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Optimization of Transethosome by Varying Dark Purple Glutinous Rice Variety Leum Phua (*Oryza sativa* var. *glutinosa*) Extracts and Rice Bran Oil under Hot Method

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Abstract

Leum Pua is native Thai glutinous rice that contains antioxidants higher than other colored rice. The purpose of this study was to develop transethosomes (TEs) extracted from Leum Phua rice by hot method. The influence of rice extract and rice bran oil concentrations was investigated on physical properties of the TEs. The formulation of TEs was performed by selecting the optimal ratio obtained from a mixture of glutinous rice extract at the concentration 20, 30 and 40% (w/v) and rice bran oil 20, 25 and 30% (w/v). Characterization of the TEs was based on results from particle size, polydispersity index, zeta potential, entrapment efficiency and stability testing. Leum Pua glutinous rice was extracted by 95% ethanol. The extracts were developed for TEs using rice bran oil as phospholipid and lecithin as permeation enhancer, Span 80 and Tween 20 as edge activator (surfactant) in their formula. Furthermore, the required size of ethosome vesicles was prepared by using sonication. Results showed that the optimized TEs formulation with particle size below 300 nm could be achieved by using rice extract at the concentration 30 and 40% (w/v) and rice bran oil 20% (w/v). At a concentration of rice extract 40% (w/v) and rice bran oil 20% (w/v) showed the highest entrapment efficiency (68.94±2.9%). The good colloidal characteristics had a particle size (PZ) of 233.0±12.9 nm, polydispersity index (PDI) of 0.314±0.078 and zeta potential (ZP) of -51.4±0.24 mV. The TEs optimized formulation was stable at room temperature and also at elevated temperature conditions (45°C/75% RH) for 3 months. Therefore, based on the current study, the potential of employing the novel carrier transethosomal-loaded nanoparticles of Leum Phua rice extracts could serve as an effective dermal delivery. The development of TEs for topical delivery can lead to value added of native Thai glutinous rice.

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Introduction

Dark purple glutinous rice has several health benefits. It contains high concentration of important bioactive compounds especially those with anti-aging properties. Other interesting properties are anti-tumor and anti-inflammatory (Seekhaw et al., 2018). Black rice extract, in particular, is a potent source of natural antioxidants, which is important for the potential to increase immune system in the body and to protect and reduce the risk of cancer and heart diseases (Pitija et al., 2013). Bioactive compounds present in pericarp include alkaloids, flavonoids and phenols. The flavonoids (particularly the anthocyanins) are basic phenolic compounds responsible for antioxidant activity (Peanparkdee et al., 2019). Leum Phua glutinous rice is a traditional purple rice known for over 100 years by the Hmong people. It is still planted in Phop Phra District, Tak Province, Thailand and can only be planted once a year. When cooked, the sticky rice is very fragrant with a soft sweet outer part. This rice has dark-purple pericarp and contains high levels of certain nutrients such as γ-oryzanol, vitamin E, omega-3-6-9 fatty acids, iron (Fe), calcium (Ca) and anthocyanins. Additionally, Leum Phua rice extracts has been reported to have antioxidant activity higher than other black rice, citing two to three times higher antioxidant activity (Wattanuruk et al., 2020; Boonsit et al., 2010). Several recent reports showed that antioxidant agents could prevent and improve many diseases such as cancer, atherosclerosis and Alzheimer's disease (Kaur & Ling, 2008; Podsedek, 2007). Therefore, with high antioxidant property, Leum Phua rice could be one of the nutraceuticals for the brain. Leum Phua rice aqueous extract (LP) has shown learning and memory enhancing effects in male mice. The effect of LP might be due to high concentration of antioxidants in Luem Phua rice (Srisuwan et al., 2013). Moreover, Leum Phua rice as an anti-aging agent due to its ability to not only counteract reactive oxygen species production induced by different challenges including UV exposure, but also to inhibit cutaneous collagenase and elastase activity (Vichit & Saewan, 2016). Although there is quite a lot of information concerning the usefulness of dark purple rice extracts available, its application as an active ingredient for cosmetics and drugs has been limited. The anthocyanins are hydrophilic and thus has poor skin permeability. Therefore, a delivery system that can facilitate the skin absorption of bioactive compounds is desirable and a formulation using nanocarrier could be among the best such vehicles for extracts-based treatment.

