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INVESTIGATION OF EFFECT OF PHOSPHOLIPIDS ON PHYSICAL AND FUNCTIONAL CHARACTERIZATION OF PACLITAXEL LIPOSOMES

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Keywords:

Liposomes, Phospholipon, Entrapment efficiency, *In vitro* release, Hemolytic activity

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ABSTRACT: The present study was assessed to determine the effect of various grades of phospholipids on liposomes. The various grades of synthetic phospholipids such as Phospholipon 90G® (PL90G), Phospholipon 80H® (PL80H) and Phospholipon 90H® (PL90H) were used along with cholesterol in stoichiometric ratio (1:0.25, 1:0.5, 1:1, 1:2, 1:3). Using these ratios the paclitaxel liposome was formulated by film hydration method. The prepared liposomes were assessed for characterization using entrapment efficiency, vesicular size, *in vitro* release and hemolytic toxicity assay. The synthetic grades of phospholipids significantly influenced paclitaxel liposomal formulations. The stoichiometric ratio (1:1) between cholesterol and various synthetic phospholipids was found to be optimized one, from rest of ratios. The characterization confirmed the formation of liposomes. The entrapment efficiency was observed to be high as increasing the ratios between cholesterol and phospholipids, but then declined suddenly as further increasing the ratio. The best liposomal formulations showed the spherical shape with size ranging from <10µm. The optimized paclitaxel liposomes % release in phosphate buffer (pH 7.4) enhanced significantly, and rest of ratios. The optimized liposomal formulation also lowers the hemolytic toxicity of paclitaxel.

INTRODUCTION: Cancer is a challenging and complex disease causes most of the population around the world. Where, the normal cell physiology altered totally and directs to malignant tumours. These unwanted tumour growth cells during their journey (normal cell conversion to abnormal cell) affects the neighbouring and dissimilar cells, results death of the cancer patient [1].

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On the basis of previous history and research output shows that, the prolong exposure of virus attack, chemicals, inflammation, radiation and proactive agents could be responsible for the initiation of this disease. Moreover, in the changes of normal cell physiology, following modifications might be accountable *i.e.* growing and signalling of tumour cells in self sufficient way, showing insensitivity to tumour growing cells, unlimited replication and finally, the metastasis [2]. Over the last three decades, the extensive research has been going on this metabolic disorder in order to understand its process, mechanism and transformation from normal growth to unwanted tumour growth cell.

The synthetic anticancer agents can be helpful in treating cancer; however prolonged exposure, accompanied with heavy dose of these agents could be caused inconvenience to normal cells [3].

The natural origin based anticancer agent like paclitaxel (PT), isolated from the bark of *Taxus brevifolia* (northwest Pacific Yew Tree), characterized it as white crystalline powder, with empirical formula ($C_{47}H_{51}NO_{14}$), and on the basis of characterization named it as Taxol. Later on, the Bristol-Myers Squibb Company developed this isolated compound commercially with the generic name Paclitaxel and then sold under the trademark Taxol. As per as anticancer activity is concerned, PT, exclusively bind to the β-tubulin subunit through N-terminal side chain with 31 amino acid, in the microtubules [4, 5] causing depolymerization of β-tubulin subunit, with inhibition of mitosis and these combined effect can be directed to induction of cell apoptosis [6]. Based on previous reports, PT shows anticancer activities towards breast cancer [7], ovarian cancer [8], lung cancer [9] and pancreatic cancer [10]. Supportive to this, previous published reports by Mullins *et al.*, [11], shows that PT gives antitumor activity by formation of macrophage IL – [12] through nitric oxide, which further caused to dysregulation of IL-12 p40 expression and finally reduces the tumour growth. Additionally, PT also exhibits anticancer activity by removing phosphor-fructokinase from melanoma cells, accompanied with reducing the level of glucose and fructose 1, 6-phosphatse, with ATP [12].

