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Original Article

Statistically Optimized Nano Emulsion Enhances Bone Regeneration Efficiency of Ipriflavone in Osteoporosis-Induced Zebrafish Model

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ABSTRACT

Background: The objective of this research was to develop a nano emulsion of ipriflavone (IP nano emulsion), a soy isoflavone to enhance its solubility, bioavailability and bone regeneration potential. **Materials and Methods:** The oil phase and surfactants for the IP nano emulsion were selected by plotting ternary phase diagrams. Optimisation of surfactant and oil concentrations was achieved using the Box-Behnken Design in Design Expert software. A monodisperse nano emulsion was produced using sonication and the globule size was below 100 nm. The zeta potential readings for the below -15 mV. The optimised formulation was tested for its morphology and examined using TEM imaging. **Results:** *In vitro* drug release studies exhibited a release of 80% of ipriflavone in 90 min. The selected formulation demonstrated stability over a three-month period in accelerated conditions of temperature and humidity. The bone regeneration capacity of IP nano emulsion was tested on a dexamethasone-induced osteoporotic zebrafish model. The Caudal vertebrae bone of treated zebrafish showed significant bone regeneration compared to untreated and was confirmed by length measurements. **Conclusion:** The nano emulsion displayed effective bone regeneration capability in zebrafish models, indicating the usefulness of nano emulsion in the treatment of osteoporosis.

Keywords: Ipriflavone, Nano emulsion, Osteoporosis, Zebrafish model, Bone regeneration.

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INTRODUCTION

According to the World Health Organization (WHO), osteoporosis is a "progressive systemic skeletal disease characterised by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture". Osteoporosis is a chronic disease mainly due to the imbalance between bone resorption and bone formation.[1] Changes during menopause cause significant bone loss and increase the risk of osteoporotic fractures. Currently, anti-resorptive agents, including oestrogen, bisphosphonates, Selective Oestrogen Receptor Modulators (SERMs), acidic oligopeptide-conjugated E2 and Tissue-Selective Oestrogen Receptor Complexes (TSECs) are used for the treatment of osteoporosis.[2,3]

The treatment costs were reduced with the classic anti-resorptive drugs such as bisphosphonates and denosumab because of the risk of osteonecrosis of the jaw and atypical femur fractures.[4] SERMs have been reported with side effects like breast cancer, menopausal hot flashes, Alzheimer's disease and prostate cancer in the long-term treatment.[5] Plant-derived SERMs are known as phytoestrogens and are structurally like endogenous oestrogen that can bind to the ER and display a mixed oestrogen agonist/antagonist activity like synthetic oestrogen with lesser side effects.[6] Ipriflavone (IP) is a synthetic phytoestrogen[7] and is reported to increase bone formation by inhibiting parathyroid hormone-stimulated bone resorption and osteoclast maturation with fewer side effects.[8] While there is little evidence for its activity in osteoporosis, its poor solubility and high first-pass results in lower oral bioavailability, making ipriflavone inefficient in the treatment of choice in osteoporosis.[9]

The effectiveness of ipriflavone on bone tissue regeneration is well demonstrated in our in silico studies and *in vitro* cell line models.[10] Whereas a study carried out with injectable nanoparticles of ipriflavone couldn't demonstrate a significant

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effect on osteoporosis in animal models, which might have caused by its poor bioavailability.[10] Researchers formulated ipriflavone in various forms, such as conventional oral emulsion, spray-dried IP with food additives and micellization using surfactants.[11-14] These formulations attempted to overcome the drawback of poor solubility of Ipriflavone. Solubility improvement is the best and easy technique to administer the drug orally. The study demonstrated that the oral absorption and bioavailability of IP is enhanced when the drug is taken along with food and fatty substances. Additionally, the nano emulsion method has been shown to improve the solubility of lipophilic drug materials.[15] With this information, we are attempting to formulate an ipriflavone nano emulsion to enhance the solubility and bioavailability.

MATERIALS AND METHODS

Materials

Ipriflavone RS was purchased from Sigma Aldrich, Oleic acid was procured from RFCL Limited, New Delhi and Miglyol 812 was procured from Yarrow Pharma, Mumbai. Corn oil, tween (20 and 80), span (20 and 80), Polyethylene glycol (PEG 400), Propylene glycol and Ethanol were procured from LobaChemiee, Mumbai. Soya lecithin was procured from Hi-Media Laboratories, Mumbai. All chemicals used were of analytical or pharmaceutical grade.

Methods

Selection of the excipients

Selection of oil phase

An excess amount of the IP was dissolved in various oils, such as oleic acid and Miglyol 812 and the mixtures were shaken vigorously in a Magnetic stirrer. The resulting mixtures were sonicated for 15 min at an amplitude of 30 and subjected to centrifugation at 3000 rpm for 15 min. The supernatant was collected and filtered through 0.45 µm syringe filter and evaluated for IP content using a UV spectrophotometer (Shimadzu, UV-19001, Japan) at 249 nm.[16]

Screening of surfactants

Migyol 812 was added into the 1 mL of 10% v/v surfactant (tween 80, tween 20, span 80) solutions in small increments until turbidity appears. The surfactant, which allowed the incorporation of the highest amount of oil was finalised for formulation.