Nanotechnology is an increasingly widespread area of research for its various applications and advantages. Nanosystems allow for a deep skin penetration and an effective release profile of ingredients, which contributes to drug and cosmetic effects. Moreover, nanoparticle that can entrap the molecule in a physiological environment and protect it against oxidative degradation. Presently various type of vesicular nanocarrier systems is available such as liposomes, ethosomes, transferosomes, niosomes, transethosomes etc. Transethosomes (TEs) are the new generation of ethosomal systems and were first reported in 2012 (Song et al., 2012). The tranethosomal system contains the basic components of classical ethosomes and an additional compound, such as a penetration enhancer or an edge activator (surfactant) in their formula. These novel lipid vesicles were developed to combine the advantages of classical ethosomes and deformable liposomes (transfersomes) in one formula to produce TEs. TEs may contain advantages of both transfersomes and ethosomes. The mechanism of skin penetration might be a fusion of both mechanisms that can improve penetration through the skin due to their deformable ability, but they are unable to penetrate deep into the stratum corneum. TEs are composed of phospholipid (PC), water, edge activator or permeation enhancer (oleic acid) and are characterized by having a high content of ethanol (up to 30%). Due to presence of ethanol the intercellular space between the corneocytes increases which increase the permeation (Ascenso et al., 2015). The PC component of the vesicles tends to evade a dry environment. Thus, to stay fully swollen, the vesicles follow the local hydration gradient and penetrate more strongly the hydrated layers of skin, reaching the epidermis and dermis (Kumar et al., 2012). Recently, the efficacy of TEs have been successfully developed for both pharmaceuticals and cosmeceuticals in enhancing the dermal and transdermal delivery (Ascenso et al., 2015; Meng et al., 2013). TEs can cross intact skin by transcutaneous hydration gradient. Drying and partial dehydration of vesicles are the initial events in skin permeation by the vesicles after topical application. As a result, the vesicles become compressed or curved. This fact may be due to the combination of ethanol and edge activator that causes a rearrangement in the lipid bilayer of these vesicles (Gondkar et al., 2017). Few studies have investigated the nanoparticle of black rice extract

(Bulatao et al., 2017). To the best of our knowledge, there is no published research about the use of TEs to enhance the transdermal delivery of black glutinous rice extract. Further, many methods have been made for formulating TEs. In hot method, a dispersion of phospholipid and water is subjected to heating at a temperature 40-80°C on a magnetic stirrer. Depending on the ability and affinity of active ingredient to bind with hydrophilic and hydrophobic solvent, it either gets dissolved in water or in ethanol. In addition, the required size of ethosome vesicles can be easily prepared by using methods like extrusion or sonication (Pandey et al., 2015; Garg et al., 2017).

This study prepared TEs from Leum Phua glutinous rice extracts. The resulting formulation of TEs is a development of a form that produces sufficiently the particles' size and ensures stability. Therefore, this affects the transdermal delivery. Thereafter, the optimized formulation was analyzed for particle size (PZ), polydispersity index (PDI), zeta potential (ZP), entrapment efficiency and stability properties.

Materials and methods

1. Preparation of crude extracts

Crude rice extract was prepared according to the methods of Plaitho, (2016) with some modifications. The glutinous rice of Leum Phua rice was purchased from Tak Province, Thailand and were dried in the oven at 60°C for 24 hours. Dried rice samples were extracted with 95% ethanol under stirring in a shaker at 120 rpm for 24 hours. The ethanol extracts were separated in the centrifuge (Becton Dickinson Dynac Centrifuge, Sparks, MD, USA) at 6,000 g for 10 min and were filtered through a paper filter (Whatman No.1). The waste from the previous extraction was re-extracted with the same method and then combined with the extract obtained from the first and mixed well. The extracts were transferred to a flat-bottomed flask. The solvents were evaporated by a rotary evaporator (Buchi R-124 Rotary Vap System, New Castle, USA) at 45°C until the samples were dry. All crude extracts were stored at -10°C in storage vials.

