Formulation of KT-Encapsulated Niosomes to Improve Behavioral Responses in Preclinical Models

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(Received on 5th May 2025, accepted in revised form 24th December 2025)

Summary: Niosomal drug delivery systems have attracted significant attention due to their potential to enhance bioavailability, drug stability, and therapeutic efficacy. In this study, a non-ionic surfactant (D1) was synthesized and characterized employing advanced techniques, including NMR and EI-MS, to elucidate its structure. D1 and lignin were employed as ketamine (KT) carriers for the preparation of two formulations, designated as KD1 and KL1, respectively. Characterisation techniques, like UV-Vis spectroscopy, FT-IR spectroscopy, and dynamic light scattering (DLS), were employed to examine the properties of formulations, such as their interactions, size in solution, polydispersity index (PDI), and particle charge. KD1 and KL1 showed outstanding drug encapsulation efficiencies (EE) of 75.0% and 80.1%, respectively. The results demonstrate the potential of these formulations for efficient delivery of KT. Moreover, the behavioral studies, including the Elevated Plus Maze (EPM) test, Social Interaction Test (SIT), and Open Field Test (OFT), were performed on rats for examining anxiety-like symptoms induced by exposure to electric shock to evaluate their therapeutic potential. The findings demonstrated that rats subjected to electric shock alone experienced considerable anxiety-like behaviors, such as less social engagement with new cage-mates and less exploration of open-arm and open arena apparatus. However, these anxiety symptoms were successfully reduced by treatment with the nanoformulations (KD1 and KL10. This suggests that the prepared formulations of KD1 and KL1 have promising potential to treat anxiety conditions. Furthermore, KD1 and KL1 were examined for drug retention using a Storage Stability (SS) study conducted over 30 days. The SS for KD1 and KL1 ranged from 96.4% to 88.04% and from 97.1% to 89.94% from the 1st to the 30th day, respectively.

Keywords: NSMs, Drug delivery, KT, Behavioral tests, Characterization.

Introduction

A fundamental aspect of pharmaceutical research is drug delivery, which aims to transport active pharmaceutical substances to the targeted site of action to optimize release, absorption, and therapeutic efficacy [1]. Drug delivery vehicles are essential to the effective administration of drugs because they serve as carriers to help distribute active ingredients throughout the body.

Oral drug delivery is recognized as the optimal method in drug delivery systems because of its multiple benefits. Its ease of administration and independence from assistance enhance patient adherence [2, 3]. Drugs with limited aqueous solubility sometimes fail to attain the necessary therapeutic concentration in the bloodstream, hence reducing their oral bioavailability [4]. This limitation may reduce the efficiency of oral drug administration. Drug absorption through the gastrointestinal barrier may be further hampered by a bigger particle size, increased lipophilicity, and low solubility in water. Furthermore, first-pass hepatic extraction and inadequate absorption time can affect the bioavailability of drugs taken orally [5].

Non-ionic surfactants have gained attention in the drug delivery domain over typical liposomes because of their superior capability for stabilizing pharmaceuticals and offering cost-effective vesicular drug delivery systems [6]. Niosomes (NSMs) are spherical, non-ionic surfactant-based vesicles that self-assemble in an aqueous medium to produce closed bilayer structures.

NSMs have become a viable option for targeted drug delivery to infected sites because of their lower toxicity and biological system compatibility [7]. NSMs have multiple benefits over traditional phospholipids, including greater physical, chemical, and biological stability. These robust structures possess the potential to serve as drug carriers. Compared to liposomal formulations, NSMs have demonstrated exceptional capability to improve drug permeability and penetration

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through biological membranes, resulting in improved bioavailability [8]. Moreover, it has been observed that NSMs are more capable than liposomes in encapsulating drugs. This indicates that NSMs may facilitate the delivery of a larger quantity of drugs, thereby enhancing therapeutic efficacy [9]. NSMs are also important because they do not require particular handling or storage conditions, which increases their usefulness and convenience [10]. Drugs that are encapsulated in NSMs have the additional benefit of stability for prolonged drug release. This characteristic makes NSMs ideal for many uses, such as peptide drug delivery, NSMs loaded with insulin, and transdermal drug administration by niosome encapsulation [11]. NSMs can be meticulously developed to interact with the target area by modifying their surface, hence improving drug localization and reducing off-target effects. This type of surface alteration generates novel possibilities for tailored drug distribution and personalized therapy [12].