Screening of Co-surfactant

Co-surfactants were selected by plotting pseudo-ternary phase diagrams. Ethanol, PEG 400 and propylene glycol were among the co-surfactants chosen and were added at ratios of 1:1, 1:2, 1:3 to Tween 80 and observed for the formation of a transparent nano emulsion. The zone of emulsion formation in pseudo-Ternary Phase Diagrams (TPD) was labelled. It was determined that the oil and S_{mix} (tween 80 and co-surfactant) should be combined in fifteen different ways (i.e.; 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1, 1:2, 1:3, 1:5, 1:6, 1:7 and 1:8) to incorporate the highest possible ratios that could accurately define the phase diagrams' boundaries of phases.[17]

Optimisation of IP nano emulsion formulation by Box-Behnken Experimental Design

The Box-Behnken experimental design was adopted to optimise the IP Nano emulsion formulations statistically. The independent variables for optimisation were the amount of oil (X1), the volume of the S_{mix} (X2) and the amount of the Soya lecithin (X3), while the globule size (Y1) and Polydispersity Index (PDI) (Y2) were dependent factors. X1 was tested at three levels of 10, 12.5 and 15 %v/v. X2 varied at concentrations of 12, 31, 50%v/v whereas X3 was tested at 0, 0.5 and 1%w/w. A design matrix with 15 experimental runs was constructed using the software Design Expert 11 as shown in Table 1.[18] The statistical significance of the constructed model was tested by ANOVA and the P values for Globule size and PDI optimization were 0.0023 and 0.0054 respectively indicating the model is significant.

Preparation of IP nano emulsion

Ipriflavone nano emulsion was prepared by magnetic stirring followed by ultra-probe sonication. The drug was dissolved in oil with continuous magnetic stirring at 1500 rpm with a slow addition of ethanol to produce the oil phase. The aqueous phase was prepared by dissolving tween 80 in distilled water with the addition of soya lecithin with the help of heating. Add oil phase into aqueous phase dropwise under continuous magnetic stirring at 1500 rpm. After complete addition, the mixture was subjected to ultra-probe sonication for 15 min at an amplitude of 30. The formulation composition is given in Table 2.

Evaluation of IP nano emulsion formulations

Thermodynamic stability of nano emulsion

Heating/Cooling cycles: All the formulations chosen from the former test were gone into six cycles of 4°C and 40°C for two days each.

Centrifugation test: All the IP nano emulsion formulations were subjected to this test. This test was performed by centrifugation at 3500 rpm for half an hour. The formulation that was still homogenous and pure without any turbidity was subjected to the next test.

Freezing/Thawing cycles: Three cycles of freeze temperature of -21°C and room temperature were passed through the formulations for two days each cycle.

The Results for IP nano emulsion thermodynamic stability are shown in Table 3.

Physical evaluation of statistically optimised nano emulsions

Determination of globule size and Polydispersity Index (PDI)

The globule size and polydispersity index of prepared nano emulsions were analysed by dynamic light scattering (Zetasizer Nano ZS90, UK). These tests were performed to ensure the homogeneity, size uniformity and stability of the prepared formulations (Table 4). The scattered light at a 90º angle is directed to the specific volume of the nano emulsion in a cuvette.[19]

Determination of Zeta potential: This test was performed using Malvern Zetasizer Nano ZS90 after suitable dilution with the supernatant. The zeta potential was recorded in mV.

pH determination: A calibrated pH meter was used to measure the pH of all prepared formulations by immersing the electrode bulb into 10 mL of each formulation.

Table 2: Formulation composition of Ipriflavone nano emulsion.

	Independent Variables			Observed data	
Run	A: Oil (mL)	B: S_{mix} (mL)	C: Soya lecithin Solution (mL)	Globule size (nm)	Polydispersity Index (PDI)
1	1.25	3.1	1	29.816±1.00	0.36±0.02
2	1.25	1.2	2	170.33±9.02	0.15±0.12
3	1	3.1	0	91.21±3.52	0.08±0.06
4	1.25	3.1	1	32.24±0.14	0.28±0.01
5	1	5	1	8956.67±3240.64	1.00±0.00
6	1	3.1	2	24.13±1.40	0.32±0.03
7	1.5	3.1	2	13.82±0.05	0.40±0.01
8	1	1.2	1	453.033±23.98	0.45± 0.01
9	1.25	5	2	8825.66±370.13	1.00±0.00
10	1.5	3.1	0	208.3±10.62	0.92±0.08
11	1.25	3.1	1	119.26±0.15	0.48±0.03
12	1.5	5	1	4556.66±3033.50	1.00±0.00
13	1.25	1.2	0	2.72±0.06	0.23±0.00
14	1.25	5	0	6499.66±19.22	1.00±0.00
15	1.5	1.2	1	72.043±0.72	0.28±0.07

Table 2: Formulation composition of Ipriflavone nano emulsion.

