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PRODUCTION AND CHARACTERIZATION OF NANOPARTICLES LIPID CARRIER (NLCS) LOADED WITH GINGEROLS EXTRACT AND THEIR EFFECT ON SERUM LIPID PROFILE IN POSTMENOPAUSAL PERIOD

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Abstract

Plant-derived edible nanoparticles (PDNPs) are nano-sized membrane vesicles released by edible plants. They are non-toxic, have tissue-specific targeting properties, and can be mass-produced. The study aimed to assess the effect of gingerols to decreased the levels of total cholesterol (TC), triglycerides (TG) and lipoproteins (HDL, LDL, VLDL) levels that associated with menopausal transition using nanoparticles lipid carrier (NLCs) loaded with gingerols extract (GE-NLCs). The study was conducted using thirty adult female mice divided into three groups (10 mice/ group) and handled as follows for 6 weeks. Group A: Control group has injected 0.1 ml D.W intraperitoneal (IP) daily. Group B: This group has injected IP daily 160 mg/ kg B.W of 4-Vinylcyclohexene dioxide (VCD) for two weeks as model for menopause, group C: Animals in this group were administered 160 mg /kg B.W of VCD IP daily for two weeks for inducing menopause, then shaved at the last third of the back and treated with dermal sticker saturated with 0.1ml of GE-NLCs for six weeks. A gingerols extract standardized by High-performance liquid chromatography (HPLC) to contain (18.3) mg/g of gingerols. The characterization of GE-NLCs was done by transmission electron microscopy (TEM), Scanning probe microscopy (SPM), Zeta potential. TEM images showed that shape of particles was mostly spherical and few cylindrical with average diameter (64.31) nm. SPM images showed the average grain size was (42.81) nm. Encapsulation Efficiency (EE) was 85%. Zeta potential was (-42.9) Mv. The results of statistical analysis showed a significant decrease ($P \leq 0.01$) in the level of estrogen in group B (23.767) ng/ml, compared with group A (33.154) ng/ml and significant increase in the estrogen level in group C (29.742) ng/ml compared with group B. While for levels of lipids, the result of statistical analysis showed a significant increase in the level of TC in group B (101.94) mg/dl, compared with group A (80.00) mg / dl, and significant decreased in group C (80.86) mg/dl, compared with group B and significant increase ($P \leq 0.01$) in the level of TG in group B (79.400) mg/dl, compared with group A (54.200) mg/dl and significant decrease in group C compared (61.158) mg/dl, compared with group B. and The result s of statistical analysis showed a significant decreased ($P \leq 0.01$) in the level of HDL in group B (18.800) mg/dl, compared with group A (23.001) mg/dl and no significant difference in group C (18.168) mg/dl, compared with group B and significant increase in the level of LDL in group B (66.88) mg/dl, compared with group A(50.36) mg/dl and significant decreased in group C (44.71) mg/dl, compared with group B and results of statistical analysis showed a significant increased ($P \leq 0.01$) in the level of VLDL in group B (16.62) mg/dl, compared with group A (10.84) mg/dl and significant decreased in group C (11.90) mg/dl, compared with group B. *Conclusion:* The results of this study revealed that administration of GE-NLCs shows an effective to regulation of lipids metabolism disorders due to hormonal changes that associated with menopausal transition via impact of VCD.

Key words: Lipoproteins, Menopause, NLCs, VCD, Triglycerides, Gingerols

Introduction

Menopause is clinically diagnosed when a woman has not menstruated for one year due to the loss of ovarian follicular activity, which typically occurs at around 45-55 years of age (Landgren *et al.*, 2004). During the menopausal transition period, there is an emergence of various lipid metabolic disorders due to hormonal changes, such as decreased levels of estrogens and increased levels of circulating androgens; these may lead to the development of metabolic syndromes including cardiovascular diseases and type 2 diabetes. (Seong and

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Hyun, 2020) Dysregulation of lipid metabolism effects the body fat mass, fat-free mass, fatty acid metabolism, and various aspects of energy metabolism, such as basal metabolic ratio, adiposity, and obesity (Lobo *et al.,* 2014). Moreover, menopause is also associated with alterations in the levels of various lipids circulating in the blood, such as lipoproteins, apolipoproteins, LDLs, HDL and TG. Alterations in lipid metabolism and excessive adipose tissue play a key role in the synthesis of excess fatty acids, adipocytokines, proinflammatory cytokines, and reactive oxygen species, which cause lipid peroxidation and result in the development of insulin resistance, abdominal adiposity and dyslipidemia (Ebtekar *et al*., 2018). In the present study, treatment of female mice with VCD to stimulate perimenopause complicated with hyperlipidaemia served as the model of perimenopause.