2. Preparation of TEs

The TEs from Leum Phua glutinous rice was prepared by hot method in 100 ml batches (Nandan & Shivalik, 2016). In a beaker the Leum Phua glutinous rice extracts was placed in specified amount of 95% ethanol, rice bran oil, lecithin, Sorbitan Oleate (Span 80) and Ceteary Alcohol (and) Ceteary Glucoside (Montanov 82) and dissolved at 80°C. Stirring was continuing through the magnetic stirrer at 300 rpm. Later, Polysorbate 20 (Tween20), Poloxamer and glyceral was dissolved in distilled water in a separate beaker at 80°C using the magnetic stirrer at 300 rpm. All chemicals' reagents were from Namsiang, Ltd., Bangkok,Thailand (analytical grade). Aqueous phase was added into the organic phase and stirring was continuing through the magnetic stirrer at 500 rpm for 30 min at 70°C. After the stirring process, sonication was done by the probe sonicator (Sonics Vibra Cell, Sonics & materials, Inc., Newtown, USA) at 8,000 rpm for 10 min (Fig 1). Finally, the TEs were prepared. For this experimental, different variable influencing vesicles' characteristics were prepared using four different amounts of Leum Phua glutinous rice extracts such as 20, 30 and 40% (w/v) and rice bran oil (20, 25 and 30 (w/v)). Among the various designs available for the purpose, the central composite design (CCD) has extensively been employed in optimization practice for identifying the best formulation (Singh et al., 2009). The compositions of each TEs formulation are presented in Table 1.

Fig. 1 Probe sonicator

Table 1 The compositions of TEs formulation

Material	Formular% (w/v)				
	TEs 1	TEs 2	TEs 3	TEs 4	TEs 5
Leum Phua glutinous rice extract	40	30	20	20	20
Rice bran oil	20	20	20	25	30
Lecithin	1	1	1	1	1
Span 80	5	5	5	5	5
Montanov 82	3	3	3	3	3
Tween 20	5	5	5	5	5
Poloxamer	3	3	3	3	3
Glycerol	2	2	2	2	2
Distilled water	increase to 100	increase to 100	increase to 100	increase to 100	increase to 100

3. Characterization of TEs

The mean particle size (PZ), polydispersity index (PDI) and zeta potential (ZP) of vesicles dispersions were measured by Dynamic light scattering technique using Malvern Zetasizer 2000 (Malvern Instruments Ltd., Malvern, UK). Physical properties were performed after 30 days, storage at 25°C. The measurements were performed after dilution. The dilute sample (50 µl) was added to 950 µl of distilled water to obtain a light-scattering substance of sufficient light intensity. (Abdelbary et al., 2015). Zeta potential evaluation was carried out by monitoring the electrophoretic movement of the particles in the electrical field using Malvern Zetasizer 2000 (Malvern Instruments Ltd., Malvern, UK). The ZP indicates charge present on the surface of TEs which is responsible for stability of the formulation and interact with membrane (Souto et al., 2004) All measurements were performed in triplicate. The optimized formulation was selected on the basis of size, extract entrapment and other colloidal characteristics like zeta potential and polydispersity index (PDI).

4. Entrapment efficiency

The percentage entrapment of the rice extracts added is called entrapment efficiency. The amount of rice extracts entrapped in the nanoparticles was determined by the separation of nanoparticles of the rice extracts from the freely suspeded rice extracts by centrifugation. Free unentrapped from TEs was separated by centrifugation at 20,000 g for 1 h at 4°C using a cooling centrifuge. The pellets that were formed after centrifugation were washed twice with 5 ml of phosphate buffer (pH 7.4) and re-centrifuged again for 1 h. The encapsulation efficiency of the extract was determined after the breakdown of the pellets with 5 ml of methanol and sonication for 10 min. The concentration of extract in methanol was determined using UV-Visible spectrophotometer (Bio-Tek, Vermont, UK) at 268 nm. Entrapment was determined using the following equation (Ramachandran & Shanmughavel, 2010).

$$\text{Entrapment Efficency (\%)} = \frac{\text{Total extract added} - \text{Free unentraped extract}}{\text{Total extract Added x 100}}$$

The entrapment efficiency or percentage of the content was estimated as the difference between the initial extract quantity and the free or unentrapped quantity of extract in the supernatant with respect to the total quantity incorporated in the nanocarrier preparation.