Ingredients	NE1	NE2	NE3
Ipriflavone (%W/V)	0.5	0.5	0.6
Miglyol 812 Oil % V/V	12.5	12.5	15
Ethanol % V/V	15.5	6	6.5
Tween 80 % V/V	15.5	6	6.5
Soya Lecithin % W/V	0.5	0.5	0.5
Water q.s. % V/V	100	100	100

Table 3: Breaking point of emulsions during preliminary stability evaluation of statistically optimised nano emulsions.

Formulation code	Breaking point of emulsion		
	Number of heating-cooling cycles	Time of centrifugation (min)	Number of freezing/ thawing cycle
NE1	6	30	5
NE2	6	30	5
NE3	6	40	5

Measurement of formulation viscosity

A digital viscometer (Brookfield LVDV-E, USA), Model-DV2TLV with spindle number SC4-18 with a small sample adapter of volume of 6.7 mL and a shear rate of e 1.32N was used to determine the viscosity of the formulation at room temperature. This test was performed in triplicate and the results were obtained as mean±SD.[20]

Results for the Physical evaluation of statistically optimised nano emulsions are given in Table 4.

Drug content estimation

The amount of IP in each formulation was calculated compared to the theoretical amount. A volume of 1 mL of each formulation was diluted with a suitable volume of methanol to dissolve all the loaded amounts of ipriflavone. The emulsion was centrifuged at 3000 rpm, the supernatant was collected and filtered through a 0.45 µm syringe filter and the filtrate was analysed in a UV-vis spectrophotometer at λ_{max} of 249nm. This test was carried out in triplicate and the results were recorded as mean±SD.[21]

Stability studies of the optimised formulation

A short-term stability study of optimised nano emulsion was performed by keeping the sample at two different temperatures 4°C and 25°C over a period of 3 months. The globule size, PDI, viscosity, phase separation and refractive index were determined at 30 days.[22,23]

Examination of the optimum formulation morphology

The morphological study of nano emulsion is carried out using Transmission Electron Microscopy (JEM2100 Electron microscopy, Made in Japan). The sufficiently diluted one-drop sample placed on a grid, air-dried and used for the morphological observation.[24]

In vitro drug release study

Dissolution apparatus type II (USP) with 900 mL purified water as dissolution media was used for the *in vitro* drug release analysis. Dissolution was carried out at 37±0.5°C at 50 RPM. A sample of 5 mL was drawn at a specific time interval and replenished with a fresh medium. Each sample was filtered with a syringe filter of 0.45 mm before being analysed with a UV-VIS spectrophotometer at λ_{max} of 249nm. Each experiment was performed three times to determine the results as mean±SD.[25]

Determination of Lethal Concentration (LC_{50}) of the nano emulsion formulation in zebrafish embryos

Zebrafish breeding setup was kept the previous day and the embryos were collected in the morning. The collected embryos were cleaned and stored in an incubator with embryonic rearing media at 28.8°C. Embryos were segregated into a 24-well plate with five embryos in each well. At 10 hpf, the embryos were exposed to Ipriflavone of different concentrations of 1 ppm, 5 ppm, 10 ppm and 15 ppm, which was observed at 24 hpf under the Stereo Trinocular microscope (Leica, Japan) according to the OECD guidelines of FET.[26] The number of deaths was recorded and probit analysis was calculated for LC_{50}.[27]

Toxicity study of ipriflavone

A five-day toxicity study of ipriflavone was carried out on zebrafish embryos to check the different toxicological points like developmental toxicity, cardiotoxicity, neurotoxicity and hepatotoxicity. At 10 hpf, five embryos were segregated into 24-well. $1/10^{th}$ of the LC_{50} value of ipriflavone was exposed and observed for five days under a Stereo Trinocular microscope (Leica, Japan) and toxicity was recorded.

Anti-osteoporotic activity on zebrafish

Adult zebrafish with an average weight of 729.6 mg and average length of 3.56 cm were selected for the study and were divided into three groups: control, induced and treated. Osteoporosis was induced by injecting 0.022 mg of dexamethasone intraperitoneally, twice daily for three days, to the zebrafish of the induced group and treated group.[28] Treated groups received 0.0057 mg/mL concentration of ipriflavone nano emulsion intraperitoneally twice daily for three days, after induction of osteoporosis. After the drug treatment, zebrafish from all the groups were kept on ice for 20-70 min for anaesthetising and performed the bone staining procedure by fixing, bleaching and staining the bones using alizarine red dye. The bones were observed under a Stereo Trinocular microscope (Leica, Japan) and fluorescent microscope (Leica, Germany) and the length of the bones were measured. The measurement of the bones of each group of fishes were recorded and ANOVA was performed to obtain the significance of the ipriflavone treatment.