Cholesterol is an additive commonly employed in niosomal drug delivery systems [13]. Its incorporation improves the drug's trapping efficiency, affects niosome binding properties [14], and establishes a vesicular bilayer architecture that enhances the stability of the niosome systems [15]. Cholesterol contributes to long-lasting therapeutic benefits by promoting the regulated release of the drug for an extended duration by enhancing fluidity.

Ketamine (KT) is a prevalent anesthetic with emerging applications in the treatment of chronic pain and depression; however, its short half-life *in vivo* complicates its use. Moreover, when administered in appropriate quantities, it has analgesic effects that are comparable to or superior to opioids, while avoiding the associated disadvantages of their usage. Unlike opioids, KT may alleviate pain without the risk of addiction or significant respiratory depression [16]. However, it is important to acknowledge that KT usage may result in certain negative consequences, like hallucinations [17], floating sensations, dizziness, and transient psychotic effects[18]. Because these negative consequences are usually dose-dependent, they can be reduced by carefully assessing the dosage before administering it. As an anaesthetic and analgesic, KT has many benefits, including a broad therapeutic window and the ability to overcome some of the drawbacks of opioids. To reduce negative effects, the dosage needs to be carefully considered before using it.

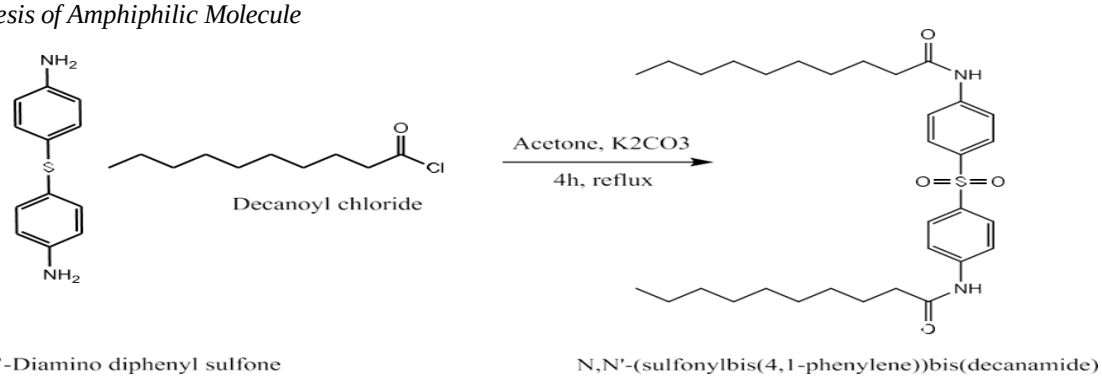
In this research work, a non-ionic surfactant (D1) was synthesized and characterized. D1 and lignin were employed to prepare two novel nanoformulations for the effective delivery of KT. The prepared formulations showed excellent drug encapsulation efficiencies of 80.1% and 75.0%, respectively. The results of behavior studies showed that KD1 and KL1 nanoformulations could mitigate anxiety symptoms, indicating potential therapeutic benefits for anxiety disorders.

Experimental

Materials

Standardized procedures and clean equipment were utilized in the entire study. Deionized water was employed for the preparation of the solutions. The reference standard was commercial Tween 80, which was purchased from Merck in Germany. The nano-vesicular formulations were prepared by adding cholesterol, which was procured from BDH, UK. Furthermore, the novel lipid-based niosomal vesicles were synthesized using Na_2SO_4, K_2CO_3, Lecithin, Decanoyl Chloride, and 4,4' Diaminodiphenyl Sulfone, which were supplied by Sigma-Aldrich, USA.

Synthesis of Amphiphilic Molecule



Yield: 38.14 %, M.P. 170- 180 ºC.

Scheme-1: Scheme for the synthesis of amphiphilic molecule **(D1).**

For the synthesis of the D1 compound, the amphiphilic molecule 4,4'-diaminodiphenyl sulfone was subjected to chemical derivatization, yielding the compound **D1**. The synthesis was carried out in a round-bottom flask connected with a condenser and a magnetic stirrer to ensure efficient mixing and reflux conditions. K_2CO_3 (1200 mg; 4.83 mmol) and 4,4'-Diaminodiphenyl Sulfone (400 mg; 1.61 mmol) were added to the flask, followed by refluxing in 15 mL of acetone at 80 °C (30 min). Thereafter, decanoyl chloride (0.835 mL; 2 mmol) was incorporated into the flask at the same temperature, followed by refluxing for 5 h. The monitoring of the reaction was carried out through TLC. When the reaction was completed, the final product was obtained by quenching and extracting the reaction mixture with hexane and then ethyl acetate. Following its extraction from the organic solvent phases, the resulting product was purified using column chromatography. The refined white product had a yield of 38.14%.