The rhizome of ginger (*Zingiber officinale Roscoe*, family Zingiberaceae) is a well-known food spice and medicinal plant over the world. (Ali *et al.,* 2008) It has long been used as a remedy for some diseases such as vomiting, joint and muscular pain, indigestion and cold-induced syndrome. The main ginger derived bioactive compounds are gingerols. (Butt and Sultan, 2011) There are some scientific evidences regarding various pharmacological activities of ginger powder, extract or its bioactive components including anti-inflammatory and antioxidant, (Mashhadi *et al.,* 2013) antifungal and antibacterial, (Gaus *et al.,* 2009) antiemetic16-18 and anti-cancer (Palatty *et al.,* 2013) effects. Moreover, ginger is generally considered as a safe herbal medicine according to existing data and also the FDA's report on its safety (Ryan *et al.,* 2011). Regarding the hypoglycemic and hypolipidemic effects of ginger, it has been shown that the ethanolic extract of ginger can decrease blood glucose and plasma lipids (Ozougwu and Eyo, 2011). Moreover, ginger maybe decrease lipid profile using decreased fat absorption (e.g. decreased pancreatic lipase), cellular cholesterol biosynthesis and increased cholesterol conversion to bile acids (Li, 2012).

Nanotechnology consists of a new model in science, in which new strategies with nanostructured drugs have been provide interesting and unique properties such transdermal absorption. Nanolipid carrier (NLC) is the latest generation colloidal nanoparticles with stability improved and drug loading efficiency. The lipids used in preparation of NLC are usually physiological lipids (biocompatible and biodegradable) so, that drugs can be delivered at the required location of action with the controlled release with reduced acute and chronic toxicity (Purohit *et al*., 2016). Transdermal treatment for delivery of drugs are as effective as oral routs in the treatment of menopause symptoms (weight gain), Given the absence of first-pass hepatic metabolism and the simple effect on lipid and coagulation system (Castelo;2014), the transdermal system could be particularly suitable for women with digestive problems.

The present study was designed to assess the efficacy and safety of a nanolipid carrier of GE in order to prevent perimenopause-induced ovarian dysfunction and hyperlipidaemia in the female mice Model for menopause.

Materials and Methods

Ginger was obtained from the local markets in Baghdad, then classified by the University of Baghdad grassland, 4-vinylcyclohexene diepoxide (VCD), standards of gingerols, Tween 80 were acquired from Sigma Aldrich, Germany. Lecithin Soybean was acquired from Santa Cruz, USA. HDL, LDL, Triglyceride, Total C - ELISA-Kit Glycerol from Merck, Germany. Virgin coconut oil from Abideen, Pakistan.

Preparation of Gingerols Extraction

The Fig. 1 shows preparation of gingerols extraction, 100g of ginger were washed and cleaned well with distilled water and then it was dried, was crushed with mortar and pestle and soaked into 500 ml ethanol (99.5 %) for 8 hrs. The extract was filtered by a Buechner funnel, repeat extraction three consecutive times after every 24 hrs. Then it was concentrated with a rotary evaporator under reduced pressure. (Ali *et al.,* 2015).

Identification and Estimation of Gingerols in the ginger extracts by HPLC technique

The quantity of Gingerols were analyzed by HPLC technique, SYKAWN, Equipped with UV detector in combination with Ezchrome software. Reverse-Phase column C18-OD5 has been employed in this technique. A mixture of Acetonitrile and double deionized water in the ratio 70:30 has been used as the mobile phase. The solvent flow rate was kept as 1mL/min and the UV detection wavelength was set at 360nm. The instrument was set as per the chromatographic condition described above. The Sample was centrifuged at 10000rpm for 10 min. and sample was filtered by 0.45µm filter. By means of a suitable syringe, 100µl of standard solution was injected and subjected to HPLC. The chromatogram is recorded. The sample is also subjected to HPLC analysis. The qualitative and quantitative evaluation of Gingerols in the sample was performed on the basis of retention time and chromatographic behavior with reference to that of authentic standards. From the peak areas of the graph, the percentage of Gingerols was calculated. Chromatograms obtained at 280 nm for Gingerols for the