Table 4: Physicochemical evaluation of statistically optimised nano emulsions.

Formulation code	Globule size (nm)	PDI	Zeta potential (mV)	pH	Drug content %	Viscosity (mP)
NE1	29.80±0.02	0.378	-10.5	6.5±0.0	90.36±3.71	2.94± 0.14
NE2	45.91±1.95	0.401	-12.6	6.5±0.0	92.86±6.19	2.96± 0.32
NE3	72.03±0.02	0.271	-12	6.5±0.0	95.42±3.83	3.02± 0.21

Statistical Analysis

Bone lengths are analysed using Analysis of Variance (ANOVA); the statistical Analysis was conducted through GraphPad Prism 6, followed by the Tukey test to determine the significant differences and the data is represented as Mean±SD.

RESULTS

Selection of oil phase and surfactant screening

Among the two oils, Miglyol 812 attained the highest concentration of 43 mg/mL of ipriflavone, followed by oleic acid producing a concentration of 30 mg/mL.

Among surfactants, Tween 80 could incorporate the highest amount of Mygliol 812, which was 50 µL, into the surfactant solution and adsorbed at the interface to form a stable nanoemulsion.[29]

Co-surfactant screening

Different ratios of oil and S_{mix} (surfactant and co-surfactants) were mixed and titrated against water. The results were constructed as phase diagrams. The phase diagram of 1:1 of Tween-ethanol showed a larger area of emulsification than the other co-surfactants.

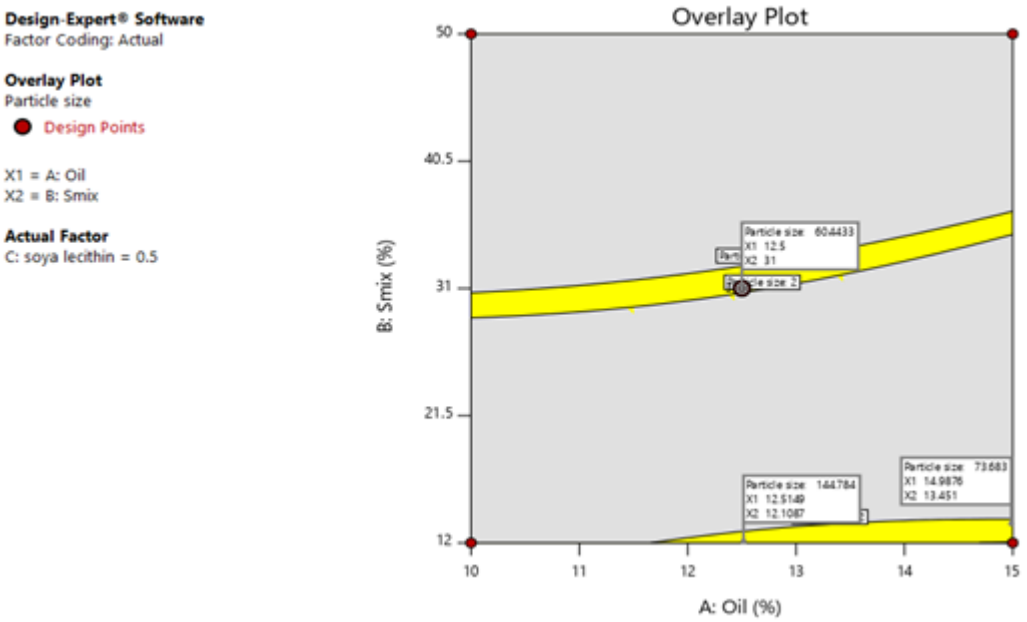

Figure 1: Overlay plot obtained by optimization showing various compositions (flagged areas) that can produce stable nanoemulsions with lowest globule size.