PT possesses such wide spectrum anticancer potential. However, PT exhibits poor aqueous solubility and permeability owing to bio-pharmaceutical classification system (BCS) class IV drug, which directs it to low bioavailability. Moreover, the low bioavailability could be caused by following reasons; a) PT, an efficient anticancer drug act as substrate for drug efflux pump transporter *i.e.* P – glycoprotein (P-gp) and multidrug resistance protein (MRP-2), these P-gp and MRP -2 with substrate, inhibits oral absorption of PT by excreting it into intestinal lumen [8, 13], b) PT follows the widespread pre-systemic first pass metabolism in liver and gut by involvement of cytochrome enzymes (CYP3A and CYP2C8) [8, 14]. c) Lower elimination half-life ($t_{1/2}$ el) [15]. Therefore, by consideration of these problems, there is a need to develop novel formulation for such effective and efficient anticancer drug.

For improving poor aqueous solubility and permeability of PT, a number of formulation strategies have been developed and used. Some of them were modified, due to some excipient – drug interactions. For improving the solubility, PT dissolved in a mixture of polyoxyethylated castor oil (Cremophore EL): dehydrated ethanol (1:1) ratio as delivery vehicle. The formulation produced hypersensitivity and non-linear pharmacokinetic behaviour after intravenous administration. The hypersensitivity reaction at site of administration could be due to inclusion of Cremophore EL [16, 17]. After that, the delivery vehicle was replaced with addition of tween 80 alone or combination of tween 80: dehydrated alcohol, and diluted with aqueous media. The diluted formulation showed the precipitation of PT from solution due to low solubility [18].

These attempted techniques, with persistent low solubility problem has been overcome by creating novel formulation with the aim of improving aqueous solubility, permeability and bioavailability of PT. It includes novel oral formulation [19], novel PT self emulsifying drug delivery system (SEDDS) [20], novel ligands based PT targeting formulation [21], miceller formulation [22], liposomal formulation [23], bioconjugates [24], dendrimers [25] and nanocarrier systems [26]. In all these formulation techniques the problem associated to PT, were shown to be improved significantly.

The liposome an emerging techniques for specialized drug delivery [27] and best suitable for lipophilic drug due to its biocompatibility and reducing drug toxicity, with maintaining efficacy of anticancer drug for maximum period of time. Some previous studies include asulacrine [28], docetaxel [29] and tamoxifen [30] with these approaches, their poor aqueous solubility and bioavailability was found to be improved. Therefore, the present work was elected to formulate and characterize the paclitaxel liposomes (PTL) using saturated and unsaturated phospholipids for topical drug delivery and studied their effect on vesicle size, drug entrapment and *in vitro* release of PT. Moreover, the PTL were also characterized and investigated for hemolytic toxicity assay.

MATERIALS AND METHODS:

Materials: Paclitaxel (PT), with purity > 90% was received as a gift sample from MAC-CHEM Products (India) Pvt. Ltd. Bhoisar, Thane, India. The phospholipids samples *viz.*, Phospholipon 90G® (PL90G), Phospholipon 80H® (PL80H) and Phospholipon 90H® (PL90H) with purity > 90%, was obtained as a free gift sample from Lipoid GmbH, Ludwigshafen, Germany. The solvents namely acetonitrile, chloroform, and methanol were purchased from Merck Ltd. Mumbai, India. Cholesterol (CH), potassium dihydrogen phosphate and sodium hydroxide pellets were obtained from Sigma Chemicals, Sigma-Aldrich Corporation, St. Louis, MO. Chemical used in these work were of analytical grade (AR).

