Original Article

# Essential Oils Enriched Self-nano-emulsifying Systems for Effective Oral Delivery of Zaleplon for Improvement of Insomnia Treatment

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## ABSTRACT

**Background:** Insomnia is usually associated with mental and physical daytime impairment. Zaleplon (Zp) is indicated in insomnia management but its limited aqueous solubility led to its low bioavailability (BAV) ~30%. Self-nanoemulsifying drug delivery system (SNEDDS) was a recommended nano-delivery system for improvement poorly soluble drugs' oral bioavailability. Consequently, this study aimed to prepare SNEDDS entrapped Zp using essential oils (EOs) have insomnia management effect. **Materials and Methods:** Different EOs, surfactants and co-surfactants in varying ratios were used in preparing Zp-loaded SNEDDS, which were chosen according to their ability to ease the emulsification and improve Zp solubility. To optimize the formulations; Zp-SNEEDS formulae were characterized for particle-size, zeta-potential, emulsification-time, and drug loading capacity. Additionally, *in-vitro* release and stability studies were performed to provide a perception on the stability and enhancing Zp release from Zp-SNEDDS formulae. To improve Zp-SNEDDS activity; BAV study and psychomotor evaluation test were carried out in albino mice. **Results:** The selected optimized formulae containing Tween80, PEG400 and anise oil had nanoparticle size (~98nm), loading capacity up to 40%, emulsification time~34sec and increased Zp dissolution rate up to 2 folds compared to pure Zp suspension. Zp-SNEEDS' BAV is 1.29 and increases in the sleep time up to 165min which equal to the synergetic effect of anise oil and zaleplon alone. **Conclusion:** The significant increase in the rate and extent of Zp oral absorption from nano-positively charge Zp-SNEEDS with high BAV and increases in sleeping time indicates the effectiveness of Zp-SNEEDS in improving Zp oral absorption and therapeutic effect in insomnia treatment.

**Keywords:** Nanoparticles, Zaleplon, Essential oils, Insomnia, SNEDDS.

## INTRODUCTION

Insomnia is sleep disorder subjective complaint in which patients have poor sleeping, inadequate amount of sleep, staying asleep throughout the night or inability to return to sleep after early morning awakenings.[1] It was usually associated with physical and mental daytime impairment, as fatigue, decreased concentration, inability to perform complicated tasks, and memory impairment.[2-3] In chronic cases, insomnia is associated with increased coronary artery disease risk, incident myocardial infarction, type 2 diabetes mellitus, obesity, patient depression and systemic hypertension which all the main mortality causes.[4-5]

Zaleplon (Zp) is a pyrazolopyrimidine hypnotic drug (new nonbenzodiazepine drug), indicated in insomnia management. It also acts as a potential anticonvulsant phenylenetetrazole against electroshock-induced convulsions by effects on GABA receptor.[6-7] Zaleplon is rapidly absorbed after oral administration with no active metabolites, and a short elimination half-life ~ 1 hr. In spite of its rapid absorption but its bioavailability is only 30% of its oral dose.

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Its low bioavailability and delayed onset of action are related to its extensive first-pass metabolism and limited aqueous solubility. The later indicated that its absorption is dissolution rate dependent as its low aqueous solubility associated with slow solubility rate and low bioavailability.[8-10] According to the bio-pharmaceutical categorization system, zaleplon is categorized as Class II medication due to its low solubility and high permeability (BCS).[1,10]

Self-nanoemulsifying drug delivery system (SNEDDS) is a nanotechnology that has been utilized to improve the solubility of poorly soluble hydrophobic medicines in order to improve their bioavailability and therapeutic effect.[11] Improvement of drugs' bioavailability via SNEDDS was not only by enhancing drugs' solubility but also by enhancing their permeability, by passing their liver metabolism, inhibiting P-glycoprotein efflux or even by encourages the lymphatic absorption capacity of the incorporated drugs.[12-14] SNEDDS is a transparent lipid-based drug delivery system formulated of oil, surfactant, and co-surfactant to create ultrafine oil/water nano-emulsion particles.[15] The used oils, surfactant and co-surfactant, and their ratios influence the construction and the stability of SNEDDS.[16]

Essentials oils (EOs) are generally reported to provide synergistic therapeutic effects of their active ingredients and can be loaded in colloidal carriers as SNEDDS.[17] They contain volatile molecules such as terpenes and terpenoids, as well as aliphatic and phenolic aromatic compounds.[18] Essential oils can cure insomnia through effecting on glutamate, gamma-aminobutyric acid, 5-hydroxytryptamine, dopamine, and norepinephrine, which are neurotransmitters pathogenesis related to insomnia.[19-20] From the essential oils used in insomnia treatment are lavender oil, lemon oil,[21] anise oil,[22] chamomile extract,[23] clary oil.[24]

In the light of the previously mentioned facts, the purpose of this study was to prepare SNEDDS entrapped with zaleplon and essential oils for improving zaleplon oral bioavailability and investigating the synergetic effect of essential oils with zaleplon on the central nervous system in order to insomnia treatment.

## MATERIALS AND METHODS

### Materials

Zaleplon was supplied from Amoun pharmaceutical company, Egypt (Zp). The other chemicals, Tween 80 (polyoxyethylene sorbitan monooleate), Cremophor EL, PEG 400 (Polyethylene glycol), PG (propylene glycol), and ethanol were obtained from Merck, Mumbai. Anise oil, lavender oil, lemon oil, chamomile extract and clary oil all were purchased from Sigma–Aldrich Company, St. Louis USA.

### HPLC Method for the Determination of Zp

Zaleplon determination was performed by a modified reported HPLC method using an Agilent 1100 HPLC system.[7,25] A Thermo Inertsil ODS 3V®, $C_{18}$ column (5 µ, 25cm × 4.6 mm, Hypersil) was used. The mobile phase consisting of acetonitrile/ 0.067 M phosphate buffer (pH 7.4); 45:55 v/v was used at a flow rate 1 mL/min. The system was set at 40°C and UV detection was performed at 304 nm. Intra- and inter-day validation was done for three days to determine the method linearity, precision, selectivity, and accuracy with respect to ICH guidelines. A calibration curves mean correlation coefficient ($R^2$) was over 0.9978. This method had an acceptable precision (C.V. %) and accuracy (relative error %) ranged from 0.021 to 10.984 and from -3.535-11.875 respectively.