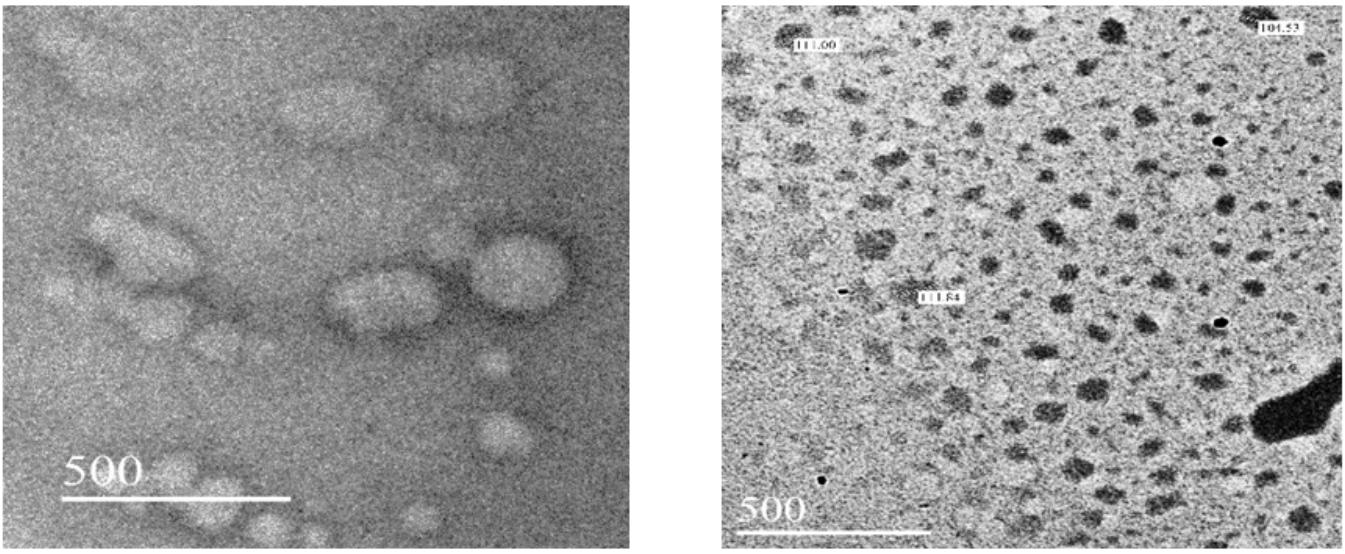

Figure 2: TEM Micrographs of formulated nano emulsion at different magnifications.

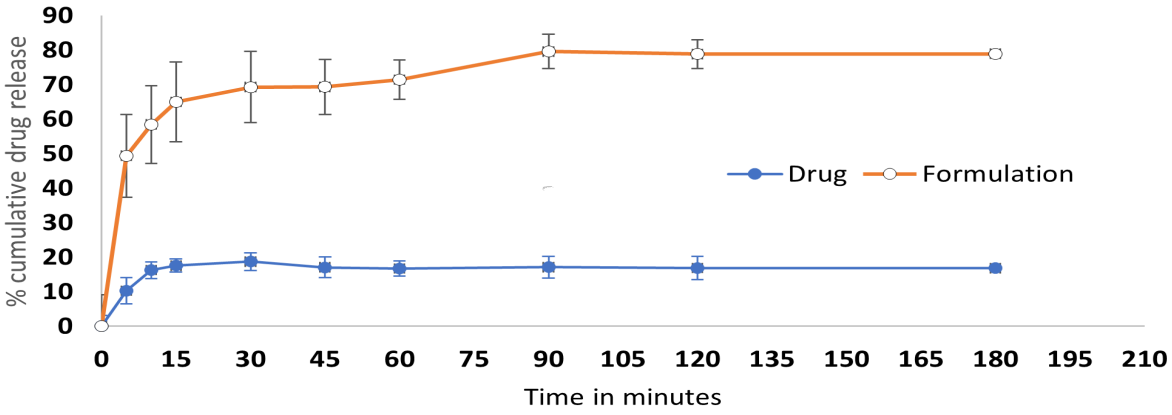

Figure 3: Drug release profile of IP Nanoemulson and ipriflavone.