Preparation of KT-Loaded NSMs **(KD1)**

The thin film hydration process was used for the preparation of the KT-loaded NSMs of D1. The preparation of **D1-based** formulation loaded with KT was carried out by dissolving **D1** (400 mg), KT (200 mg), and Cholesterol (200 mg) in 50 mL of MeOH: DCM ratio 6:4 v/v in a round-bottom flask. A rotary evaporator (BUCHI, 131 Rotavapor, Switzerland) was employed for the evaporation of the organic solvent, leading to the development of a thin layer of niosomal vesicles on the flask walls. The rotary evaporator was used to further dry the flask containing the film for two hours at a lower pressure. The film was rehydrated by adding distilled water (100 mL) to the flask and then putting the mixture in a bath sonicator set to 25 ℃ for 15 min. To reduce the particle size, the resultant suspension was sonicated for two min at 25 ℃ with a 5-second on/off cycle. Thereafter, the suspension was kept at 4 ℃ for subsequent studies.

Preparation of KT-Loaded NSMs **(KL1)**

The preparation of KL1 was carried out by dissolving lecithin (600 mg), cholesterol (300 mg), and KT (300 mg) in 50 mL of a MeOH: DCM ratio 6:4 v/v in a round-bottom flask. A rotary evaporator was employed to evaporate the organic phase, leading to the development of a thin film of niosomal vesicles. The rotary evaporator was used to further dry the flask containing the film for two hours at a lower pressure. After adding 150 mL of distilled water to the flask, the mixture was left in a bath sonicator set at 30 ℃ for 12 to 15 min for the rehydration of the film. The resulting suspension was then sonicated for two minutes at 25 ℃ with a 5-second on/off cycle using a probe sonicator for reducing the particle size. For subsequent studies, the suspension was then kept at 4 ℃.

Characterization of the prepared NSMs

Size, Zeta Potential (ZP), and PDI

A Zetasizer analyzer (Malvern Instruments, Worcestershire, UK) was employed to ascertain the average diameter, PDI, and charge on the drug-loaded and empty NSMs. The formulations of nanoparticulates were diluted with distilled water before analysis.

FT-IR spectroscopy

The developed formulations were mixed with KBr powder. Thereafter, a Shimadzu IR-470 spectrometer (Shimadzu, Kyoto, Japan) was employed to obtain IR spectra in the 400 to 4000 cm^{-1} range.

Entrapment Efficiency (EE%) of KT

A UV-visible spectrophotometer (Shimadzu, UV-240, Hitachi U-3200, Japan) was employed to examine the developed niosomal formulations to ascertain the entrapment effectiveness of the encapsulated drug. The KD1 and KL1 niosomal formulations with a particular concentration of KT underwent centrifugation for 30 min at 12,000 rpm. After being dissolved in a predetermined amount of distilled water, the separated pellets comprising the drug-loaded vesicles were measured using a UV spectrophotometer. The wavelengths I 220 and II 269 nm were employed for the quantification of KT.

The EE% of each formulation of KT was determined employing the given expression:

EE% = (Amount of KT entrapped / Total amount of KT) × 100**.**

Anxiolytic effects of KT formulations in the rat model of anxiety

All animals were procured from the animal housing facility of the University of Karachi, Karachi, Pakistan, and adhered to the NIH pointers and were conducted with approval from the institutional Ethics and Animal Care Committee (Approval No. IBC KU-340/2023). KD1 and KL1 NSMs were tested in different trials. Experiment 1 included 25 rats separated into 5 groups (n=5) – Control, Anx, Anx+KD1 (10 mg/kg), Anx+KD1 (50 mg/kg), and KD1 (50 mg/kg). Two different dosages (10 and 50 mg/kg) of KT were selected for conducting the animal

studies. These dosages were selected based on previous reported findings. Reports showed that a dosage of 10 mg/kg KT elicits observable anxiolytic or antidepressant-like effects with no sedation[19, 20], whereas higher doses near 50 mg/kg are generally tolerated efficiently and safely employed in rat behavioral studies to assess dose-dependent performance[21]. After acclimatization, intraperitoneal (i.p.) drug treatment was carried out for 3 days. All rats were subjected to electric foot shocks on the fourth day, except the Control and KD1 (50 mg/kg) groups. Each 10-second electric foot shock was administered at 0.8 mA intensity, with a 10-second gap between shocks. During every session, fifteen shocks were given. These traits are consistent with accepted methods for assessing anxiety-like behavior in rats using the foot-shock paradigm, guaranteeing the repeatability of the experiment [22]. The treated rats got their appropriate doses after shock, while the control and Anx groups were given empty KD1. Behavioral assessments, like the elevated plus maze (EPM), open field test (OFT), and social interaction test (SIT), were carried out after 30 min. During KD1 testing, one rat in the Anx+KD1 (50 mg/kg) group died.