5. Accelerated stability study

The optimum physical stability testing of TEs were exposed to various temperatures like 45 ± 2°C and room temperature at 25°C±2°C for three months. The humidity level was kept at 75% in dark conditions. Optimized TEs were evaluated mainly for their physical characteristics at 0 and 3 months to check for the storage physical stability. Vesicles were examined visually for aggregation and change in their appearance such as texture and phase separation (Li et al., 2012).

6. Data and statistical assessments

The experiments were performed in triplicates and the results were presented as mean ± standard deviation. The statistical differences were determined at $P \leq 0.05$. Data were subjected to Duncan's post hoc test and the differences were detected for homogenous subsets. All statistical analyses were performed using SPSS® software 22.0.

Results and discussion

TEs extracted from Leum Phua glutinous rice extracts were effectively produced by using the hot method. This method is simple and does not require highly specialized equipment or extreme conditions. Various TEs formulations were produced by varying the amount of Leum Phua rice extracts and rice bran oil in their compositions. (Table 1) The optimized formulation was selected based on size (below 300 nm), entrapment efficiency and other colloidal characteristics like zeta potential and polydispersity index (PDI). The size of the colloidal carrier system is an important parameter of investigation as it affects the penetration of carrier into the skin especially in deeper layers. Prior research on transdermal systems revealed that vesicular carrier having a size below 500 nm effectively penetrated the skin and formed holes in the deeper layers of the skin (Zakir et al., 2010). Size of all TEs formulations was found in the range of 141–280 nm (Kohli & Alpar, 2004). Table 2 and graphically illustrated in Fig. 2 describes particle size (PZ), polydispersity index (PDI), zeta potential (ZP) and% entrapment efficiency of different TEs formulations.

The present work developed TEs using rice bran oil as phospholipid and lecithin as permeation enhancer. The size of TEs extracted from Leum Phua rice increased with the increase amount of rice bran oil. The vesicular size of ethosomal systems plays an important parameter that should be considered in the preparation of

Table 2 PZ, PDI, ZP and Entrapment efficiency of formula variables

Formular	PZ (nm)	PDI	ZP (mV)	% Entrapment efficiency
TEs 1	233.0±12.9[a]	0.314±0.078	-51.41±0.24	68.94±2.9
TEs 2	256.7±18.9[a]	0.364±0.036	-52.08±2.13	66.21±3.2
TEs 3	391.2±.21.9[b]	0.427±0.029	-57.35±2.93	59.63±2.5
TEs 4	415.2±14.9[c]	0.450±0.064	-58.62±1.15	56.07±2.8
TEs 5	435.8±15.5[c]	0.474±0.026	-58.69±2.38	50.58±2.2

Remark: Mean values for each parameter followed by a different letter within each column are significantly different ($p \leq 0.05$)