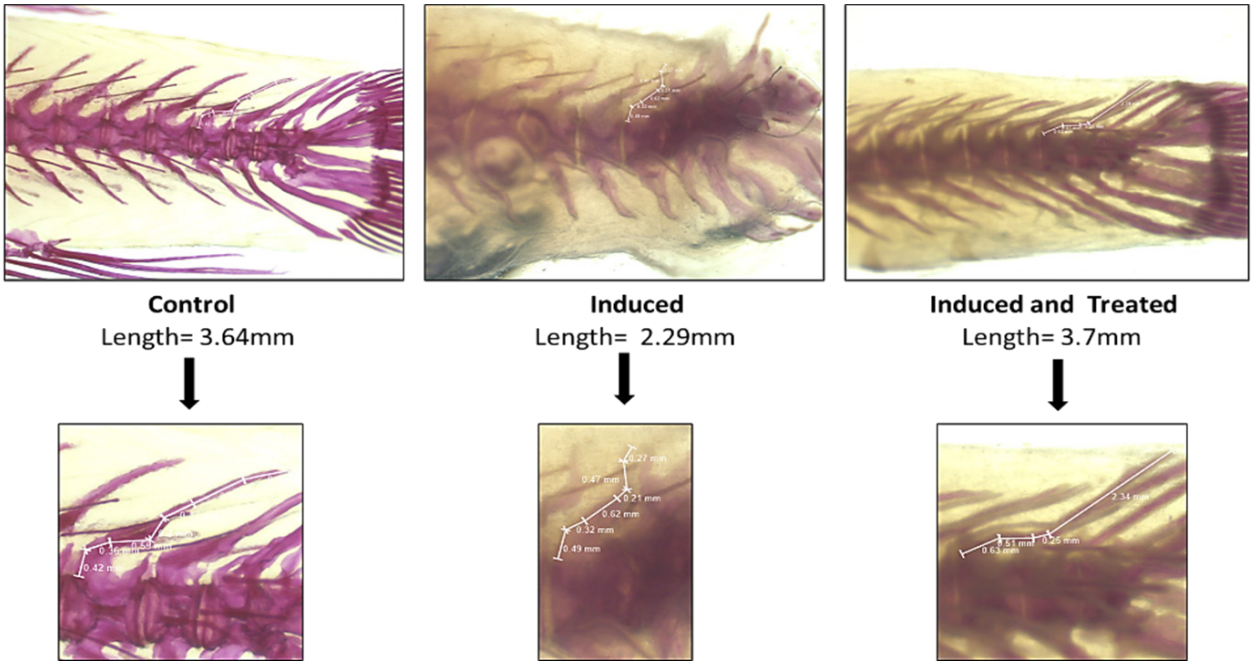

Figure 4: Measuring the length of the vertebrae of control, induced and treated fish.

Optimisation of IP nano emulsion formulation by Box-Behnken Experimental Design

Table 1 illustrates the responses observed throughout the 15 experimental runs to optimise IP nano emulsion. The various responses (Globule size and PDI) were fitted to the multiple variables.

The final Equation in Terms of Coded Factors is:

Globule size=60.44-584.28A+3517.57B+279.01C-1004.8AB-31.85AC+539.6BC-170.54A2 +3619.7B2 +194.46C2

The final equation in terms of coded factors for polydispersity index is given below.

PDI=0.5293+0.0936A+0.3623B-0.0443C

From the overlay plot, the best three formulations (Table 3) were prepared and evaluated for further studies. An overlay plot indicating the optimum composition of nano emulsion is depicted in Figure 1.

Evaluation of IP nano emulsion

Thermodynamic stability test

Thermodynamic stability of all the selected formulations were tested and reported in Table 3. NE3 formulation showed separation after 30 min. which is the highest among the three formulations.[31]

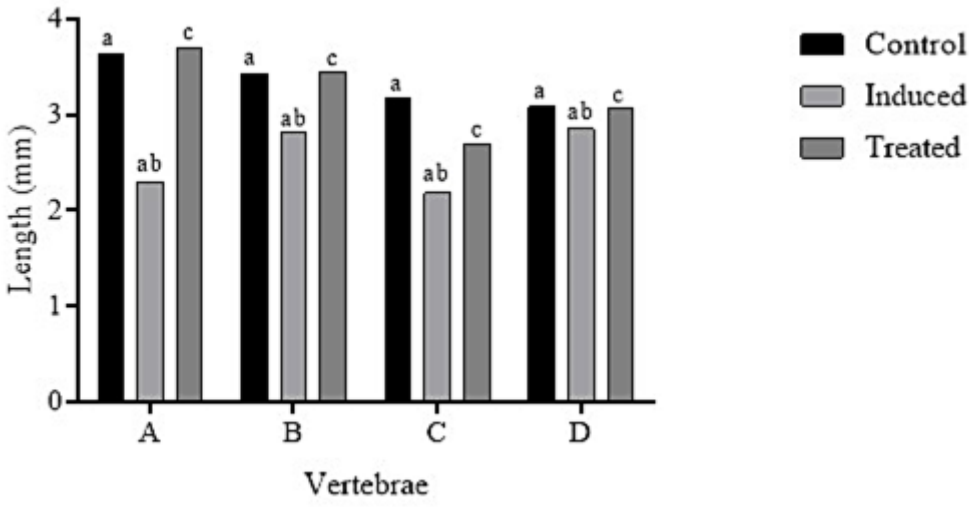

Figure 5: Anti-osteoporotic effect on zebrafish showing caudal vertebrae length of induced and treated groups. Caudal vertebrae bone of treated zebrafish showed significant (p<0.05.) bone regeneration compared to untreated. The data represented in Mean±SD, subscripts a, ab and c indicates the significance.

Globule size and Polydispersity Index (PDI) and Zeta potential

The globule size distribution of the nano emulsions and PDI and zeta potential are represented in the Table 3. NE3 formulation had the good distribution of globule size and that was assumed to be the stable emulsion.[29]

Examination of the optimum formulation morphology

TEM images (Figure 2) showed nano emulsion globules with sizes average of 100 nm.