Fig. 1: Ginger Extract: (A) Ginger soaked in ethanol (B) Ginger after filter (C) ethanol

authentic as well as the samples gave the best compromise between sensitivity and baseline noise. (Aly *et al.,* 2013)

Preparation of NLC

One hundred mg of solid lipid (glyceryl monostearate) and liquid lipid (virgin coconut oil) range from 60% to 40%, w/w were dissolved in 8.5 ml of Dichloromethane (DCM) were blended and melted at 40°C to form a uniform and clear lipid phase. 10 mg of gingerols extract (Mahmoud *et al.,* 2008) subsequently added to the lipid phase and ensures heating temperature always maintained at 10°C above melting temperature of solid lipid. Meanwhile, the aqueous phase was prepared by blending 200 mg of Tween 80 and 80 mg of soy lecithin were prepared in 50ml D.W (Negi *et al.,* 2014). Immediately, the aqueous mixture was added onto lipid mixture. The pre-emulsion was homogenized using Homogenizer at 11 000 rpm for 15 minute. The emulsions were ultrasonicated using probe sonicator for (5 to 20) min. durations at 40 amplitudes. To evaporate of DCM, the obtained nanoemulsion was stirred at 400 rpm for 3 hrs. NLC was cooled in ice water bath to room temperature and stored at 4°C. (Amr *et al.,* 2019)

Characterization of GE-NLCs

1- Atomic Force Microscope (AFM)

The granularity accumulation distribution of GE-NLCs by using Scanning probe microscope (SPM)AA3000.

2- Transmission Electron Microscopy (TEM)

The surface morphology of the formulation was investigated using transmission electron microscopy (TEM, FEI TECNAI G220 TWIN MODEL 94320502221).

3- Zeta Potential (ZP): ZP was determined by using Particle size analyzer (Delsa Nano C Beckman Cutler).

4- Encapsulation Efficiency (EE): The EE of GE-NLCs was estimated after separation of free plant extract and lipids from the aqueous phase by ultrafiltration. The concentration of loaded plant extract in the aqueous phase were then evaluated by HPLC. The Concentration of the loaded RC extract in the NLC was calculated according to the following equation. (Jaber *et al.,* 2018)

EE (%) = (Total concentration of drug content- free drug / Total drug concentration of content) × 100

Experimental animals

A total 15 female albino mice were used in this study were albino at the age of (8-10) weeks and average weight (21±6g), the mice were housed in polypropylene cages under controlled conditions at temperature (25-28)°C with a 12/12 h light/dark cycle. Mice were acclimatized condition for 7 days before commencement of the experiment.

Experimental design

A total of 30 female mice was divided into 3 groups randomly (n = 10/group), two groups saved as negative control and positive control as follows:

Group A: Control group **(-)** has injected 0.1 ml D.W by intraperitoneal injection (IP) daily for two weeks.

Group B: Control group **(+)** has injected 0.1 ml 160 mg/kg B.W of VCD (Wright *et al.,* 2011) by IP daily for two weeks.

Group C: The group have injected IP daily 160 mg/kg B.W of VCD daily for two weeks for inducing menopause, then shaved at the last third of the back and treated with dermal sticker saturated with 0.1 of GE-NLCs for six weeks.

Blood samples: After six weeks, animals were sacrificed by cervical dislocation. Then open the inverted T-shaped cavity and draw blood directly from the heart by stabbing the heart to get as much blood as possible. One mL of blood was collected from each mouse in test tubes. Serum separated from coagulated blood sample by centrifugation at 2500 rpm for 15 min and kept it by freezing at -20 C until used. (Parasuraman *et al.*, 2017)

Statistical analysis: The Statistical Analysis System (SAS; 2012) was used to affect different factors in study parameters. Least significant difference (LSD) at P>0.01 test was used to significant compare between means in this study.

Results and Discussion

The Fig. 2 showed concentrations of gingerols in ginger extract by HPLC technique were (18.3) mg/g in retention time (10.75). Fig 3 shows concentration of standard.

Characterization of NLC Formulation:

Fig 4 shows GE-NLCs with milky white solution, the proportions used in the preparation of GE-NLCs were increased volume of aqueous phase, increase in drug content of particles and high concentration of surfactant.