Experiment 2 included a KL1 trial, distributing 25 rats into 5 groups: Control, Anx, Anx+KL1 (10 mg/kg), Anx+KL1 (50 mg/kg), and KL1 (50 mg/kg). Procedures for testing and treatment were similar to those employed in Experiment 1.

Statistical Analysis

GraphPad Prism 8 was employed to analyze the data employing a one-way ANOVA and the Bonferroni post-hoc test. *P* values below 0.05 were believed significant, and values were displayed as mean±SEM.

Results and Discussion

Characterization

Synthesis of Non-Ionic Surfactant (D1

^{1}HNMR ppm (in $CDCl_3$): 7.79 (4H, dd, carbon (aromatic)), 7.81 (4H, dd, Aromatic carbon), 4.70 (2H, s, NH-CO), 2.40 (4H, t, CH_2-CO), 1.61 (4H, m, CH_2), 1.19 (24H, CH_2, m), and 0.79 (6H, CH_3, t). The D1 compound was characterized through ^{1}H NMR and EI-MS. The EI-MS spectrum of D1 showed a noticeable molecular ionic peak located at 556 m/z value. This peak confirmed the molecular weight of D1. The ^{1}H NMR spectral analysis confirmed the successful synthesis of D1. The ^{1}HNMR spectrum revealed separate peaks, documenting molecular constitution. The CH_3 terminal protons showed outstanding distinguishability, establishing a triplet with 6 protons, exactly confined to 0.79 ppm. On the other hand, the residual 6 protons of methylene demonstrated a multiplet comprised of 24 protons, at 1.19 ppm. Methylene assembly near the nucleus of the carbonyl carbon showed a distinctive triplet configuration, concisely defined through 4 protons vibrating at 2.40 ppm. Moreover, the residual lonely methylene group near the carbonyl-bound methylene was discriminated as a quartet echoing at 1.61 ppm, highlighting its structural uniqueness. Protons related to amide, significant for D1's chemical structure, unveiled the resonance as odd and ultimate singlet with 4.70 ppm, authenticating their presence inside the molecule. Aromatic contents within D1 were similarly obvious in the ^{1}HNMR spectrum, captured in doublets observed at 7.81 and 7.79 ppm, jointly confirming aromatic protons with precise surroundings and interfaces. The ^{1}HNMR and EI-MS spectra confirmed the effective synthesis of the D1 compound.

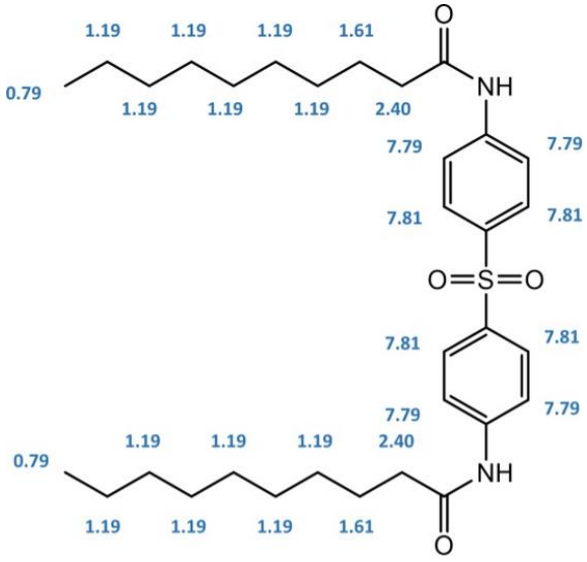


Fig. 1: NMR Study of D1.