In vitro drug release study

As depicted in Figure 3, the maximum release of Ipriflavone from its pure form was achieved within 30 min, which was 20%. Whereas 70% of the drug was released from the nano emulsion in 30 min and the maximum release of 80% was attained within the next hour.[33]

Stability studies of the optimised formulation

Based on the stability data provided (Table 4), it can be observed that the mean globule size increased with time at both 4°C and 25°C. The globule size increased to 91.04±0.17 nm in duration of 3 months were stored at 4°C. Whereas globules grown into 85.03±0.01 nm after 3 months storage at 25°C. The Polydispersity Index (PDI) remained consistent over the study period for both temperatures, so as the viscosity.

Toxicity studies of IP nano emulsion

The study indicates a positive correlation between the concentration of IP nano emulsion and the death of the embryo, with the maximum percentage of embryo death observed at 15 PPM. The LC_{50} of the nano emulsion is calculated to be 5.7 ppm by plotting the concentration of nano emulsion (ppm) vs % probit of death.[34]

Anti-osteoporotic activity on zebrafish

To evaluate the anti-osteoporotic effect in zebrafish, their Caudal vertebrae (A, B, C and D) were focused. As shown in Figure 4, the size of the bones was measured from its micrographic images. The result showed that the dexamethasone-induced fish bones were degraded (reduced in length) compared to the control and the IP nano emulsion-treated groups.[35]

DISCUSSION

The oil was selected based on the solubility of ipriflavone into it. Miglyol 812, being a mixture of fatty acid, dissolved more amount of IP compared to oleic acid. The non-ionic surfactants were preferred because of their neutral charge. The HLB value of Tween 80 is more than 10, which was suitable for the preparation of an oil-in-water emulsion. Moreover, it could incorporate highest amount of oil into it. The co-surfactant was added to reduces the surface tension further by lowering the interfacial energy between the oil globules in water and making the system thermodynamically stable.[30]

The equation in terms of coded factors derived for optimization of nano emulsions were used to predict the response of each factor at given levels. By default, the high levels of the factors are coded as +1 and the lowest levels are coded as -1. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients.

Based on the acceptable range of globule size and PDI, there formulations were selected for further stability evaluations. The globule size increase during the period of stability studies

were not significant. The Polydispersity Index (PDI) remained consistent over the study period for both temperatures, indicating uniformity in globule size distribution. NE3 is considered as more stable compared to NE1, NE2 and considered for the further studies after thermodynamic stability testing. The globule size and size distribution confirmed the stability of the nano emulsions for further research. The oil globule size found increasing with increase in the ratio of oil phase and drug, decreasing the S_{mix} ratio. all the three formulations were within the required nanoscale range and PDI was 0.4 or less indicating a uniform distribution. The viscosity remained relatively constant over the study period suggesting no significant changes in the chemical composition or stability of the formulation. These findings indicate the formulation is stable over time and within the studied temperature range. However, further studies are recommended to evaluate the long-term stability and performance of the formulation.

The initial fast release of the drug from nano emulsion might be due to the pre-solubilised form of Ipriflavone in the emulsion globule, which could enhance the aqueous solubility of the compound to a greater extent. Since the drug exists in solubilised form in the oil, the migration into the aqueous phase would have been easier. The oil, surfactant mixture and drug formed a matrix, which delayed the release of the drug further, which showed a sustained pattern in the release graph.[33] Additionally, the release media saturation by Ipriflavone can also result in retardation of the drug release and any excess drug might have precipitated. In the *in vivo* conditions, faster solubilisation may enhance the rate of absorption, which helps in maintaining a sink condition within the GI tract thereby limiting the chances of precipitation.

The DLS measure the zetapotential of IP nano emulsions accurately. The higher the charge on the globules higher its stability in the nano emulsion. The zeta potential value of ±30mV is considered as the requirement for at most stability of a nano system. At times a steric stabilization is observed in nano systems with lower zeta potential. A negative charge on the globules is due to the presence of anionic groups in the oil and the hydroxyl group from the ethanol. In this study, despite having a low zeta potential, the system exhibited good stability maintaining the small globule size, due to the precise concentration of surfactant ratio employed. The surfactant may be producing a steric effect on the surface of the globules which helps in the stabilisation of the system. While zeta potential plays a role in the solubility and interaction of charged globules in the Gastrointestinal Tract (GIT), its influence on globule size was negligible in this case. However, it is important to note that zeta potential may still impact other aspects of the formulation's behaviour, such as its interaction with the GIT and subsequent absorption. Therefore, while globule size is crucial, considering the zeta potential along with other factors is essential for a comprehensive understanding of the oral absorption of nanoglobules.[32]

The Probit of Death percentage highlighted that the percentage of embryo death increases with the increasing concentration of the drug. These findings provide valuable insight into the toxicity of the tested compound and can be useful in determining the safe dosage for potential applications in various fields. In the 5 days of toxicity studies, it was observed that all the embryos were alive. The samples showed no signs of general as well as organ toxicity. Ipriflavone is target-specific to produce its mechanism of action and it undergoes efficient metabolism and causes no damage to DNA. LD_{50} of aglycone part of ipriflavone is reported 2 g/Kg body of mice.[34] This shows that the nano emulsion is safe to use for the anti-osteoporotic study.