Preparation of Paclitaxel Liposomes (PTL): PTL's were prepared according to previous reported procedure described by Dua [31]. Briefly, the cholesterol and phospholipids (*i.e.* CH:PL90G, CH:PL80H and CH:PL90H) at various stoichiometric ratios of (1:0.25, 1:0.5, 1:1, 1:2 and 1:3) accompanied with PT was accurately weighed and placed into 100mL round bottom flask. The weighed ingredients were dissolved in 10mL of chloroform. The round bottom flask was then fixed to Rotary vacuum evaporator (Model: PBV – 7D, Vertical condenser, Rotavap, SuperfitTM Continental Pvt. Ltd., Mumbai, India) at inclined position and temperature of the flask was kept constant at 400 °C using water bath for three hours. The organic solvent was evaporated off under reduced pressure and thin film was obtained. The obtained thin film was hydrated by addition of 30mL of phosphate buffer (pH 7.4) and vertoxed for 15 min. After vertexing, the whole liposomal formulation was centrifuged at 20,000 rpm for 45 min and separated the supernatant part that contains free (non-incorporated) PT. The settled sediment containing PT were further redispersed into phosphate buffer (pH 7.4) up to 25mL in order to achieve a lipid content of 1mg/ml and then transferred to a amber colour glass vials and stored at 40 °C. The formulation batches for PTL using different ratio of cholesterol: phospholipids *viz.*, PL90G, PL80H and PL90H are shown in **Table 1**.

Characterization of PTL:

Entrapment Efficiency: The entrapment efficiency (EE) is defined as the ratio of the amount of the PT encapsulated in the liposome to that of the total PTL dispersion. The amount of PT encapsulated in liposomes was measured by using ultracentrifugation method [32]. Briefly, the 2mL of PTL dispersion was placed into 10mL of volumetric flask and diluted up to the mark with a mixture of acetontrile: phosphate buffer (pH 7.4) and then sonicated. After complete disruption of PTL vesicles, the reaction mixture was centrifuged at 10,000 rpm for 5 min to separate the supernatant. The collected supernatant was suitably diluted and the absorbance of the resulting solution was recorded at 217nm using UV-visible spectro-photometer (Model: SPECTRO 2060 PLUS, Analytical Technologies Ltd., Gujarat, India).

Vesicular Size and Distribution: Motic Digital Microscope (type DM-1802) was used to characterize the vesicles size and size distribution of PTL. Briefly, 2mL of PTL dispersion was placed over the clean slide and covered with cover slip. The microscopic characterization of PTL was examined at magnification of (×40) using calibrated eyepiece micrometer. The images were recorded using Motic Image Plus 2.0 ML software, accompanying with instrument.

***In vitro* Dissolution Studies:** The *in vitro* dissolution study for PT from PTL formulation was carried out as per procedure described by Utreja [33] with little modifications. In brief, the Franz diffusion cell apparatus (Details) was employed for this study. The apparatus is consisted of donor and receptor compartment, with effective surface area for dissolution was (2.303 cm^2). The dialysis membrane (LA395, Dialysis Membrane – 110 AV, flat width ~ 31.12 mm, Average diameter ~ 21.5 mm, and approximate capacity is ~ 3.63 mL/cm; Himedia laboratories, Mumbai, India) was employed and pretreated as per the directions given by manufacturer. After proper pretreatment, the membrane was cut into desired size and shape, then mounted between effective surface area of donor and receptor compartment. The PTL dispersion (2 mL) was placed over the membrane, accompanied with addition of phosphate buffer (20 mL, pH 7.4) as dissolution media in the receptor compartment. The contents of receptor compartment were stirred at 100 rpm using magnetic stirrer at 37 ± 1.00 °C. At specified time intervals, 2mL aliquots were withdrawn from sampling port of apparatus, diluted

suitably with fresh media and the absorbance of resulting solution was read at 217nm using UV-visible spectrophotometer (Model: SPECTRO 2060 PLUS, Analytical Technologies Ltd., Gujarat, India).

Hemolytic Toxicity Assay: The hemolytic toxicity for prepared PTL dispersion was estimated by means of Red Blood Cell (RBC) lyses assay procedure as described by Utreja and Reed *et. al.*, [33, 34]. Briefly, the prepared PTL dispersion was diluted with phosphate buffer saline (PBS, 2.5 mL, pH 7.4) to suitable concentration range. The blood samples were obtained from tail vein of albino rats then centrifuged at 3000 rpm for 10 min and discarded the supernatant part. Then, the settled sediment of RBC suspension was further diluted with normal saline solution (0.9% w/v) to get concentration up to 5 % w/v. Form these solution, 0.5 mL suspension was mixed with distilled water and then incubated at 370 °C for 1 h to get complete (100%) hemolysis. After incubation, the contents were centrifuged and separated the supernatant (containing non-lyses RBC).