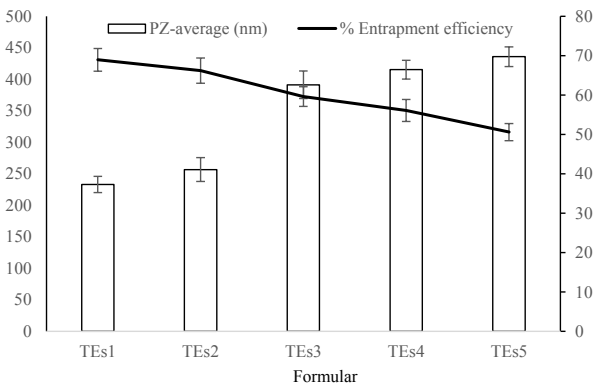

Fig. 2 PZ-average diameter and entrapment efficiency of formula variables

nanocarriers. Smaller vesicular size facilitates the TEs to pass through the small pores of the skin leading to more enhanced skin permeation. It has been reported that the vesicular size should be <300 nm to be suitable for this route of administration (Verma & Pathak, 2012). The optimized TEs formulation with particle size below 300 nm with low PDI could be achieved by using rice extract at the concentration 30 and 40% (w/v) and rice bran oil 20% (w/v) (TEs1 and TEs2). There was no significant difference in the particles size of all vesicles in both formulations ($p<0.05$) . Vesicle size of TEs1 and TEs2 formulation had an optimized range of 233.0±12.9 nm and 256.7±18.9 nm, respectively. It was observed that rice bran oil concentrations >20%(w/v), the size of the vesicles was >300 nm. The vesicular size of the TEs increased significantly ($p<0.05$) by increasing the rice bran oil concentration. The polydispersity of TEs1 and TEs2 formulation was 0.314±0.078 and 0.364±0.036, respectively. PDI≤0.3 are considered ideal and indicate a narrow size distribution (Pathak & Nagarsenker, 2009). Moreover, TEs1 and TEs2 formulations showed high entrapment efficiencies (more than 65%) indicating their capability to produce the desired therapeutic effect. The%entrapment efficiency of TEs1 and TEs2 formulation was found to be 68.94±2.9% and 66.21±3.2%, respectively. The rice extracts of TEs3, TEs4 and TEs5 formulation were also excluded from the selection due to the associated increase in the vesicular size. Therefore, formulation were TEs1 and TEs2 formulation considered to be optimized. The phospholipid type had a significant effect on the ethosomal size, but not the entrapment efficiency. Increasing phospholipid concentration will increase vesicular size slightly or moderately but will increase entrapment efficiency significantly (Prasanthi & Lakshmi, 2012). The selection of a proper edge activator or penetration enhancer is a critical step in the formulation of TEs, as they have profound effects on the properties of the ethosomal system.

The results of the zeta potential analysis revealed that all the prepared TEs had negatively charged zeta potential, ranging from −51.41 to −58.69 mV. All the prepared TEs nanovesicles showed zeta potential values that were significantly affected by the percentage of rice extract and rice bran oil. As the concentration of rice extract was decreased and rice bran oil was increased, the obtained zeta potential value was increased owing to further deposition of the charge-inducing agent on the vesicles' outer surface. Zeta potential involved with surface charges of particles can be positive or negative. Ogiso et al., (2001) reported that the negatively charged vesicles had better skin permeation properties than the positively charged ones. The zeta potential of vesicles in formular TEs1 and TEs2 showed higher negative value compared to other vesicles (TEs3, TEs4 and TEs5). Zeta potential of TEs1 and TEs2 formulation was found to be -51.41±0.24 mV and -52.08±2.13 mv, respectively. Zeta potential is an important and useful indicator of particle surface charge, which can be used to predict and control the stability. High zeta indicates high repulsive force, resulting in prevention of particle aggregation. In general, nanoparticles with good physical stability should have zeta potential higher than +20 mV or lower than -20 mV due to the electric repulsion between particles. However, some studies mentioned that this was not always true and the stability studies should be established (Das et al., 2012).

TEs represent novel lipid formulation which contains phospholipids, ethanol in high concentration of 30 to 40% (Prasanthi & Lakshmi, 2012). The negative charge of the zeta potential of ethosomal systems is attributed mainly to the high ethanol content in these nanocarriers. Ethanol is a central character of TEs system giving unique identity to it as a vesicular system. Presence

of high content of ethanol imparts a negative charge on the surface of vesicles which promotes reduction of its size, thereby avoiding aggregation of the vesicular system due to electrostatic repulsion (Lopez-Pintoet et al., 2005). Additionally, ethanol was also reported to have stabilizing effect and efficient penetration enhancer (Finnin & Morgan, 1999; Dubey et al., 2007) Ethanol also has a significant effect on ethosomal system entrapment efficiency and in general increasing ethanol concentration will increase entrapment efficiency (Bhadra et al., 2004).