The dexamethasone-induced fish bones were degraded (reduced in length) compared to the control and the IP nano emulsion-treated groups, indicating bone regeneration in the treated group. Dexamethasone leads to osteoporosis by influencing enzymes involved in bone resorption, such as cathepsin K and Tartrate-Resistant Acid Phosphatase (TRAP), as well as enzymes related to bone formation, such as Alkaline Phosphatase (ALP). The higher measurement of vertebrae as compared to the induced and control groups indicates that the IP nano emulsion treatment may have a significant effect (Figure 5) on bone regeneration by impacting the enzymes responsible for both bone resorption and formation. Studies have shown that ipriflavone can intervene the P13K/AKT signalling pathway to influence the bone cell proliferation and inhibit the apoptosis of the osteoblast cells.[35] Therefore, the IP nano emulsion can help bone remodelling thereby it can be an ideal treatment for osteoporosis.

CONCLUSION

In this study we concluded that the successful formulation of ipriflavone into nano emulsion can be helpful in increasing the drug solubility. The increased solubility may enhance the bioavailability of the drug provided its hepatic first-pass metabolism is controlled, which needs advanced evaluation. A pivotal component of our formulation was the inclusion of Miglyol 812, which was instrumental in achieving nanoemulsion stability. The unique properties of Miglyol 812 contributed substantially to the enhanced solubility and overall stability of the nanoemulsion. We demonstrated the effectiveness of ipriflavone in bone regeneration in zebrafish model, which is the first of its kind. Further studies are required to quantify the bioavailability and the extent of metabolism. The strong evidence for its effectives generated in this study can be helpful in future research to bring up ipriflavone as a mainline therapy in osteoporosis.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

WHO: World Health Organization; **ER:** Oestrogen Receptor; **SERMs:** Selective Oestrogen Receptor Modulators; **TSECs:** Tissue-Selective Oestrogen Receptor Complexes; **IP:** Ipriflavone; **RS:** Reference Standard; **UV:** Ultraviolet; **PDI:** Polydispersity Index; **USP:** United States Pharmacopeia; **RPM:** Revolutions Per Minute; **SD:** Standard Deviation; **ANOVA:** Analysis of Variance; **S_{mix}:** Surfactant and Co-surfactant; **GIT:** Gastrointestinal Tract; **LC_{50}:** Lethal Concentration 50; **OECD:** Organisation for Economic Co-operation and Development; **FET:** Fish Embryo Toxicity.

SUMMARY

This study explores the development of an ipriflavone nano emulsion to improve its solubility and bioavailability in treating osteoporosis. Osteoporosis, known for low bone mass and increased bone fragility, is a chronic condition frequently managed with anti-resorptive medications. Nevertheless, these medications come with drawbacks such as side effects and limited bioavailability.

Ipriflavone, a synthetic phytoestrogen, has demonstrated promise in enhancing bone formation while causing fewer adverse reactions. Although ipriflavone is effective, its therapeutic use is limited by its poor solubility and bioavailability. The study seeks to address these constraints through the development of an ipriflavone nano emulsion.

Choosing excipients like oleic acid, Miglyol 812, tween 80, ethanol, PEG 400 and propylene glycol is part of the formulation process to improve solubility and stability. Utilising the Box-Behnken experimental design helps in fine-tuning the formulation parameters such as oil quantity, S_{mix} volume and soya lecithin amount.

After examining the nano emulsions, which involved assessing factors like globule size, polydispersity index, zeta potential and viscosity, it is evident that the formulations are appropriate. Drug release studies conducted *in vitro* show a sustained release pattern, suggesting the possibility of improved absorption and bioavailability. Experiments conducted on zebrafish embryos reveal concentration-dependent toxicity, with an LC_{50} of 5.7 ppm for the nano emulsion. After a five-day toxicity study, there were no signs of toxicity, suggesting the nano emulsion is safe. Research in zebrafish shows that the IP nano emulsion has promising anti-osteoporotic properties, suggesting its effectiveness in addressing osteoporosis.

Conclusively, the research effectively develops an ipriflavone nano emulsion with improved solubility and bioavailability. This nano emulsion demonstrates encouraging outcomes in bone regeneration, indicating its promise as a treatment for osteoporosis. Additional research is required to assess its effectiveness and safety in human subjects.

AUTHOR CONTRIBUTIONS

Anish John: Data analysis, manuscript writing; Sreehari: Data collection; Masmarika Mohan: Data collection; Anoop Narayanan V: Conceptualization, study design, Supervision, Data analysis, Gunimala Chabraborthy: Supervision, Data Analysis, Manohar M: Data Analysis.

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