The aliquot of supernatant was diluted with same quantity of phosphate buffer saline (PBS, 20.5 mL, pH 7.4) and measured the absorbance of the resulting solution at 540nm using UV-visible spectrophotometer (Model: SPECTRO 2060 PLUS, Analytical Technologies Ltd., Gujarat, India) by taking supernatant normal saline solution as blank. The hemolytic activity for each sample was expressed as % hemolysis by taking absorbance of distilled water as 100% hemolytic sample.

TABLE 1: COMPOSITION AND CHARACTERIZATIONS OF PTL

Formulation Code	Formulation Ratios (CH:PL)	PL (mg) 90G	80H	90H	CH (mg)	Drug (mg)	EE (%)	Avg. VS* (µm)	In vitro Release (%)	HTA (% Hemolysis)
L1-90G	1:0.25	19	-	-	75	2	60.21	3.18 ± 0.90	62.59	5.93
L2-90G	1:0.5	38	-	-	75	2	65.62	3.21 ± 0.96	76.92	4.02
L3-90G	1:1	75	-	-	75	2	86.67	2.52 ± 0.86	86.22	2.70
L4-90G	1:2	150	-	-	75	2	68.33	2.81 ± 1.07	64.79	4.66
L5-90G	1:3	225	-	-	75	2	62.08	3.11 ± 0.97	60.38	7.62
L6-80H	1:0.25	-	19	-	75	2	53.96	2.99 ± 1.09	58.11	8.60
L7-80H	1:0.5	-	38	-	75	2	59.58	2.85 ± 1.29	65.53	7.83
L8-80H	1:1	-	75	-	75	2	74.58	2.31 ± 0.70	79.25	6.77
L9-80H	1:2	-	150	-	75	2	61.46	2.86 ± 1.39	62.45	7.41
L10-80H	1:3	-	225	-	75	2	57.92	3.18 ± 0.96	53.71	9.53
L11-90H	1:0.25	-	-	19	75	2	58.12	3.92 ± 1.09	60.69	8.47
L12-90H	1:0.5	-	-	38	75	2	61.04	3.03 ± 0.89	70.88	7.21
L13-90H	1:1	-	-	75	75	2	80.62	3.18 ± 0.96	82.64	4.44
L14-90H	1:2	-	-	150	75	2	65.00	3.49 ± 0.96	61.70	11.01
L15-90H	1:3	-	-	225	75	2	60.63	2.92 ± 0.76	57.23	7.83

*Values represented as mean ± SD.
CH = Cholesterol,
PL=Phospholipids (PL90G = Phospholipon 90G®, PL80H=Phospholipon 80H®; PL90H= Phospholipon 90H®)
CH: PL = Paclitaxel liposomes containing different concentration of phospholipids.
EE = Entrapment efficiency; VS = Vesicular size; HTA = Hemolytic toxicity assay.

RESULTS AND DISCUSSION:

Entrapment Efficiency: According to previous published reports shows that, mainly cholesterol and phospholipids are employed in different concentration, molar and stoichiometric ratios for the formulation of liposomes [35]. So in the current study, the PTL's were formulated using stoichio-metric ratio and also studied the effect of various phospholipids at different concentrations on EE. It is observed that, the EE *i.e.* highest incorporation of PT in liposomal vesicles were found to be enhanced as increasing the ratios between cholesterol and phospholipids, but then declined suddenly as further increasing the ratio. The obtained results (**Table 1**) demonstrated that, by using PL90G, PL80H and PL90H the EE were found approximately 86.67, 74.58 and 80.62% at 1:1 ratio of CH: PL. The results were consistent with previous published reports [36]. Thus, it can be concluded that, stoichio-metric ratio between cholesterol and phospholipids, in addition to selection of proper phospholipids with highest phosphatidylcholine content might be responsible for obtaining highest EE.