### Preliminary Investigation

#### *Selection of the suitable media based on saturated solubility studies*

The saturated solubility of Zp in various media was measured using a modified shake flask method.[26] An excess Zp was sealed in vials each with (2 mL) of the different media, mixed in a vortex mixer (Snijders, Holland) to facilitate the solubility then stirred at 40°C for 24 hr in a water bath. The vials allowed for reaching equilibrium at 25°C for 72 hr. To remove excess undissolved Zp, the vials were centrifuged at 15,000 rpm for 10 min (Jouan centrifuge, France), then filtered the supernatant through a membrane filter (0.45 m). The resulting filtrates were diluted with ethanol, and the drug concentration was determined using a validated HPLC procedure previously reported.

#### *Emulsification study (Screening of Surfactant and Co-surfactant based on emulsification ability)*

Surfactants (Tween® 80 and Cremophor EL) and co-surfactants (PEG 400, PG, and ethanol) were screened for possibility of SNEDDS' formulation. Ease of emulsification and transparency percentage (% transparency) will be the basis of selection. In brief, 100 mg of each surfactant was added to 100 mg of the selected oils and 100 mg of each co-surfactant. Each mixture was gently heated separately at 40°C for components homogenization. In a stoppered conical flask, 100 mL of each combination was diluted with 100 mL of distilled water. The number of flask inversions recorded to produce a homogeneous phase was used to assess the ease of emulsification data (emulsion). After 2 hr, the prepared formulae's transparency % was

**Table 1: Selected Ratio of Variables for the Zp-SNEDDS Design.**

| Component | Ratio (% w/w) | |
|---|---|---|
| | Low | High |
| A: Anise oil % | 10 | 30 |
| B: Tween 80% | 20 | 60 |
| C: Polyethylene glycol 400% | 10 | 70 |

determined using a spectrophotometer (at 304 nm) against distilled water as a blank. For any turbidity or phase separation, SNEDDS formulae were also visually monitored.

## Development of Zaleplon-SNEDDS

Anise oil, Tween 80, and PEG 400 were chosen as the oil, surfactant, and co-surfactant phase, respectively, based on emulsification studies (saturated solubility and ease of emulsification tests). Different combinations at various oil, surfactant and co-surfactant concentration were mixed separately with a fixed drug concentration (10% w/w) using vortex mixer to prepare Zp-SNEDDS' formulations as represented in Table 1. The Design-Expert 13® (Software, Version 13.2.03, 2021, Stat-Ease, USA), was employed to determine the regression equations and to calculate the recorded responses.

The self-emulsifying capacity of the prepared pre-concentrates was performed by; 0.5 milligrams of each system were introduced into 50 ml phosphate-buffer PH 6.8 at 37°C and the contents were stirred using a magnetic stirrer at 100 rpm. They were investigated for self-nano-emulsification efficiency, clear appearance (transparent), and phase separation soon after dilution. Then, the prepared formulae were categorized as (clear), not completely clear (Translucent), and phase separation (not clear). The resultant nanoemulsion was evaluated by visual inspection after 24hr as stable (no precipitation occur even after 24 hr), or unstable (precipitation appearing within 24hr).[27] Due to the difficulty of measuring the degree of turbidity by visual evaluation, it was measured by turbidimeter HACH 2100P (HACH Co, Dusseldorf, Germany). To optimize the prepared Zp-SNEDDS; the rapid dispersability with maximum drug loading capacity and nanoparticle size with a high zeta potential system will be selected.

## *In vitro* Zp-SNEDDS' Formulation Characterization and Optimization

### *Visual inspection particles size and zeta potential determination*

The prepared SNEDDS were visually checked regularly for 24 hr at room temperature to notice any phase separation.[28] The droplet size distribution (polydispersity index, PDI) of the optimized SNEDDS formulation was recorded using a particle size analyzer (Horiba LA920, Japan). A 50 mg of the optimized SNEDDS formulations was added to 100 mL water in a flask, and hand stirred gently. The electrode of a portable digital conductemeter (Hanna, Hungaria) was dipped in the sample until equilibrium. The recorded readings were those which stable for 20 min. All studies were repeated in triplicates, with a significance level $p < 0.05$.

## Drug Loading Capacity

One gram of the selected prepared Zp-SNEDDS formulations was shaken with excess zaleplon for 24hr. The ultrafiltration method (3500 Da) was used to isolate the unincorporated drug. The incorporated drug was measured in the filtrate after certain dilution by the HPLC method discussed earlier.

## Assessment of Emulsification Time

The emulsification time of the optimized Zp-SNEDDS formulations was determined using a USP dissolution tester (Dissolution tester, Pharma test, PTZ, Germany). Amount of Zp-SNEDDS formulae equivalent to 1 mg of Zp was added drop-wise to 0.5 L distilled water and stirred by paddle (50 rpm) at 37 ± 0.5°C. The emulsification time was manually recorded.[29]

## Transmittance Percent Determination

The selected Optimized Zp-SNEDDS formulations were diluted (1 to 100 times using phosphate-buffer PH 6.8). Transmittance percent was determined at 304 nm using Synergy 2 UV Microplate Reader by BioTek Instruments.[30]

## Cloud-point Measurement

Cloud-point value was assessed to optimize different SNEDDS formulae. Each formula was diluted using phosphate buffer PH 6.8 (1:100) and employed in a water bath with gradual temperature increases. The cloud point, is the temperature at which the sample transmittance percent drop from zero points using a spectrophotomet measurement at 304 nm.[31]

## Stability Study

Stability study was done by subjected the optimized SEDDS formulae to freeze-thaw cycles, centrifugation and heating–cooling cycle thermodynamic stability studies. The freeze - thaw cycles were done on the selected formula and were stored between -4°C and 40°C temperature for at least 48hr. Three cycles were applied at each temperature. Samples were withdrawn at regular intervals and were considered for visual analysis

(*e.g.*, drug precipitation or phase separation). The same method was used for heating–cooling cycle but by incubation at 4 and 45°C for 48 hr. For centrifugation; samples were centrifuged at 5000 rpm for 10 min and re-observed for any phase separation. The chemical stability of the optimized Zp-SNEDDS formulae was done by measuring the Zp amount by HPLC at the end of the stability time (12 months at 25°±2 C and relative humidity 60%±5%). All measurements were repeated for three independent samples.[32]

