successfully developed a vaginal film containing lupeol which incorporated a solubilizer system consisting of Capryol 90®, Transcutol P® and PEG 400 for improvement of drug dispersion and final film properties. As a result, a thin, soft, and flexible lupeol film was developed using a solvent casting technique. Future studies should evaluate the stability of the optimized lupeol film as well as the efficacy of both lupeol original and optimized prototype films and their pharmacokinetics in animal models such as the non-human primate model.

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5.0 Conclusion

The present study successfully developed a prototype of lupeol immediate release vaginal film and evaluated the essential film properties that contributed to the performance of the product. During pre-formulation studies, a reliable HPLC assay was developed and can support further studies associated with the project. To assess the drug content and content uniformity of the film products, an efficient drug extraction method using SPE was developed and validated. Oasis® HLB cartridge was chosen based on the material of the sorbent that is ideal for acidic, basic and neutral analytes. Efficient drug loading and content uniformity were achieved by introducing plasticizers and solubilizers into the formulation. The cellular toxicity study showed that the drug loading dose of lupeol in the film (1mg) is biocompatible with the VK2/E5E6 cells for up to 72 hours. The short-term stability study of lupeol original prototype films in 25°C/60% RH and the accelerated stability study in 40°C/75% RH conditions revealed that the drug content and the uniformity of the films remained stable during the 3-month time period relative to initial time zero testing. The physicochemical characterizations of the films, including drug content, content uniformity, water content, disintegration time, puncture strength and contact angle, were assessed. Significant reductions in water content and disintegration time were observed over time ($P<0.001$). On the contrary, the puncture strength and the contact angle were significantly increased over the 3-month duration. This could be due to the solvent evaporation within the products leading to the variation in the physical properties of the films. Although the films were stored in sealed aluminum foil packages, there might be package failures causing solvent evaporation.

A dissolution study was developed as a quality control method using USP I apparatus and organic solvent as dissolution media. The results demonstrated the immediate release profile as

well as the consistency of the film products. However, only 88% of lupeol were release from the original prototype films due to solubility issue of polymers in 80% MeOH. The result from the dissolution study does not fully represent the product properties *in vivo* due to the usage of organic solvent as the dissolution media. Therefore, to predict *in vivo* profile, further studies should develop a biorelevant *in vitro* dissolution study to build an *in vitro-in vivo* relationship model.

To increase the release of lupeol from the film, optimization of formulation was achieved by introducing solubilzers system consisting of Capryol 90®, Transcutol P® and PEG 400. This system significantly improved the physicochemical properties of optimized prototype films compared to the original prototype films. With the reduction in disintegration time and contact angle, the hydrophilicity of optimized prototype films was increased. These findings suggested that the optimized prototype films should have achieved increase in the extent and rate of lupeol release compared to the original prototype films.

6.0 Discussion

6.1 The challenges of developing the lupeol immediate-release vaginal film and performing formulation assessment

There were several challenges encountered during pre-formulation, formulation and characterization stages of lupeol film development. Hydrophobicity of lupeol was the major barrier encountered in the process of developing the formulation and extraction method for analyzing drug content. Since lupeol is hydrophobic, it precipitates upon contact with the hydrophilic polymer solution, leading to inadequate dispersion of lupeol in the film formulation and resulting in poor drug content uniformity. To overcome the uniformity issues, plasticizers and solubilizers were included in the formulation, which helped to achieve acceptable content uniformity. Because solubilizers dissolved lupeol prior to the addition of water, the precipitation of lupeol was avoided in hydrophilic formulation. At the same time, plasticizers improve miscibility of drug, reduce interaction between molecules, and improve flexibility of the film by reducing glass transition temperature [153]. The extraction of lupeol from the polymer film base was met with difficulties. Therefore, three procedures were tested including solid-liquid extraction, liquid-liquid extraction and SPE. Various solvents, including ACN, MeOH, and EtOH were evaluated for their potential use in lupeol drug extraction. Although ACN efficiently precipitated film polymers, the observed extraction efficiency was insufficient. MeOH and EtOH not only extracted lupeol but also dissolved the film polymer. In this case, the solubilized polymer in the samples interfered with HPLC analysis by obstructing the column. A liquid-liquid extraction method was employed due to the high solubility of lupeol in chloroform and the precipitation property of polymers in the

organic solvent. Yet, during the extraction process, the polymers formed into a layer of barrier between the organic solvent layer and the aqueous layer, prohibiting the partitioning of lupeol into chloroform. Finally, the SPE method was introduced and successfully extracted lupeol without obstructing the HPLC column.