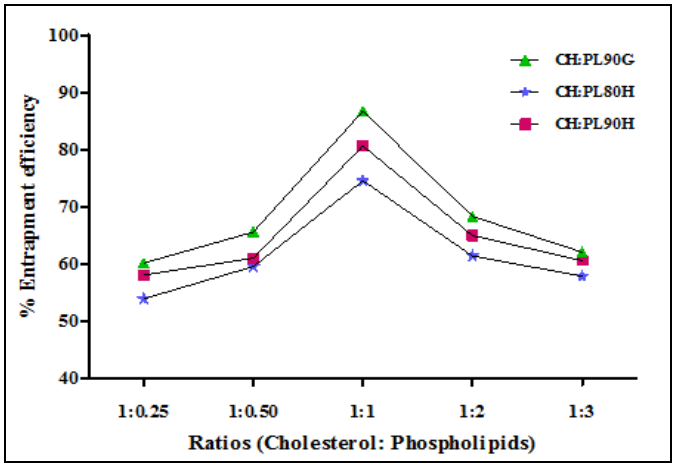

FIG. 1: % ENTRAPMENT EFFICIENCY FOR PTL CONTAINING DIFFERENT RATIO OF CHOLESTEROL: PHOSPHOLIPIDS (CH: PL90G, CH: PL80H, CH: PL90H)

Vesicular Size and Distribution: As shown in **Fig. 2**, the surface morphology of PTL was observed by Motic Digital Microscope (type DM-1802). The liposomes were spherical in shape with a smooth surface, size was appropriate and uniform (CH: PL90H).

Fig. 3 shows the average vesicular size and size distribution of PTL prepared with varying ratio of cholesterol: phospholipids. The average vesicular size of PTL prepared with varying ratio of CH: PL90G, CH: PL80H and CH: PL90H was found to be in the range of 2.96 ± 0.2955 µm, 2.83 ± 0.323 µm and 3.18 ± 0.222 µm respectively. The results are presented in **Table 1**. As clearly seen the size distribution (72%) was in the range of 2.1-4.0 µm for CH: PL90G, (97%) was in the range of 1.1-5.0 µm for CH: PL80H and (87%) were in the range of 2.1-5.0 µm for CH: PL90H.

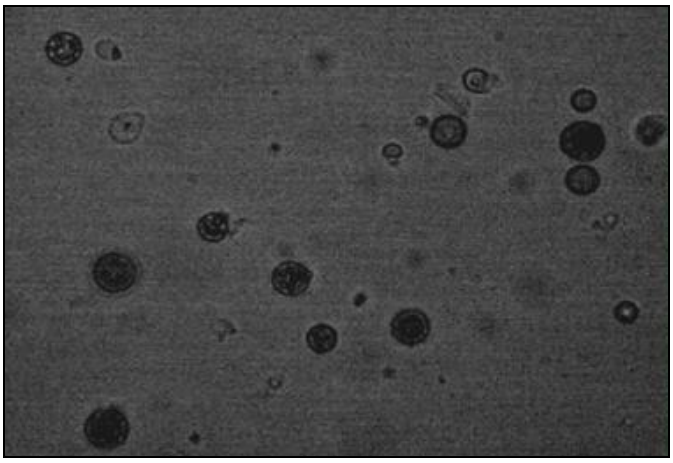

FIG. 2: MICROPHOTOGRAPH OF PTL BY MOTIC IMAGE PLUS 2.0 ml SOFTWARE

On the basis of consideration of above results it suggests that PTL with cholesterol: phospholipids ratio (1:1) demonstrated a smaller vesicle size and CH: PL90G shows greater uniformity in vesicle size as compare to CH: PL80H and CH: PL90H.