Size, ZP, and PDI

The average size of liposomal-based formulations is a vital factor that affects their performance for drug-delivery systems. Liposome sizes play a noteworthy part in determining stabilization, biodistribution, and uptake in cellular transport. Table 1 denotes the size, PDI, and ZP values of KD1 and KL1. These parameters propose valuable insights regarding physical as well as electrostatic features of these samples. KD1 shows an average size

of around 189 nm and a comparatively greater PDI value of 0.41, demonstrating a wider distribution of sizes. The ZP value was -19.5 mV, which exhibits negative charge due to repulsive forces among particles, essential for stabilization. KL1 displays a bit larger size, of 213 nm, as compared to KD1 with a PDI value of 0.39, showing somewhat narrow size distribution for KL1 with a ZP value of -18.5 mV, which is less negative as compared to KD1, however, still showing repulsion among particles, hence contributing to the stability of the sample. These factors are decisive for applications, like drug delivery, stability in solution form, and communications among biological arrangements.

Table-1: ZP, Size, and PDI of KD1 and KL1.

Samples	Size	PDI	ZP
KD1	**189 ± 7.8**	**0.41**	**-19.5**
KL1	**213 ± 6.3**	**0.39**	**-18.5**

Percent Encapsulation Efficiency of Drug (%DEE)

Drug encapsulation efficiency (%DEE) is supposed to be an extremely critical condition, and it quantifies the quantity of drug effectively captured within liposomes. Increased %DEE shows extra effective drug loading, thus suggesting the encapsulation of a larger part of the drug inside the liposomal frame rather than actual loss during the formulation method. Figs. 2 and 3 exhibit the UV spectra and calibration plot of KT. Fig. 2 shows typical shapes of KT I at a wavelength of 220 nm and KT II at around 269 nm.

Formulation KD1 reveals a very inspiring DEE with 75.0%, which proposes that a considerable amount of drug anticipated for delivery was effectively encapsulated inside the liposomes, making KD1 a striking candidate for controlled release of the drug. Formulation KL1 even exhibits a higher %DEE of 80.1%, establishing an outstanding capability to capture the drug proficiently.

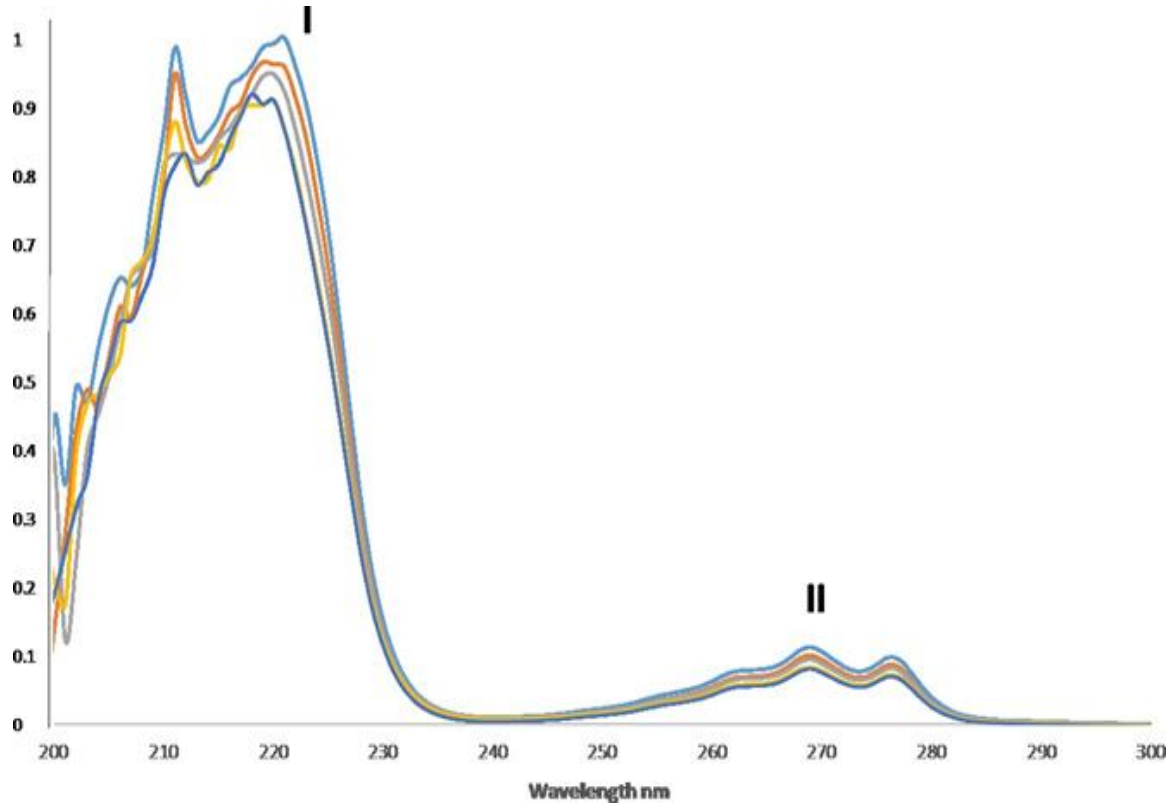


Fig. 2: UV spectra of KT.