### *In vitro* Release Studies

*In vitro* release studies of Zp-SNEDDS were conducted using the USP apparatus II (Dissolution tester, Pharma test, PTZ, Germany) at a rotation speed of 50 rpm. Selected SNEDDS formulae and Zp-suspension were filled in hard gelatin capsules (size # 3), all equivalent to 5mg Zp separately. The used dissolution media was 1000 mL of simulated intestinal fluid (SIF, phosphate buffer PH 6.8, enzyme-free) at 37 0.5°C. Samples were withdrawn at certain time intervals for 120 min, filtered with a 0.45 µm syringe filter (Microsart® Hannover, Germany), and analyzed for drug content by the previously mentioned HPLC method.[33]

### Morphological Examination

Transmission electron microscope (TEM) was used to determine the selected Zp-SNEEDS formulation morphological aspects, which was conducted using a (TEM) (JEOL JEM1230, Tokyo, Japan). Dried film of the sample on a carbon-coated copper grid was exanimated under a transmission electron microscope (Jeol JEM 1230, Tokyo, Japan). The system was operated at an accelerating voltage of 80 kV. The film was prepared by adding a drop from the selected formulation on a copper grid then negatively stained with 1% phosphotungstic acid (PTA). The excess was removed with filter paper and was allowed to dry in the air.

### *In vivo* Evaluation of the Selected Zp-SNEEDS

### Experimental Animals

This effectiveness of the selected Zp-SNEDDS in improving the Zp bioavailability and hypnotic effect were carried out in accordance with the recommendations of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978). Healthy albino mice weighing 20–25 g of either sex were provided by the veterinary service NODCAR (National organization of drug control and research, Egypt) used in the present study. Before the studies, the animals were kept and acclimatized to standard laboratory settings. The mice were admitted daily fresh food and water and kept on natural light and dark cycle. The mice were screening for righting reflex 1 day prior to doing any psychological tests. The animals that show positive righting reflex was selected for the psychomotor evaluation studies. Each animal was used only once in each experiment. All experiments were carried out in daylight.

### Pharmacokinetic parameters of oral administration of Zp-SNEDDS determination

The animals were randomly distributed into two groups (six mice) each and were administered separately with each treatment. Group I received zaleplon oral suspension (zaleplon suspended in phosphate buffer pH 6.8). The other group received the selected Zp-SNEDDS, all at an equivalent dose of 5 mg of zaleplon. At the selected time intervals, blood samples (500µL) were collected from retro-orbital plexus into heparinized micro-centrifuge tubes[34] at different time intervals (0.5, 1, 2, 3, 4, 6, and 8hr). Then samples were centrifuged for 10 min at 5000 rpm and the plasma was collected and kept at - 4°C until analysis. The drug concentration in the plasma was quantitatively determined by the modified HPLC method.[35] Sample (200 µL) was vortexed for 10 sec after adding 50 µL of acetonitrile then filtered by 0.45 mm filter paper and the supernatant was injected into the Agilent 1100 HPLC system; column: $C_{18}$ analytical column (250x4.6 mm) with 5µm particle size using UV and the absorbance was at 304 nm. The mobile phase was composed of acetonitrile with 5% acetic acid and run at flow rate 1.0 mL/min. Different pharmacokinetic parameters; the highest concentration ($C_{max}$) and the time it takes to reach it ($T_{max}$), the area under the curve ($AUC_{(0-t)}$), the $AUC_{t-\infty}$ were determined. The relative bioavailability was determined by dividing the $AUC_{0-\infty}$ of Zp-SNEEDS formulation to the $AUC_{0-\infty}$ of Zp oral suspension.

### Psychomotor Evaluation Tests

### Hypnotic effect and Sleep induction test

The sleep evaluation method was established on the base of prolongation of pentobarbital-induced sleeping time.[36] Briefly, the animals were divided into four groups of six mice each randomly. The animals were given a single dose of the anise oil (0.3 mg/kg), SNEDDS free drug equivalent to (0.3 mg/kg) anise oil, zaleplon (5 mg/kg), or the Zp-SNEDDS at the same equivalent dose. Diazepam was given i.p. dose (3 mg/kg) as a positive control. After 30 min, thiopental was injected (i.p) at a dose 50 mg/kg to induce sleep. After the mice remained stationary and lost their righting reflex when positioned on their back, the sleeping induction time

was recorted. The sleep latency was considered as the interval period between thiopental injection and the sleep starting.

### $LD_{50}$ Determination

An acute toxicity study was conducted by using a previously described Akhila *et al* method.[37] Six groups of six animals each were used. Six different concentrations of Zp-SNEDDS were tested in increasing order (2.5mg/Kg, 5mg/Kg, 7.5 mg/kg, 10 mg/kg, 12.5mg/kg and 20 mg/kg). Monitoring for mortality of the treated animals was for 24 hr. The highest dose that resulted in no animal deaths and the lowest dose that resulted in one mouse fatality were recorded. The median lethal dose ($LD_{50}$) was recorded as a mean of these two doses.

### Statistical Analysis

The results of all tests were expressed as the mean± SD of triplicates conductions at $p < 0.05$ level of significance. The significance of the impact of storage on the tested formulae and the fresh formulae was determined using one-way ANOVA (In All experiments) while two-way ANOVA was applied to assess the significance of the formulations' factors effect on SNEDDs formulae characters and the formulation effect on the pharmacokinetics' parameters and psychomotor effect at $p< 0.05$.

## RESULTS AND DISCUSSION

### Saturated solubility study (screening of oil)

Saturated solubility studies were done to identify a suitable dissolution media and choosing oily phase, surfactant and co-surfactant that suitable for Zp-SNEDDS development. The selection was based on improving zaleplon solubility due to its poor solubility results in its poor oral bioavailability. The later may be due to its lower dissolution rate in the gastrointestinal tract, and its lower intestinal permeability.