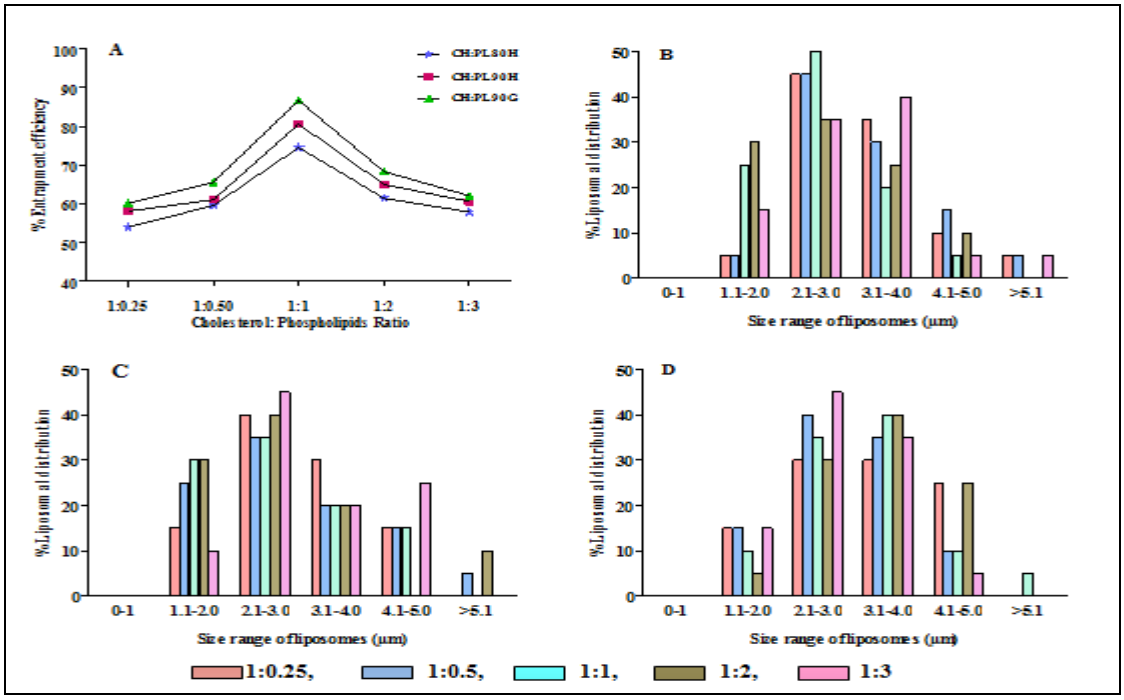

FIG. 3: AVERAGER VESICULAR SIZE (A) AND VESICULAR SIZE DISTRIBUTION (B, C, D) OF PTL CONTAINING DIFFERENT RATIOS OF CHOLESTEROL: PHOSPHOLIPID. (B = CH: PL90G; C = CH: PL80H; D = CH: PL90H)

***In vitro* Release Studies:** The releases of PT from PTL in phosphate buffer saline (PBS, pH 7.4, 20 mL) are shown in **Fig. 4**. From the figure, it shows that the highest % of drug release (86.22%) was obtained with liposomal formulation having ratio (1:1) of CH:PL90G. The PT release from liposomal formulation is increased as the concentration of the PL90G was increased [From 1:0.25 (62.59%) to 1:1 (86.22%)] and further increases to this ratio resulting into decline of release rate [From 1:2 (64.79%) to 1:3 (60.38%)].

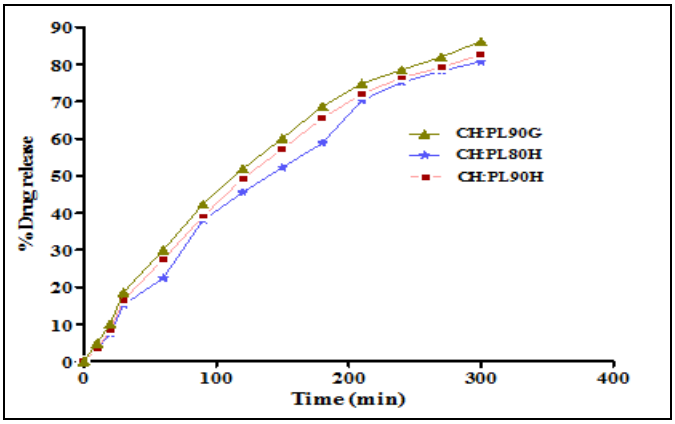

FIG. 4: % *IN VITRO* DRUG RELEASE BY PTL CONTAINING 1:1 RATIO OF CHOLESTEROL AND PHOSPHOLIPID [CH: PL90G (1:1); CH: PL80H (1:1); CH: PL90H (1:1)]