Atomic force microscopy (AFM):

Fig. 5, A and B shows images at two and three

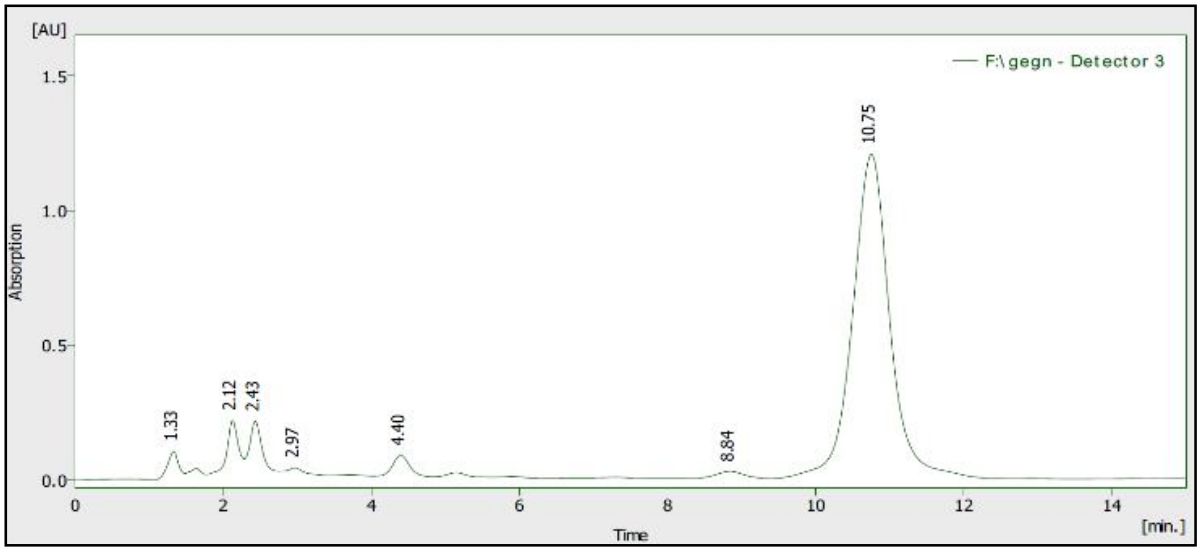

Fig. 2: Chromatographic HPLC of gingerols.

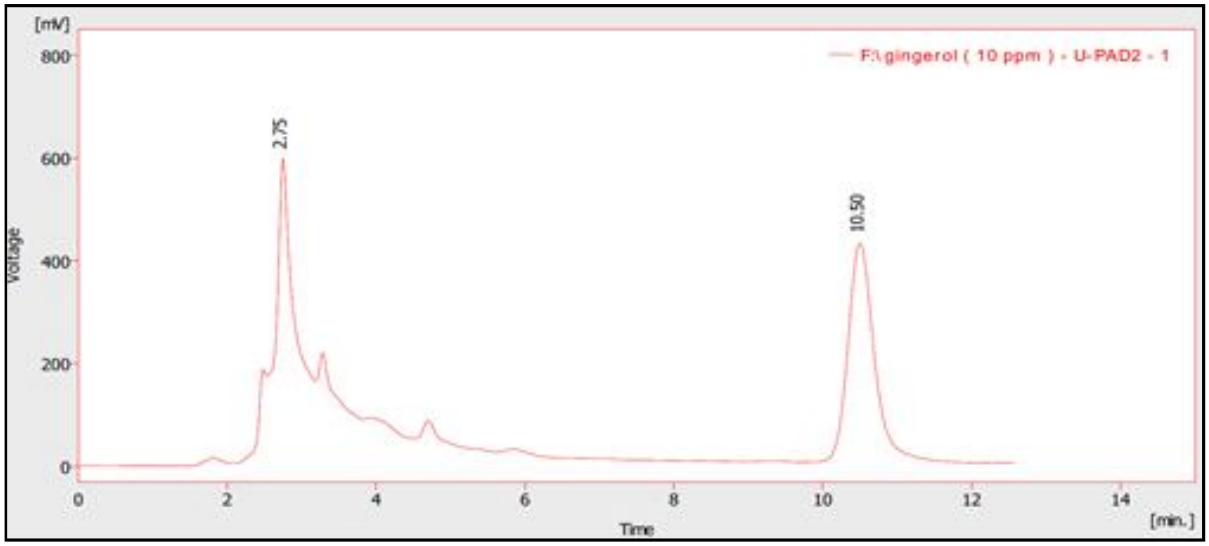

Fig. 3: Chromatographic HPLC of standard gingerols.