During the development of the dissolution study, the low solubility of lupeol in water limited the choice of dissolution medium. Generally, to enhance the solubility of a poorly soluble drug in a dissolution study, surfactants such as SDS, Tween80, and CTAB are added. The addition of surfactants in dissolution media is preferable compared to organic solvent as the latter is not relevant to *in vivo* conditions. Unfortunately, these surfactants were not able to significantly improve the solubility of lupeol in aqueous based solutions and thus, the low concentration of lupeol in samples was not detectable and quantifiable by the HPLC analysis. Therefore, organic solvent was chosen as the dissolution media due to sufficient solubility of lupeol. The use of organic solvent as the dissolution media lacks physiological relevance but it is suitable as a quality control test for detecting product consistency and stability.

6.2 Limitations of lupeol immediate-release vaginal film

A limitation of our studies is the lack of long-term stability data for the developed products. Stability tests were performed only for 3 months and longer stability testing will be required to establish adequate product shelf life. According to ICH guidelines, the long-term stability testing should cover a minimum of 12 months' duration and should be continued until the duration covers the proposed shelf life. These studies are planned.

Moreover, although immediate drug release rate was achieved, complete release of the entire loaded amount of lupeol was not achieved as only 88% of the drug was released in the dissolution study. The 88% drug release may have resulted from inefficient film disintegration in organic solvent. It has been observed that hydrophilic films show inadequate disintegration when a high percentage of organic solvent is present. However, if the films are wetted with the water directly and the organic solvent is added afterwards, the films can disintegrate completely and dissolve in the solution. However, this strategy is not applicable to a dissolution scenario. Therefore, alternate dissolution conditions may need to be developed.

Lupeol is a BCS class II drug due to its low solubility and high permeability properties. Because of its low solubility, *in vivo* precipitation after application in the vaginal vault is possible and concerning. Precipitation of the drug could potentially lead to tissue irritation and tissue toxicity, resulting in reduced efficacy and bioavailability. It is to be noted that vaginal films have been previously reported for hydrophobic drug delivery without safety concerns [150, 154, 155].

6.3 Significance and contribution to the field

Compared to the amount of studies that have been reported to evaluate the pharmacological activities of lupeol, there are only a few studies dedicated to the formulation of lupeol dosage forms [156]. This thesis work pioneered development of a film formulation of lupeol. The methods developed in this work can be used to further advance a lupeol containing pharmaceutical product. Moreover, we have successfully incorporated lupeol into a polymeric film platform and achieved sufficient drug loading and content uniformity. The methods to incorporate highly hydrophobic

API into hydrophilic polymer formulations can be used as a precedence in further studies broadly applicable to other poorly soluble drugs.

6.4 Innovation of lupeol non-hormonal contraceptives

Lupeol immediate-release vaginal film is a hormone-free contraceptive that could provide immediate protection for women from unintended pregnancy. Unlike oral hormonal contraceptives that require women to take them every day to achieve contraception, lupeol vaginal film is an on-demand product that releases the contraceptive agent upon contact with vaginal fluid and immobilizes the sperm cells. Women can insert the product right before intercourse without the need for continual administration. They use the product only when they need it. Lupeol is believed to have minimal side effects compared with hormonal contraceptives due to the target site of lupeol, which is located on the flagellum of the sperm cells. Finally, the vaginal film is convenient for women, discrete, self-applied and its use does not result in product leakage. It is reported that contraceptive methods influence women's well-being. Their overall satisfaction with the methods, affects their continuation and compliance, which eventually affects their risk of unintended pregnancy. Therefore, the development of a novel lupeol immediate-release vaginal film as a contraceptive provides women with an alternative choice to prevent pregnancy.

6.5 The potential health benefits of lupeol contraceptives

The primary indication of hormonal contraceptives is to prevent pregnancy through inhibiting ovulation; however, they also possess several health benefits. COCs treat acne vulgaris through their anti-androgenic properties and are proven to reduce facial and truncal total lesion count (TLC) after the treatment [157] [158]. COCs can also relieve dysmenorrhea, regulate irregular menstrual cycles [159], and mitigate polycystic ovarian syndrome (PCOS) [160]. Therefore, there exists multiple non-contraceptive uses for hormonal contraceptives. Lupeol may also potentially benefit women in additional ways due to its wide range of pharmacological activities. If the bioavailability of lupeol is improved and can be successfully delivered to the target site, it is worth evaluating the capability of lupeol contraceptives to exhibit non-contraceptive indications, such as anti-cancer,anti-inflammatory, and anti-infective effects. Multiple modifications can be made to enhance the bioavailability of lupeol, including decreasing the particle size of the compounds, using solubilized liquid formulations or generating different synthetic derivatives of lupeol without affecting the pharmacophore. In a study evaluating potential agents for skin damage treatment, lupeol derivatives were synthesized through an esterification process. It demonstrated that the modification of the lupeol structure improved the penetration of lupeol esters through the stratum corneum exhibiting better efficacy [161].