Bragagni et al., (2012) introduced Tween 20 in an ethosomal system of celecoxib at 15% of the total phospholipid concentration. It was found that Tween 20-containing TEs had smaller vesicular size (258.4±3.3 nm), higher entrapment efficiency (54.4%) and better ex vivo skin permeation through human skin compared to TEs containing Tween 80. The effects of Tween 20 on the ethosomal system are mainly due to its solubilizing property and the prevention of vesicle fusion. In another study, addition of Tween 20 formed an unstable formulation. Morever, Spans 20 were successful in producing homogeneous and stable TEs (Ascenso et al., 2015)

The optimized formulation (TEs1 and TEs2) was evaluated after storage at room temperature and after accelerated stability studies at elevated temperature (45°C/75% RH) in stability chamber. Stability profile of TEs loaded with Leum Phua rice extracts were checked by determining their appearance for 3 months. The results of stability studies show that the formulation was stable at room temperature and at elevated temperature conditions. The formulation TEs1 and TEs2 revealed its excellent colloidal stability. Physical appearance showed no change in color and odor on TEs both before and after of storage. It was found that the appearance of the TEs1 and TEs2 formulation was unchanged during the 3 months storage (Fig. 3). However, Optimum TEs were stored at 4-25°C. The TEs can be explored for transdermal delivery of various bioactive molecules due to their high entrapment efficiency and excellent colloidal stability (Verma & Utreja, 2018).

Fig. 3 Colloidal stability of TEs1 formulation (left) and TEs2 formulation (right)

Conclusion

Leum Phua glutinous rice has been studied and concluded for its high nutrition value. TEs are elastic vesicles composed of phospholipid, ethanol, and edge activator (surfactant). Preparation of TEs from Leum Phua glutinous rice extracted by heating method to be effective can be explained by setting the appropriate formula ratios, the resulting particle characteristics and the stability test. The optimized formulation had suitable characteristics for the transdermal delivery of extracts, such as small vesicular size, negatively charged ZP and high% entrapment efficiency. The TEs formulation was optimized using rice bran oil as phospholipid. The study found that the ratio between the glutinous rice extract at 40% (w/v) and rice bran oil at 20% (w/v) was the most suitable optimization. The results suggested that vesicle size and% entrapment efficiency of the optimized TEs formulation was found to be 233.0±12.9 nm and 68.94±2.9%, respectively. Polydispersity index and zeta potential of the optimized TEs formulation was found to be 0.314±0.078 and -51.4±0.24 mV, respectively. Nevertheless, The TEs optimized formulation was stable at room temperature and at 45°C for at least 3 months.

References

Abdelbary, A., Al-Mahallawi, A., Abdelrahim, M., & Ali, A. (2015). Preparation, optimization, and *in vitro* simulated inhalation delivery of carvedilol nanoparticles loaded on a coarse carrier intended for pulmonary administration. *International Journal of Nanomedicine, 10,* 6339–6353.

Ascenso, A., Raposo, S., Batista, C., Cardoso, P., Mendes, T., Praça, F.G., ... Simões, S. (2015). Development, characterization, and skin delivery studies of related ultradeformable vesicles: transfersomes, ethosomes, and transethosomes. *International Journal of Nanomedicine, 10,* 5837–5851.

Bhadra, D., Jain, N.K., Umamaheshwari, R.B., & Jain, S. (2004). Ethosomes: a novel vesicular carrier for enhanced transdermal delivery of an antiHIV agent. *Indian Journal of Pharmaceutical Sciences, 66*(1), 72–81.

Boonsit, P., Pongpiachan, P., Julsrigival, S., & Karladee, D. (2010). Gamma oryzanol content in glutinous purple rice landrace varieties. *Chiang Mai University Journal of Natural Sciences, 9,*151-157.

Bragagni, M., Mennini, N., Maestrelli, F., Cirri, M., & Mura, P. (2012). Comparative study of liposomes, transfersomes and ethosomes as carriers for improving topical delivery of celecoxib. *Drug Delivery, 19*(7), 354–361.