Fig. 4: Nanostructured lipid carrier loaded with ginger extract.

dimensions of producing sample GE-NLCs. From Figure can note that the shape of particles was a mix between spherical and cylindrical. The grain size distribution of surface was about from (16 to 74) nm at average 42.81 nm, Fig. 5, C shows the histogram of grain size distribution on the surface.

Transmission electron microscopy (TEM):

Fig. 6, shows the image of TEM obtained from GE-NLCs. It can be shown the shape of particles was mostly spherical and few cylindrical with average diameter (64-35) nm and maximum distribution 70. Also the figure notice that the prepared GE-NLCs was highly dispersed and this indicates the quality of the prepared nanomaterial.

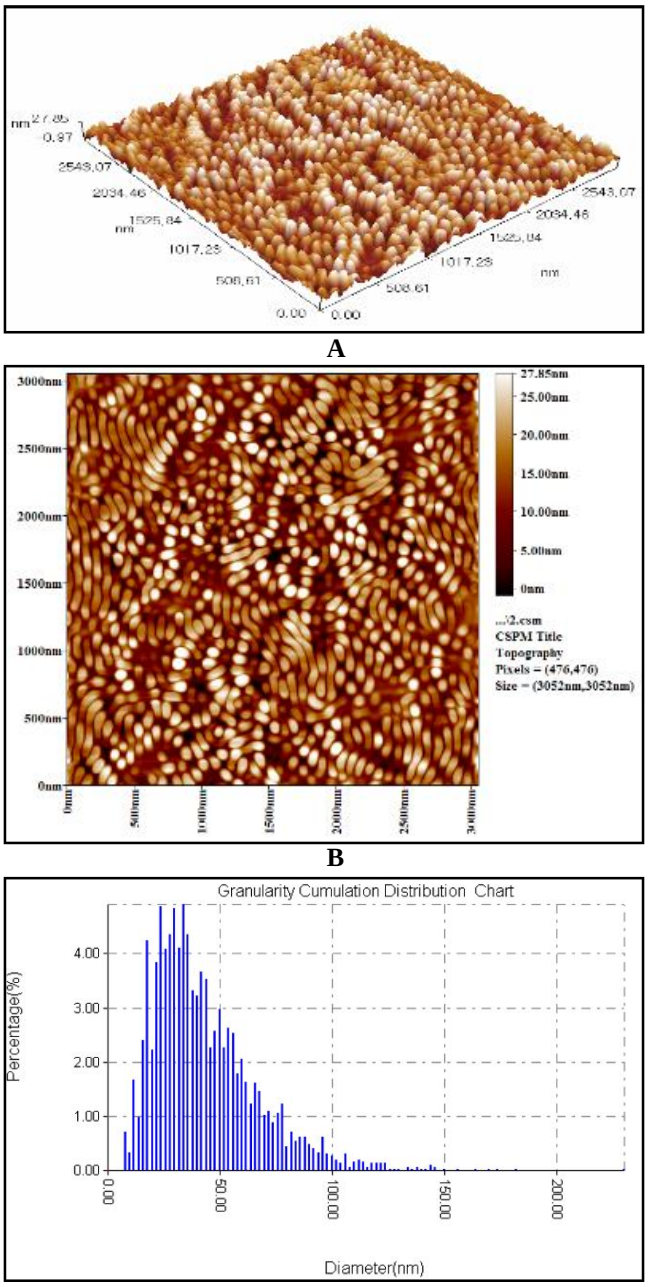

Fig. 5: Images of (AFM) for GE-NLCs (A) two dimensions, (B) three dimensions, (C) histogram of the distribution of grain size.