Identifying oil had maximum drug solubilizing potential achieves optimum drug's loading capacity and prevent drug precipitation.[38-39] The Zp solubility in different essential oils (Anise oil, Lemon oil, Chamomile oil, Lavender oil and Clary oil) was represented in Table 2. When compared to vegetable oils, essential oils were observed to be easier to emulsify, to generate SNEDDS, and to have a variety of medicinal potentials. They contain insoluble hydrophobic non-glycerol lipids (terpenes) moiety as menthol, menthone, cineol and others,[40] which affect mainly on enhancing the drug solubility. As noticed from Table 2, anise oil shows higher drug solubility. The ranking of increasing drug solubility of the used oil is proportion to increasing the non-glycerol lipids percent in the used oil.[41] The Natural anethole occurs at a high concentrations in anise oil about 80-90%.[22] The Zp solubility was about (30.026 mg/mL) in anise oil. The lowest drug solubility was noticed in lavender oil which has the lowest free alcohol group percentage 63%.[42]

**Table 2: The saturated solubility of Zp in the tested media.**

| The tested media | Solubility results (mg/ml) 0 |
|---|---|
| Water | 0.120 ± 0.01 |
| Phosphate buffer pH 7.4 | 0.570 ± 0.02 |
| Phosphate buffer pH 6.8 | 0.987 ± 1.01 |
| 0.1 N HCL pH 1.2 | 0.887± 1.12 |
| Anise oil | 30.065± 0.23 |
| Lavender oil | 0.367± 0.031 |
| Lemon oil | 25.21± 1.18 |
| Chamomile oil extract | 4.04± 2.07 |
| Clary oil | 19.68± 1.98 |
| Chremophor-EL | 0.0975 ± 0.04 |
| Tween 80 | 0.078± 0.056 |
| PG | 9.27±0.76 |
| PEG 400 | 9.56±0.55 |
| Ethanol | 5.73±0.35 |

As the drug basic nature, its solubility in 0.1N HCL was higher than in water. The drug exhibits the highest solubility was in phosphate-buffer PH 6.8 (simulated intestinal fluid enzyme–free). Therefore phosphate-buffer PH 6.8 was choosen as a dissolution media to study the release rate of Zp from the prepared Zp-SNEDDS formulae especially it was significantly higher than in phosphate buffer PH 7.4 ($p < 0.05$).

The Zp solubility was 73.8 µg/mL and 97.45 µg/mL for T80 and Cremophre El respectively. For oral ingestion, nonionic surfactants are accepted as they are considered lesser toxicity than ionic surfactants.[43] Choosing T80 and Cremophor EL in preparing SNEDDS was because it was reported that self- emulsifying preparation usually required using a hydrophilic surfactant with HLB > 12 (HLB value of T80 and Cremophor EL was 15 and 12-14 respectively). The high solubilization capacity of the used surfactant and oil led to incorporation a high drug amount furthermore; they could reduce the viscosity of the final SNEDDS

Using co-solvents as alcohols, polyethylene glycols, and propylene glycol has an efficient effect in reducing the oil-water interface interfacial tension, reducing the total surfactant amount needed for maintaining self-

emulsification and improving the solvent capacity of SNEDDS.[44-46] Co-surfactants' solubility was (9.27 mg/mL, 9.56 mg/mL and 5.73 mg/mL) for PG, PEG400 and Ethanol respectively. As there was no significant difference in the PG and PEG 400 solubility results, therefore surfactant and co-surfactant selection will be governed by their emulsification efficiency.

## Preliminary Screening of Surfactants and Co-surfactants

The anise oil exhibits the highest emulsification efficiency with Tween®80 by transparency percent: 93.65%, and 5 times flask inversions; (5s) to form a homogenous emulsion compared to Cremophor EL, which shows poor emulsification properties, thus it demands a greater number of flask inversions, Table 3. Transmittance percent values greater than or equal to 90% reflects the small droplet sizes could be prepared.[47] The fewest inversions suggested well-formulated SNEDDS, which dispersed in seconds under mild stirring conditions. The later mainly depends on the used surfactant and its emulsification ability.[48] The aforementioned results suggested the suitability of using anise oil with Tween®80 for SNEDDS formulation.

PEG 400 and PG were compared to choose the suitable co-surfactants. As shown in Table 3, anise oil exhibits a good emulsification capacity with all co-surfactants. PEG 400 had a maximum transmittance (92.965%) followed by PG (92.567%). The statistical analysis shows no significant difference with either co-surfactant used. The lower inversion times was observed from PEG 400, because it has a higher hydrophilic-lipophilic balance value (HLB) than PG. A higher HLB value indicates more hydrophilicity and a shorter emulsification time.[47] The combination effect of using T80 and PEG400 gave the highest transparency percent and the lowest number of inversions (99.87%, 4S). Therefore, PEG 400 will be chosen as a co-surfactant in SNEDDS preparation, along with T80 as a surfactant.

**Table 3: Emulsification efficiency of different surfactants and co-surfactants.**

| Surfactants and co-surfactants | %Transparency | No, of inversions(sec) |
|---|---|---|
| Cremophor-El | 84.45 | 12 |
| Cremophor-El / Ethanol | 87.98 | 6 |
| Cremophor-El / PEG 400 | 89.11 | 9 |
| Cremophor-El / PG | 88.95 | 12 |
| Tween 80 | 93.65 | 5 |
| PG | 92.765 | 9 |
| PEG 400 | 92.967 | 6 |
| Ethanol | 77.3 | 11 |
| T80 / Ethanol | 94.87 | 8 |
| T80 / PG | 95.5 | 5 |
| T80 / PEG 400 | 99.87 | 4 |

## Optimization of Zp-SNEDDs

A series of SNEDDSs were prepared consisting of anise oil as oil phase, Tween 80 as a surfactant, and PEG 400 as co-surfactant. The system shows a clear solution after titration with water will be selected. T80 was used in the ratio 20-60% w/w as it was reported that using T80 at a concentration more than 20% helps in preparing oral SNEDDS.[38] SNEDDS could improve the drug solubility and distribution by converting the drug from crystalline form into amorphous solid dispersions form.[49]

On the other hand, using PEG 400 (10-70% w/w) was because it was previously noted that incorporation of a high amount of PEG 400 as a co-surfactant, increased the spontaneity of the self-emulsification process. Additionally, using surfactant/co-surfactant concentration more than 75% w/w is efficient in ease emulsification of the SNEDDS formulation.[50]

Table 4 demonstrated the total output of 18 formulae and their particle size and Z-value changes according to the Zp-SNEDDS' components change. Tables 5A and 5B summarize the statistical analysis of variance for the ongoing preferred special cubic model of measuring responses (particle size and zeta potential value).