Bulatao, R.M., Samin, J.P.A., Salazar, J.R., & Monserate, J.J. (2017). Encapsulation of anthocyanins from black rice (*Oryza sativa* L.) bran extract using chitosan-alginate nanoparticles. *Journal of Food Research, 6*(3), 40-47.

Das, S., Ng, W.K., & Tan, R.B.H. (2012) Are nanostructured lipid carriers (NLCs) better than solid lipid nanoparticles (SLNs): Development, characterizations and comparative evaluations of clotrimazole-loaded SLNs and NLCs. *European Journal of Pharmaceutical Sciences, 47,* 139-151.

Dubey, V., Mishra, D., & Jain, N.K. (2007). Melatonin loaded ethanolic liposomes: Physicochemical characterization and enhanced transdermal delivery. *European Journal of Biopharmaceutics and Biopharmaceutics, 67*(2), 398–405.

Finnin, B.C., & Morgan, T.M. (1999).Transdermal penetration enhancers: Applications, limitations, and potential. *Journal of Pharmaceutical Sciences, 88*(10), 955–958.

Garg, V., Singh, H., Singh, B., & Beg, S. (2017). Systematic development of transethosomal gel system of piroxicam: Formulation optimization, in-vitro evaluation , and ex-vivo assessment. *AAPS PharmSciTech, 18,* 58–71.

Gondkar, S.B., Patil, N.R., & Saudagar R.B. (2017). Formulation development and characterization of etodolac loaded transethosomes for transdermal delivery. *Research Journal of Pharmacy and Technology, 10*(9), 3049-3057.

Kohli, A.K., & Alpar, H.O. (2004). Potential use of nanoparticles for transcutaneous vaccine delivery: Effect of particle size and charge. *International Journal of Pharmaceutics, 275*(1-2), 13-17.

Kumar, A., Pathak, K., & Bali, V. (2012). Ultra-adaptable nanovesicular systems: A carrier for systemic delivery of therapeutic agents. *Drug Discovery Today, 17*(21–22), 1233–1241.

Kaur, C., & Ling, E-A. (2008). Antioxidants and neuroprotection in the adult and developing central nervous system. *Current Medicinal Chemistry, 15*(29), 3068-3080.

Li, S., Qiu, Y., Zhang, S., & Gao, Y. (2012). Enhanced transdermal delivery of 18β-glycyrrhetic acid via elastic vesicles: In vitro and in vivo evaluation. *Drug Development and Industrial Pharmacy, 38*(7), 855-865.

Lopez-Pintoet, J.M., Gonzalez-Rodriguez, M.L., & Rabasco, A.M. (2005). Effect of cholesterol and ethanol on dermal delivery from DPPC liposomes. *International Journal of Pharmaceutics, 298*(1), 1-12.

Meng, S., Chen, Z., Yang, L., Zhang, W., Liu, D., Guo, J., ... Li, J. (2013). Enhanced transdermal bioavailability of testosterone propionate via surfactant-modified ethosomes. *International Journal of Nanomedicine, 8,* 3051–3060.

Nandan, D., & Shivalik, P. (2016). Ethanol based vesicular carriers in transdermal drug delivery: Nanoethosomes and transethosomes in focus. *NanoWorld Journal, 2*(3), 41-51.

Ogiso, T., Yamaguchi, T., Iwaki, M., Tanino, T., & Miyake, Y. (2001). Effect of positively and negatively charged liposomes on skin permeation of drugs. *Journal of Drug Targeting, 9*(1), 49–59.

Pandey, V., Golhani, D., & Shukla, R. (2015). Ethosomes: Versatile vesicular carriers for efficient transdermal delivery of therapeutic agents. *Drug Delivery, 22*(8), 988-100.

Pathak, P., & Nagarsenker, M. (2009). Formulation and evaluation of lidocaine lipid nanosystems for dermal delivery. *An Official Journal of the American Association of Pharmaceutical Scientists, 10*(3), 985–992.