Zeta potential (ZP) determination:

Zeta potential of the formulation was determined to study the stability behavior of the formulation in vitro and in vivo which was found to be (-42.9) may as show in Fig. 7. It confirmed the stability of the colloidal system which is high enough to keep the particles aside and prevent the aggregates formation (Korolev; 2014), also, the negative charge of the nanoparticles will delay their protein binding and thereby results in longer circulation half-life of the nanoparticles. Stability increased with

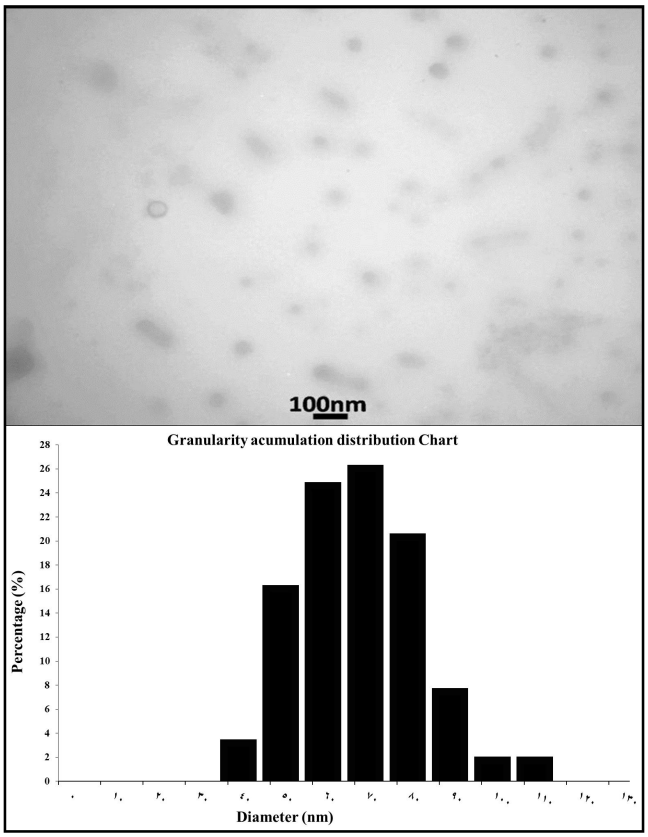

Fig. 6: Transmission electron microscopy image of GE-NLCs formulation, with X34000.

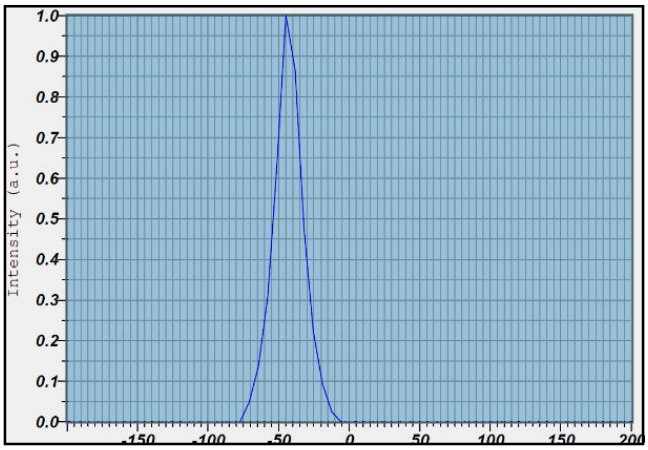

Fig. 7: Zeta potential of GE-NLCs.

increasing concentrations of surfactant. (Ramaiyan and Subramanian; 2016) Values of ZP greater than ±30 mV were generally good indicators of static stabilization of the dispersion system. (Wissing and. Muller ; 2002).

Encapsulation Efficiency (EE):

The current study showed that concentration of gingerols was (8.5) mg/g in the GE-NLCs, when 10 mg of gingerols was used to load the NLC, approximately (1.5) mg of free gingerols was detected in an aqueous GE-NLCs dispersion, suggesting that 8.5 mg (85%) of gingerols was successfully encapsulated into the NLCs. NLCs were preferred over the SLNs because of their higher entrapment efficiency and more stability of the formulation. The liquid lipids present in the formulation were able to carry more drug as compare to solid lipids alone. Increasing the liquid lipid content in the formulation could enhance the EE of the formulation (Ghanbarzadeh; 2015). The ratio of oil phase and aqueous phase showed a great impact on the EE of NLC (Yaowaporn and Ruedeekorn; 2015 (increase aqueous phase volume results in an increase in EE. This could be due to lesser aggregation of the particles in a larger space. Also increase in concentration of surfactant resulted in increase in EE. (Ramaiyan and Subramanian; 2016) Lesser surfactant concentration and higher lipid concentration will cause increase in viscosity of the formulation which will result in higher viscous resistance against a shear force which will hinder the formation of nanodroplets and also the lesser amount of drug will get solubilized into viscous lipid matrix which ultimately results into decrease EE (Sharma *et al.*, 2016). Increase in drug content is expected to raise the EE by providing more space to incorporate the drug. Increment of the lipid content also reduces the escaping of drug into the external phase (Shah *et al.*, 2007). On the other way, higher amount of organic solvent will lead to leaching of the drug from the lipid core which ultimately will decrease the EE of the formulation (Ekambaram and Sathali; 2011).