As noticed in equation 1 of the fitted model about the particle size with $R^2 = 0.9923$ and from Figure 1 indicated that; oil, surfactant and co-surfactant have a significant effect on the particle size. Increasing anise oil%, T80% or PEG 400% combinations significantly decreased the formulations' particle size. Increasing oil, T80 or PEG 400 concentration, increases the particle size of the prepared Zp-SNEDDS (Table 4). Generally, the formulae which have nanoemulsions' droplets sizes <100 nm tend to be transparent and clear while droplets with particles sizes > 150 nm and less than 200 nm tend to be translucent.[46] Therefore, formula contains 30% anise oil, 60% T80 and 10% PEG 400 will be selected as it has a smaller particle size < 100 nm. Nanoemulsions with smaller particle sizes are more stable, better absorbed and higher bioavailability compared to those having larger particles size.[51-52]

Particle size (PS)= 243.31149× anise oil+243.31149× T80+245.31672× PEG 400-1228.46533× anise oil× T80-429.10570× anise oil× PEG 400-522.59630× T80× PEG 400+1463.45146× anise oil× T80× PEG 400. Equation 1

**Table 4: The compositions and the observed responses of the tested 18 formulae in different evaluation tests.**

| Run | Tested Components | | | Response | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | A:Anise oil | B:T80 | C:PEG 400 | PS nm | Z value nV | PDI | Visual appearance | Visual appearance After 24 hr | DL mg/g |
| 1 | 10 | 20 | 70 | 160 | 13.9 | 0.22 | Translucent | Un stable | - |
| 2 | 20 | 20 | 60 | 190 | 125.2 | 0.17 | Translucent | Un stable | - |
| 3 | 30 | 60 | 10 | 110 | 133.89 | 0.2 | clear | Stable | 33.61 |
| 4 | 30 | 30 | 40 | 215 | 56.7 | 0.23 | Not clear | Un stable | - |
| 5 | 20 | 40 | 40 | 93 | 33.09 | 0.27 | clear | Stable | 15.06 |
| 6 | 30 | 20 | 50 | 160 | 38.09 | 0.2 | Translucent | Un stable | - |
| 7 | 10 | 50 | 40 | 98 | 115.3 | 0.22 | clear | Stable | 40.41 |
| 8 | 10 | 60 | 30 | 113 | 25.6 | 0.12 | clear | Stable | - |
| 9 | 20 | 60 | 20 | 90 | 14.2 | 0.16 | clear | Stable | - |
| 10 | 20 | 30 | 50 | 130 | 65.9 | 0.12 | clear | Un stable | - |
| 11 | 20 | 50 | 30 | 124 | 19.84 | o.16 | clear | Stable | - |
| 12 | 10 | 30 | 60 | 98 | 113.9 | 0.12 | clear | Stable | 40.21 |
| 13 | 30 | 20 | 50 | 203 | 117.5 | 0.10 | Not clear | Un stable | - |
| 14 | 30 | 40 | 30 | 129 | 21.78 | 0.2 | Translucent | Stable | - |
| 15 | 20 | 40 | 40 | 152 | 36.89 | 0.19 | Not clear | Un stable | - |
| 16 | 10 | 40 | 50 | 129 | 42.6 | 0.22 | Translucent | Stable | - |
| 17 | 10 | 30 | 60 | 120 | 44.76 | 0.25 | Translucent | Un Stable | - |
| 18 | 30 | 50 | 20 | 75 | 16.16 | 0.15 | clear | Stable | 25.21 |

**Table 5: ANOVA for Special Cubic model.**
**Table 5A: Particle size response.**

| Source | Sum of Squares | $d_f$ | Mean Square | *F*-value | *p*-value | |
|---|---|---|---|---|---|---|
| Model | 17562.98 | 6 | 2927.16 | 3.35 | 0.0396 | significant |
| Residual | 9610.64 | 11 | 873.69 | | | |
| Cor Total | 27173.61 | 17 | | | | |

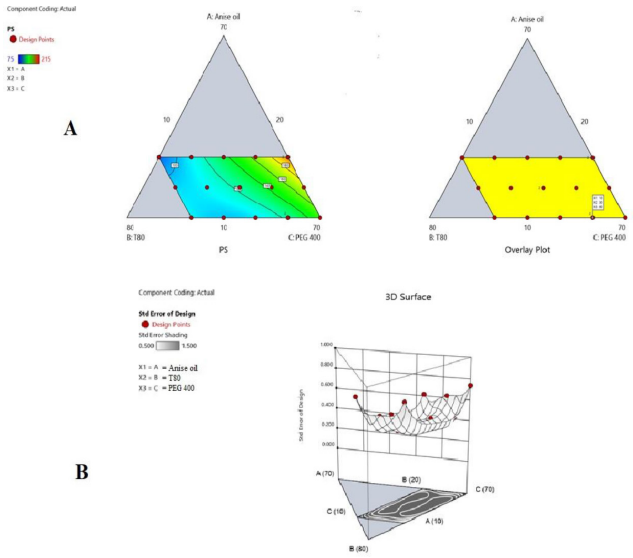


**Figure 1: A. Estimated response surface as a contour plot. B. Estimated 3D response surface effect for particle size.**

Efficient nano-emulsions should be safe, stable homogenous and monodispersed. Polydispersity index is a parameter that indicates formulations' homogeneity at a certain size.[53] All formulae show a narrow PDI ranging from 0.1 to 0.26. A PDI of 0.3 or less shows that the particle distribution in the system is homogeneous.[54] Nano-dispersions are regarded strongly cationic and anionic when the zeta potential value ranges from -30 mV to 30 mV and are considered a mono-dispersed system. The high Z values indicate a high repulsive electrostatic forces, which reduces the particle aggregation probability and improves globules stability.[55] Therefore, formulae number 1, 12 and 13 will be selected as the have the highest; Z-value =133.9, 113.7 and 117 respectively. The increasing in the Z-positive value enhances the cellular uptake of the nanoparticles drug molecule. The encapsulated drug