Peanparkdee, M., Patrawart, J., & Iwamotob. S. (2019). Effect of extraction conditions on phenolic content, anthocyanin content and antioxidant activity of bran extracts from Thai rice cultivars. *Journal of Cereal Science, 86,* 86-91.

Pitija, K., Nakornriab, M., Sriseadka, T., Vanavichit, A., & Wongpornchai, S. (2013). Anthocyanin content and antioxidant capacity in bran extracts ofsome Thai black rice varieties. *International Journal of Food Science and Technology, 48,* 300–308.

Plaitho, P. (2016). Preventive effect of oxidative stress in human intestinal cell line (Caco2-cell) of Kao Mak made from black glutinous rice (*Oryza sativa* L. variety Leum Phua). *Science and Technology Journal, 24*(5), 813-830.

Podsedek, A. (2007). Natural antioxidants and antioxidant capacity of Brassica vegetables a review. *Food Science and Technology, 40*(1), 1–11.

Prasanthi, D., & Lakshmi, PK., (2012). Development of ethosomes with Taguchi robust design-based studies for transdermal delivery of alfuzosin hydrochloride. *International Current Pharmaceutical Journal, 1*(11), 370-375.

Ramachandran, R., & Shanmughavel, P. (2010). Preparation and characterization of biopolymeric nanoparticles used in drug delivery. *Indian Journal of Biochemistry and Biophysics, 47*(1), 56–59.

Seekhaw, P., Mahatheeranont, S., Sookwong, P., Luangkamin, S., Na Lampang Neonplab, A., & Puangsombat, P. (2018). Phytochemical constituents of thai dark purple glutinous rice bran extract (Cultivar Luem Pua (*Oryza sativa* L.). *Chiang Mai Journal of Science, 45*(3), 1383-1395.

Singh, B., Pahuja, S., Kapil, R., & Ahuja, N. (2009). Formulation development of oral controlled release tablets of hydralazine: optimization of drug release and bioadhesive characteristics, *Acta Pharma. 59,* 1–13.

Song, C.K., Balakrishnan, P., Shim, CK., Chung, S.J., Chong, S., & Kim, D.D. (2012). A novel vesicular carrier, transethosome, for enhanced skin delivery of voriconazole: Characterization and in vitro/in vivo evaluation. *Colloids Surf B Biointerfaces, 92,* 299–304.

Souto, E.B., Wissing, S.A., Barbosa, C.M., & Muller, R.H., (2004). Valuation of physical stability of SLN and NLC before and after incorporation into hydrogel formulations. *European Journal of Pharmaceutics and Biopharmaceutics,* 58, 83- 90.

Srisuwan, S., Sattayasai, J., Arkaravichien, T., Wongpornchai, S., Luangkamin, S., Seekhaw, P., & Na Lampang Noenplab, A. (2013). The effects of dark purple glutinous rice variety leum phua on scopolamine-induced memory deficits in mice. *Srinagarind Medical Journal, 28*(4), 219–222.

Verma, P., & Pathak, K. (2012). Nanosized ethanolic vesicles loaded with econazole nitrate for the treatment of deep fungal infections through topical gel formulation. *Nanomedicine, 8*(4), 489–496.

Verma, S., & Utreja, P. (2018). Transethosomes of econazole nitrate for transdermal delivery: Development, in-vitro characterization, and ex-vivo assessment. *Pharmaceutical Nanotechnology, 6*(3), 171-179.

Vichit, W., & Saewan, N. (2016). Effect of germination on antioxidant, anti-infammatory and keratinocyte proliferation of rice. *International Food Research Journal, 23*(5), 2019-2028.

Wattanuruk, D., Phasuk S., Nilsang, P., & Takolpuckdee, P. (2020). Total phenolics, flavonoids, anthocyanins and antioxidant activities of Khaow-Mak extracts from various colored rice. *Journal of Food Health and Bioenvironmental Science, 13*(1), 10-18.

Zakir, F., Vaidya, B., Goyal, A.K., Malik, B., & Vyas, S.P. (2010). Development and characterization of oleic acid vesicles for the topical delivery of fluconazole. *Drug Delivery, 17*(4), 238-248.