Effect of VCD on Estrogen Level:

Ovarian dysfunction is a prognostic marker for perimenopause, which leads to the reduced production of estrogen (Vicennati *et al.*, 2015). Administration of VCD induces ovarian ageing that mimics the physiological

Table 1: Mean value ± standard of serum estrogen level and lipid profile among experimental groups.

Groups	Mean ± SE					
	ES ng/ml	TC mg/dl	TG mg/dl	LDL mg/dl	VLDL mg/dl	HDL mg/dl
Group A	33.154±2.735 a	80.00±2.92a	54.200±5.404A	50.36 ±1.28A	10.84 ±0.48a	23.001±2.925A
Group B	23.767±2.127 b	101.94±6.97b	79.400±4.782B	66.88 ±2.17B	16.62 ±0.22b	18.800±1.643B
Group C	29.742±2.795 c	80.86±3.68a	61.158±3.121C	44.71±2.03C	11.90 ±0.50a	19.168±1.912B
LSD value	2.001 **	5.463 **	4.357 **	5.355 **	1.144 **	2.254 **

Means having with the different letters in same column differed significantly, ** (P≤0.01).
Note: Similar letters within one column indicate that there are no significant differences in the parameters

and pathological conditions of omen with premature ovarian failure by impairing original and primary follicles (Hoyer *et al.,* 2001). Table 1 shows the effect of VCD which caused a decreased in estrogen level in group B compared with group A. The results of statistical analysis showed a significant decrease (P≤0.01) in the level of estrogen in group B (23.767) ng/ml compared to groups A (33.154) ng/ml. The VCD mouse model of menopause has been required repeated daily intraperitoneal injections of VCD to cause loss of primordial and primary follicles in the ovary that produces estrogen hormone via accelerating atresia in primordial and primary ovarian cells by altering the expression and distribution of the Bcl-2 family of proteins that regulate apoptosis(Brooks *et al.,* 2016) and by decreasing ovarian mRNA, protein, and/or activity of the enzymes responsible for generating estradiol and its precursor sex steroid hormones (Lee *et al.,* 2017) and via the direct inhibition of auto phosphorylation of the survival receptor c-kit, located on the plasma membrane of the oocyte, within 14 days after the cessation of daily dosing, VCD has depleted all primordial follicles. During this time frame of impending ovarian failure, there is an increase in cycle length, estrogen levels fluctuate until they reach very low levels, thus mimicking perimenopause in humans. (Mark *et al.,* 2011).

Effect of GE-NLCs on Estrogen Levels:

The present study showed increased in estrogen level after treated with GE-NLCs in group C compared with group B. The results of statistical analysis showed a significant increase (P≤0.01) in the level of estrogen in group C (29.742) ng/ml compared with group B (23.767) ng/ml. The reason which can be stated for an increase of estrogen level by CE-NLCs is that gingerols reduce the secretion of FSH and LH via an effect on pituitary-gonadal axis (Rahmanian *et al.,* 2012) via inhibition of cyclooxygenase and lipooxygenase pathways, cause to inhibition of the arachidonic acid and prostaglandin synthesis. Considering the role of prostaglandins in gonadotropin synthesis, gingerols can decrease gonadotropin levels in this way. (Shukla *et al.,* 2007) Other studies showed that, due to its action as a serotonin receptor antagonist, ginger can decrease the secretion of gonadotropin. Serotonin mediates the secretion of GnRH by direct stimulation of hypothalamus to release GnRH through the phospholipase C (PLC) pathway. (Kim *et al.,* 2006) All of this leads to elevate estrogen levels. Many studies showed that some flavonoids bind to the benzodiazepine site of the GABAA receptor. (Hanrahan *et al.,* 2011) Thus we can say the flavonoids of ginger act as GABA receptor agonists and cause to increase estrogen level. Biosynthesis of estrogens from androgens are done by Aromatase enzyme. In fact, Aromatase is a cytochrome P450 enzyme, and activated by 6-gingerol causing to the elevated of estrogen levels. (Li *et al.,* 2013) Ginger and its active components like 6-gingerol cause to decrease in testosterone levels via reduction of blood glucose and insulin levels, and also through the decrease of LH levels and changes in pituitary-ovaries axis (Khaki *et al.,* 2014).