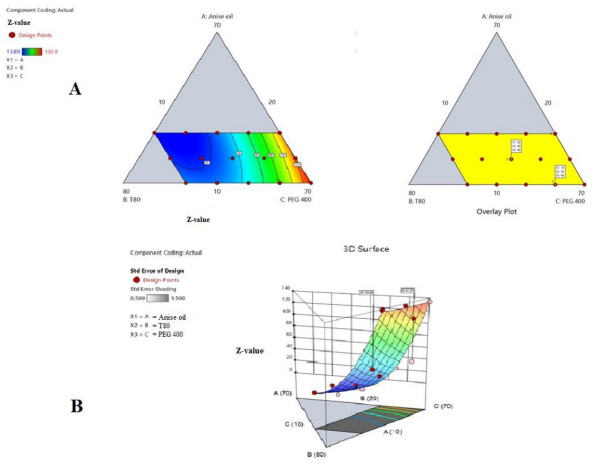


**Figure 2: A. Estimated response surface as a contour plot. B. Estimated 3D response surface effect for *Z*-value.**

molecule in positive charge nanoparticles improves their interact with the negatively charged intestinal mucosa.[56] Statistical examination of the fitted model's Z-value, as given in equation (2), with R2 = 0.9172 and from Figure 2, suggested that oil, surfactant, and co-surfactant had a significant effect on the Z-value. Increasing the oil concentration, surfactant and even the co-surfactant led to a significant ($p$<0.05) decreasing in the Z-value. As the anise oil% and PEG400 increases, the Z-value will be increased which is desirable in SNEDDS formation in compared to increasing the oil% with T80% which led to decreasing the Z-value. Increasing the *Z*-value increases the system stability. Therefore, formula 12 will be chosen, which comprises Anise oil %, T80%, and PEG 400% at 10, 30, and 60%, respectively.

Z-value = 173.18429× anise oil+ 177.89097×T80+ 267.66945× PEG 400 -638.42839× anise oil× T80 +235.23732× anise oil× PEG 400 -694.36374× T80× PEG 400 -1112.56759× anise oil× T80× PEG 400.

Equation 2

The change in the formula 10 appearance from clear to unstable and vice versa for formulae 14 and 16 was related to the change of T80 concentration. Increasing the surfactant amount can reduce the emulsification free energy by reducing oil and water interface surface tension which led to decreasing the prepared SNEDDS droplets size.[57] However, in certain instances, increased particle size was caused by an increase in surfactant concentration. The later facilitate the water molecules entrapment into the lipid droplet and led to massive distraction of the oil/water interfacial and relaxation of highly polydisperse nano-emulsion droplets.[58]

Based on the previous results; formulae 3, 5, 7, 12 and 18 (Table 5) were chosen for further studies. To determin the maximum formulations loading capacity of Zp, increasing amount of the drug was added to each formula. The drug loading capacities of the selected Zp-SNEDDS formulae were represented in Table 5 (ranged from 15.06 ± 1.03 mg/g to 40.41 ± 0.55 mg/g). One-way ANOVA showed that formulae 7 and 12 had significantly higher loading capacity than formulae 3, 5 and 18, with F-value 18.5 ($p$<0.05) while formulae 7 and 12 show no significant difference. In contrast, there was a significant difference between formulae 3, 5, and 18 concerning the drug loading capacity with F-value 88.3 ($p$<0.05). The higher Zp-SNEDDS formulae loading capacity was related to the relative drug solubility in various SNEDDS' components.[27]

Herein, formulae 7 and 12 were chosen as they had the highest solubilization capacity towards Zp among all other formulae. In addition, it was found that the maximum amount of Zp could be loaded in SNEDDS was 40.41 mg/g (w/w). A fixed concentration of 10 mg/mL of Zp will be used in the following studies.

## *In vitro* Characterization of the selected Zp-SNEDDS Formulae

### *Assessment of Emulsification Time*

A well designed SNEDDS should completely and quickly disperse when diluted using a mild agitation within 120 sec as the shorter emulsification time the more efficient emulsion formed.[59] Formulae 7 and 12 have emulsification times 30 sec and 34 sec respectively (formulae 7 and 12 content represented in Table 5). The high emulsification time of formula 12 than formula 7 was agreement with previously reported that, a high concentration of high HLB surfactant (T80; 59%) as in formula 7 than formula 12 facilitate the emulsification efficiency through facilitating the oil dispersion in the aqueous phase rapidly and ease o/w nanoemulsion formation.[60]

## Percentage Transmittance

There is no significant difference in the percentage transmittance value ($p$>0.05) for formulae 7 and 12 (99.7835%±1.058% and 99.35%±0.586% respectively). The highest transmittance percentage value attributed to their small size as reported by Sarkar and Hardenia, 2011,[61] who found that the transparency of clear light transmitted SNEDDS should be close to 100% and more than 75%.

## Cloud-point Measurement

Phase behavior is a common problem associated with self-nanoemulsions formulation, which considers

| Table 5B: Zeta potential response. | | | | | | |
|---|---|---|---|---|---|---|
| **Source** | **Sum of Squares** | **$d_f$** | **Mean Square** | ***F*-value** | ***p*-value** | |
| Model | 28975.92 | 6 | 4829.32 | 20.30 | < 0.0001 | significant |
| Residual | 2616.53 | 11 | 237.87 | | | |
| Cor Total | 31592.45 | 17 | | | | |

temperature dependent, especially when non-ionic surfactants, was used. The cloud point of the optimized formula preferred to be more than 37°C (body temperature).[62] The high cloud point temperature maintains the SNEDDS stability, prevents the system dehydration, drug precipitation and prevents phase separation. Factors such as drug hydrophobicity, the type and different ratios of the used oils, surfactant and co-surfactant are affected the cloud point value.[30] Formulae 7 and 12 exhibited cloudiness at 90°C and 93°C respectively, with a drop in percentage transmittance from 99.783%±1.058% to 78.89% ± 0.345% for formula 7 and from 96.15% ± 0.071 to 65.28% ± 2.24 for formula 12 which indicated the stability of Zp-SNEDDS formulae against phase separation at GIT temperature. The difference in cloudiness value for both formulae regarded to the higher T80 ratio in formula 12 than formula 7 as the polyethylene oxide moiety of the nonionic surfactant usually suspected to dehydration at high temperature.[63-64]

## Stability Study

SNEDDS was considering a thermodynamically stable nanoemulsion system. The tested formulations (7 and 12) show no sign of precipitation, phase separation, creaming or cracking even upon dilution. In addition, there was no statistically significant difference in the quantity of Zp in the Zp-SNEDDS fresh and stored formulae. This result was in agreement with previously reported that when Tween 80 was used as a surfactant in SNEDDS, it improves the system stability and maintains its clarity.[46]