Comparing the cumulative amount (**Fig. 4**) after 300 minutes, the highest % drug release (79.25%) and (82.64%) was obtained with liposomal formulation having ratio (1:1) of CH: PL80H and CH: PL90H respectively. The PT release from liposomal formulation (CH: PL80H) is increased as the concentration of the PL80H was increased [From 1:0.25 (58.11%) to 1:1 (79.25%)] and further increases to this ratio resulting into decline of release rate [From 1:2 (62.45%) to 1:3 (53.71%)]. Same result was obtained in liposomal formulation (CH: PL90H) the PT release is increased as the concentration of the PL90H was increased [From 1:0.25 (60.69 %) to 1:1 (82.64%)] and decrease release rate [From 1:2 (61.70%) to 1:3 (57.23%)] further increases the ratio. It indicates that the release rate of PT from PTL was found to be best when ratio between cholesterol: phosphor-lipids were (1:1).

Hemolytic Toxicity Assay (HTA): The effects of PTL formulation on hemolysis of RBC are shown in **Fig. 5**. The cholesterol: phospholipids ratio (1:1) showed the best hemolytic activity. The hemolytic activity with CH: PL80H (1:1) was found to be 6.77%. In case of CH: PL90H (1:1), the hemolytic activity was found to be 4.44%.

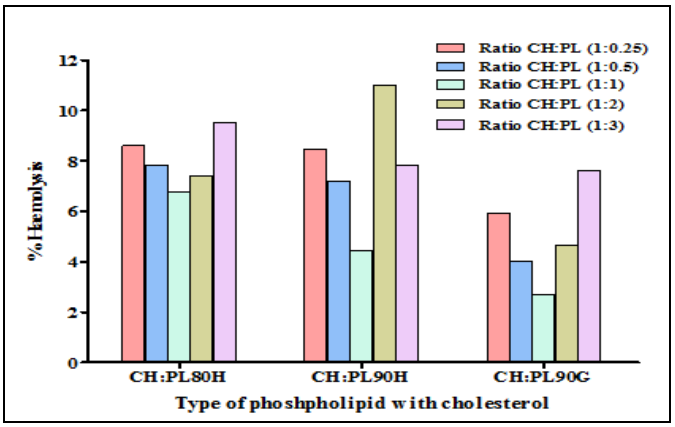

FIG. 5: % HAEMOLYSIS OF RBCs BY PTL CONTAINING DIFFERENT RATIO OF CHOLESTE-ROL: PHOSPHOLIPID (CH: PL90G; CH: PL80H; CH: PL90H)

In contrast to this, the least hemolytic activity was found to be 2.70%, when CH: PL90G ratio was (1:1). Similarly, the significant result of lower toxicity was found to be for PT solid dispersion in comparison to commercial Taxol formulation [37]. Based on above discussion, it can be concluded that the (1:1) ratio of CH: PL90G might be responsible for excellent hemolytic activity. Significantly it happens due to its composition. These vesicles are made from biocompatible phospholipids as a major constituent.

CONCLUSION: The presents work reveals the excellent effect of phospholipids on PTL. The PTL's were prepared by film hydration method, with incorporation of various phospholipids. The PT liposome's prepared with cholesterol: PL90G ratio (1:1) was found to be optimized one. The EE was found to be 86.67%. Whereas, particle size with these ratio showed to be uniform size distribution. The in *vitro* release rate in phosphate buffer saline (PBS, pH 7.4) was also found to be enhanced. The PTL formulation showed a least hemolytic activity. Hence this formulation might be accountable for better alternative of existed form of PT dosage form.

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