Effect of VCD and GE-NLCs on Lipids Profile Levels

Hyperlipidaemia is a common symptom in menopausal women and has been associated with ovarian dysfunction during perimenopause, VCD accelerate progression of metabolic syndrome (Berger *et al.,* 2012). As shown in group B, where we noticed significant increase (P≤0.01) in TC, TG, LDL and VLD levels (101.94, 79.400, 66.88, 16.62) mg/dl respectively, compared with control group (80.00, 54.200, 50.36, 10.84) mg/dl respectively. The present study shows decreased in TC, TG, LDL and VLD levels in group treatment with GE-NLCs compared with VCD group. The results of statistical analysis showed a significant decrease (P≤0.01) in the level of TC and TG in group C (80.86 and 61.158) mg/dl, respectively compared with group B (101.94 and 79.400) mg/dl, respectively. And significant decrease (Pd"0.01) in the level of LDL and VLDL in group C (44.71 and11.90) mg/dl, respectively compared with group B (66.88 and 16.62) mg/dl, respectively and showed non- significant increase in HDL level in group C (19.168) mg/dl, compared with group B (18.800) mg/dl. In this study we examined the GE-NLCs could improve lipid regulation after VCD administration. The observation that serum concentrations of TC, TG, LDL and VLDL increased in the VCD only compared with the GE-NLCs and Control groups suggests that GE-NLCs improves lipid metabolism in perimenopause complicated with hyperlipidaemia in mice. The hypocholesterolemic effect of ginger might be due to the inhibition of cellular cholesterol biosynthesis (Fuhrman *et al.,* 2000), increased hepatic cholesterol 7a-hydroxylase enzyme activity (Srinivasan and Sambaiah, 1990), increased activity of LDL receptor as the result of reduced cellular cholesterol biosynthesis (Ness *et al.,* 1996), reduced lipid peroxidase, increased pancreatic lipase and amylase, increased conversion of cholesterol to bile acids, increased intestinal peristalism and inhibited lipid hydrolyze in intestinal tract (Han *et al.,* 2005). The hypotriglyceridemic effect of ginger could be explained by increasing the activity of lipoprotein lipase enzyme which leads to the hydrolysis of circulatory TG and its subsequent decreasing serum TG (Shirdel *et al.,* 2009). Ginger also reduces the ChREBP gene expression in the

liver. Reduction of ChREBP expression decreases fatty acid synthase, and glucogenic as well as lipogenic proteins. It also reduces fat accumulation in the liver which results in reduced levels of serum TG (Gao *et al.*, 2012). Intact nanoparticles sized above 100 nm are not considered to permeate the skin surface because of their dimensions and rigidity (Cevc; 2004). Since epidermal lipids are rich in skin surface, lipid nanoparticles attaching to the skin surface would allow lipid exchange between skin surface and the nanocarriers (Müller *et al.*, 2007). Lipid nanoparticles have the potential to deliver drugs via the follicles (Chen *et al.*, 2006). Furthermore, each follicle is associated with sebaceous glands, which release sebum, creating an environment enriched in lipids. This environment is beneficial for trapping of lipid nanoparticles. Some glyceride lipids present in NLCs may accelerate the entrance into the follicles/sebaceous glands (Sarabjot *et al.*, 2015) and this is agreeing with our formulation GE-NLCs.

Conclusion

Menopause may lead to various changes in lipid metabolism due to reduced estrogen secretion. These changes include enhanced fat mass and decreased fat-free mass, which effects the basal metabolic rate. This work we used GE-NLCs to evaluate the efficacy and safety of a novel protocol of nano-transdermal treatment based on a nanostructured formulation of gingerols to regulation of lipids metabolism. The results of this study revealed that administration of GE-NLCs for six weeks as transdermal treatment shows significantly decrease the serum LDL, TC, TG levels and increase serum HDL level, and shows an effective in compensating for estrogen deficiency resulting from loss of primordial and primary follicles in the ovary via impact of VCD, these activities could support the continued investigate on of GE-NLCs as a potential therapeutic agent in HRT. The results may have an important impact in order to create in a close future an effective agent for use in the government women health programs, thus further studies are warranted to clarify its usefulness in women.

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