## *In vitro* Dissolution Studies

The *in vitro* release rate study of the selected formulae: formulae 7 and 12 were compared with respect to the plain Zp-suspension. The SNENDDs have a significantly higher dissolution rate than the plain drug suspension in phosphate buffer pH 6.8 as presented in Figure 3 ($p$ values < 0.01). After 10 min, more than 50% Zp was released from the Zp-SNEDDS formulae but ~16% of Zp-suspension released. The Zp-SNEDDS formulation enhances the cumulative drug released percent approximately 1.5 to 2-fold (~80–90%) at the end of the testing period compared to those of

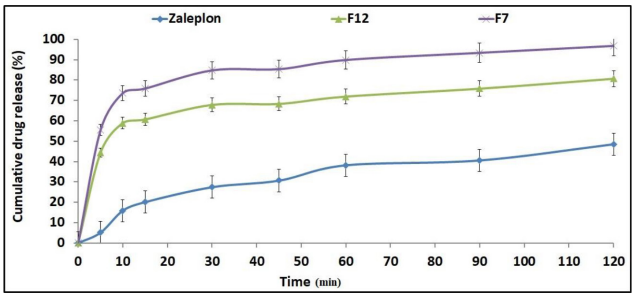


**Figure 3: The mean cumulative zaleplon % release from Zp-SNEDDS formulae compared with Zp suspension, data presented as mean ± SD, *n* = 3.**

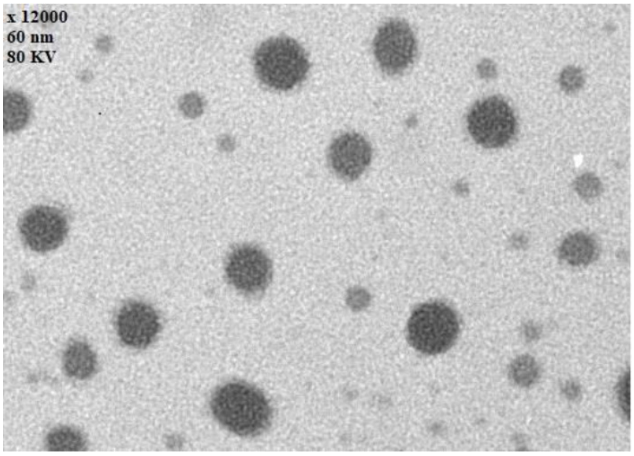


**Figure 4: TEM of the selected Zp-SNEDDS (F7).**

cumulative free Zaleplon released percent (~48%). The higher release rate was related to the nanoparticle size of Zp-SNEDDS formulae, which provided a large surface area for the drug release and thus permitted a faster drug dissolution rate. There was a statistically significant difference between different Zp-SNEDDS formulae ($p$-value < 0.05). The maximum release observed at the end of the testing period was 96.8% from formula 7 which will be selected to *in vivo* studies.

Transmission electron micrograph of Zp-SNEDDS (F7) as represented in Figure 4 showed scattered non-aggregated spherical particles with particle size range of 60-70 nm. The non-aggregated particles could result from electrostatic repulsion done by the surface negative charge. The spherical nano-size Zp-SNEDDS might increase medication permeability and retention effects.[27]

## Determination of Pharmacokinetic parameters after oral administration of Zp-SNEDDS

The mean plasma concentration versus time profiles following orally administration of the selected Zp-SNEDDS formula and zaleplon suspension was presented in Figure 5 while the PK data was illustrated in Table 6. As shown the Zp-SNEEDS has a higher rate and extent of absorption than free zaleplon. The Zp-SNEDDS' $C_{max}$ (62.37 ± 0.32) was significantly higher than the free Zp-suspension (40.31 ± 0.47) at ($p < 0.05$). It was found that $T_{max}$ (0.516 ± 0.058) at the peak concentration ($C_{max}$) following Zp-SNEDDS administration was significantly extremely lower than the $T_{max}$ value following Zp-suspension administration (1.027 ± 0.028) at ($p < 0.003$). The half-life ($t_{1/2}$) for both tested groups remained not-significantly different (31.09 ± 0.22 hr). The extent of absorption areas was recorded by recording the area under the concentration-time curve ($AUC_{0-\infty}$). $AUC_{0-\infty}$ was 250.82 ± 1.14 ng.h/mL and 193.8 ± 1.54 ng.h/mL following Zp-SNEDDS and Zp suspension administration respectively ($p < 0.002$). From the area under the concentration-time curve ($AUC_{0-\infty}$) values, the relative BAV of Zp-SNEDDS was calculated and was found that it was equal to 1.29. The BAV value higher than 1 means increases in the rate and extent of drug absorption from Zp-SNEDDs than free Zp-suspension. It was reported that the higher BAV indicates the direct transfer of the formulation at the GI tract, which may be due to avoidance of the first-pass metabolism.[7,65] Additionally, the higher Zp absorption rate and extent after oral Zp-SNEDDS could be relayed to the electrostatic attraction between the positive charge containing vesicles and the intestinal cell surface.

## Psychomotor Evaluation tests

The hypnotic effect of Zaleplon was evaluated using sleep induction method as described before.[66] The hypnotic effect of anise oil, Zp and Zp-SNEDDS

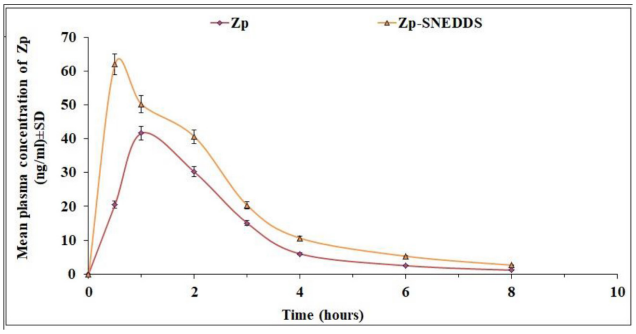


**Figure 5: Pharmacokinetic profiles of zaleplon in serum following oral administration of Zp-SNEDDS compared with Zp suspension (mean ± SD; *n*=6).**