Lupeol is reported to possess anti-bacterial and anti-fungal properties, which cover a wide range of bacteria and fungus, including *S. aureus*, *E. coli*, *S. typhi*, *K. pneumonia*, *S. schenckii*, and *Candida albicans* [162]. The most common diseases caused by infection in humans are urinary tract infections (UTIs), vaginal infections, and toxic shock syndrome. The majority of UTIs are caused by *E. coli*, and most of vaginal yeast infections are associated with fungus *Candida albicans*. A study has investigated the composition of *Ficus sycomorus*, which had isolated lupeol

from the root bark of the plant [163]. *Ficus sycomorus* is commonly known among the Hausa people of Northern Nigeria as Farin Baure and is from the Moraceae family. In traditional medicine, the root bark of the *Ficus sycomorus* is used for the treatment of inflammation, ulcers, painful urination, urinary and vaginal infections [164]. Moreover, studies have shown that lupeol as well as other pentacyclic triterpenoids exhibited anti-HIV activity by inhibiting the HIV-1 RT-associated RNase H function and the HIV-1 replication via HIV-1 RT-associated RNA-dependent DNA polymerase inhibition [165] [166]. Since the lupeol vaginal film is designed to achieve contraception via local delivery in the cervicovaginal environment, the product may also prevent vaginal infections.

6.6 Future directions for advancing the lupeol immediate-release vaginal film

Future, development of the lupeol immediate-release film should primarily focus on evaluating the dissolution profile of the lupeol optimized prototype films in a biorelevant *in vitro* dissolution method. If necessary, modification of the film formulation should be further investigated to improve its dissolution profile. The challenges of developing a biorelevant method include the low solubility of lupeol and the high LOQ of the analytical method. Therefore, if the solubility of lupeol is improved, the media of choice will not be limited to organic solvent. Also, the HPLC method for dissolution study should be validated as different calibration curve range was used. In this work, we have generated an initial optimized film formulation. This formulation can be further improved upon to obtain the ideal release profile. Once a new formulation is obtained, the drug extraction method for drug content and uniformity as well as the HPLC analysis methods must be validated to ensure that the presence of the modified film matrix does not interfere

with the original method. Depending on the level of formulation modification needed, new processing conditions may be required to ensure the efficient-loading of the drug and the uniformity within the film. A long-term stability study of the film products should be performed for at least 12 months, including the drug content, uniformity, and physicochemical characterizations. *Lactobacillus* compatibility and cellular toxicity studies for the modified formulation should be conducted to evaluate the toxicity of the newly added excipients before proceeding to animal studies. Moreover, sperm motility studies using the developed lupeol prototypes film should be conducted to evaluate *in vitro* efficacy. These studies are planned as immediate next steps. Finally, the film product will proceed to macaque intrauterine insemination studies to evaluate the tolerability and efficacy of lupeol vaginal films.

Appendix A : Abbreviation used

ABHD2: Abhydrolase domain-containing protein 2

ACN: Acetonitrile

API: Active pharmaceutical ingredients

AUC: Area under curve

CTAB: Cetyltrimethylammonium bromide

CMA: Chlormadinone acetate

CMC: Sodium carboxymethylcellulose

CPA: Cyproterone acetate

COC: Combined oral contraceptives

DMBA: 7, 12-dimethylbenz[a]anthracene

ECP: Emergency contraception

EtOH: Ethanol

FSH: Follicle-stimulating hormone

GnRH: Gonadotrophin-releasing hormone

HDL-C: high-density lipoprotein cholesterol

HEC: Hydroxyethyl cellulose

HFF: Human foreskin fibroblast

HPMC: Hydroxypropyl methylcellulose

IL: Interleukin

IVIVC: *In vitro-in vivo* correlation

IUD: Intrauterine contraceptive device

KSFM: Keratinocyte serum-free medium

LARC: Long-acting reversible contraceptive

LDL-C: Low-density lipoprotein cholesterol

LNG: Levonorgestrel

LH: Luteinizing hormone

LOD: Limit of detection

LOQ: Limit of quantification

MEC: Minimum effect concentration

MeOH: Methanol

MPA: Medroxyprogesterone acetate

N-9: Nonoxynol-9

NET: Norethindrone

NETA: Norethindrone acetate

NP-HPLC: Normal-phase high-performance liquid chromatography

PARA: Aldosterone receptor antagonism

PET: Polyethylene terephthalate

PTFE: Polytetrafluoroethylene

RAAS: Renin-angiotensin-aldosterone system

RH: Relative humidity

RP-HPLC: Reversed-phase high-performance liquid chromatography

SDS: Sodium dodecyl sulfate

SEDDSs: Self-emulsifying drug delivery systems

SHBG: Sex hormone-binding globulin

s/n: signal-to-noise ratio

SPE: Solid phase extraction

STDs: Sexually transmitted diseases

TAFI: Thrombin activatable fibrinolysis inhibitor

TPA: 12-O-tetradecanoylphorbol-13-acetate

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