**Table 6: Pharmacokinetic parameters of oral zaleplon in serum after administration of Zp-SNEDDS compared with Zp suspension (mean±SD; *n*=6).**

| Pharmacokinetic parameters | Tested Formulae | |
|---|---|---|
| | Zaleplon | Zp-SNEDDS |
| $C_{max}$ (ng/mL) | 40.31±0.47 | 62.11 ± 0.32 |
| $T_{max}$ (h) | 1.027±0.028 | 0.506 ± 0.01 |
| $T_{1/2}$ (h) | 31.19 ± 0.22 | 31.19 ± 0.36 |
| K ($h^{-1}$) | 0.02216 ± 0.16 | 0.02216 ± 0.11 |
| $AUC_{0-t}$ (ng h $mL^{-1}$) | 101.46± 1.25 | 150.72± 1.85 |
| $AUC_{0-\infty}$ (ng h $mL^{-1}$) | 193.84± 1.54 | 250.82 ± 1.14 |
| R-BAV | ------- | 1.29 ± 0.261 |

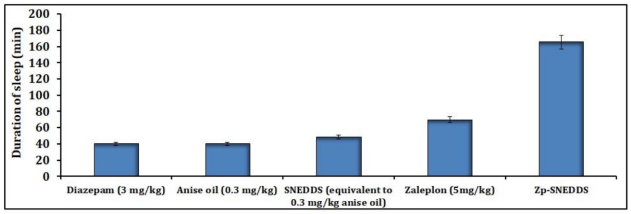


**Figure 6: The penthobarbital – induced sleeping time in mice effect of Zp-SNEEDS compared with Zp-suspension and anise oil, data represent mean ± SD, *n*=6 for each group.**

was tested by measuring the latency of Sleep duration (Figure 6). It was found that the sleeping time after anise oil administration was not significantly different from the positive control (40.25 min±1.21). The anise oil result was indicating that EO did not cause central nervous system distribution but it effects on sleeping time. The later could be explained by trans-anethole; the main component of anise oil which effects on induction of microsomal enzymes and increases the metabolism of pentobarbital in the liver and its analgesic effect.[67-68] The hypnotic effect of the prepared SNEDDS free drug exceeded that of anise oil alone (48.45 ±. 1.76). This is due to the nano particle size of SNEDDS, which facilitates anise oil absorption and dispersion, improving its hypnotic effect.

The animals who received Zp show a significant increase in the sleep time than the positive control up to 69.76±1.76 min ($p<0.05$). Drake *et al* found that zaleplon produced a persistent sleep that was comparable with that of the other benzodiazepine derivative.[69]

The last group receiving Zp-SNEDDS showed significantly the highest increases in pentobarbital induced sleeping time compared to other tested groups (165±2.76 min at $p<0.05$). The Zp-SNEEDS result is approximately showed a synergetic effect of anise oil and zaleplon effect. The preparation of zaleplon as a SNEDDS enhanced its solubility by a notable increase in the surface area by the formation of nano-

sized emulsion droplets. Besides the surfactant and co-surfactant in the formulation enhances the GIT tissue permeability that facilitates drug absorption and reduced the gastric emptying rate.[70] The $LD_{50}$-value for Zp-SNEEDS was found to be 14.8 g/Kg. This value is so far from the effective dose of zaleplon, 5 mg/Kg.

## CONCLUSION

From this study, the advantages of nano-positively surface charge SNEEDS particle containing essential oils has been investigated to improve zaleplon oral delivery BAV and insomnia curing effect. Anise oil could increase zaleplon solubility therefore, it was preferred to be used as oil phase in SNEEDS preparation. Nonionic surfactant as tween 80 was preferred as it eases the emulsification of the SNEDDS formula. Using co-solvent as PEG 400 has an efficient effect in tumbling the interfacial tension at the oil-water interface. Using anise oil as an oily phase (10-30%) with Tween® 80 (20-60%) as a surfactant and PEG 400 (10-70%) produces a well-formulated Zp-SNEDDS can disperse within seconds under gentle stirring. To be more precise; formula contains 30% anise oil, 60% T80 and 10% PEG 400 will be selected as it has the smaller droplets size < 100 nm, smaller emulsification time within 30sec, higher percentage transmittance (99.35%±0.586) and higher cloud point temperature (93°C) to maintain the SNEDDS stability. Additionally, higher positive Z-value =133.9 enhances the cellular uptake of the encapsulated molecule, maintains the nano particle stability through preventing them aggregation. The nanoparticle size of Zp-SNEDDS provides a large surface that maximizes the release at the last time point up to 96.8% and significantly enhances cumulative amount released up ~1.5 to 2-fold than pure drug. Zp-SNEDDS significantly improved pharmacokinetic parameters. The bioavailability is 1.29 times higher than pure Zp. The higher the BAV value than one means increasing the rate and extent of drug absorption from Zp-SNEDDs than free Zp-suspension and indicates the direct transfer of the formulation at the GI tract may be due to avoidance of first-pass metabolism. Zp-SNEDDS also shows a significant increase in pentobarbital induced sleeping time which approximately equal to the synergetic effect of anise oil and zaleplon effect separately (165±2.76 min at $p<0.05$). It increases the lethal dose ($LD_{50}$) up to 14.8 g/Kg. This value is so far from the effective dose of zaleplon, 5 mg/Kg. Therefore, Zp-SNEDDS containing anise oil as oily phase nanoparticles could be considered a good choice in insomnia treatment. However, further clinical studies on humans should be done in order to ensure the feasibility of these formulations for improvement zaleplon oral delivery.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## ABBREVIATIONS

**SNEDDS:** Self-nanoemulsifying drug delivery system; **BAV:** Bioavailability; **Zp:** Zaleplon; **EOs:** Essential oils; **PG:** Propylene glycol; **PDI:** Polydispersity index; **SIF:** Simulated intestinal fluid; **TEM:** Transmission electron microscope.

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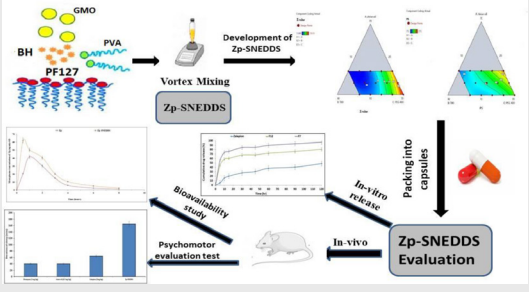
## PICTORIAL ABSTRACT

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