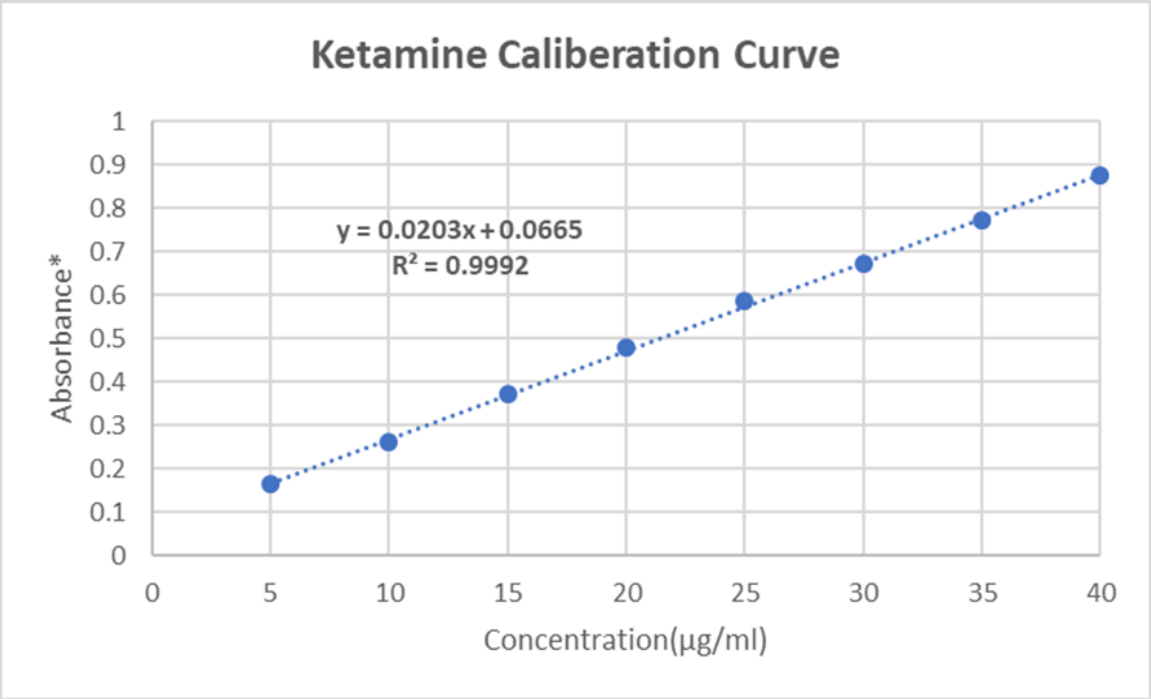


Fig. 3: Calibration curve for KT.

The loading efficiency of the KT-laden formulation was found by following the formula:

%DEE = ((Total quantity of drug – Free drug in suspension) x 100)/ Total quantity of drug

The results produced via this work focus on the changing features of KD1 and KL1 as liposomal formulations. KD1, having a fairly even size ranging between 75 and 110 nm with a DEE value of 75.0%, validates encouraging potential for efficient drug delivery. Formulation KL1, having a wider size distribution of 250 nm, demonstrates a higher DEE value of 80.1%. These results highlight the implication of formulation-specific features to ascertain the fitness of the liposomal-based system regarding drug delivery. Further studies through in vitro and in vivo procedures of the mentioned formulations will deliver more inclusive indulgences for their prospective therapeutic applications.

FTIR Analysis

In the FTIR study, KD1 displayed important features that clarify its molecular make-up. Fig. 4 shows an absorption signal at a frequency of 3294 cm^{-1}, which defines the stretching vibrations of the O-H group. This was concomitant with the manifestation of previously observed robust properties. A diverse and sharp signal with a frequency of 1724 cm^{-1} can be associated with C=O stretching, suggesting the participation of the carbonyl in the molecule. The extra distinctive group at 1668 cm^{-1} demonstrates asymmetric stretching of the carbonyl group. Furthermore, the signaling frequency of 1305 cm^{-1} gives bending vibrations of a methyl group, CH_2, and CH_3.

In the case of the FTIR study of KL1, we notice diverse peaks in its molecular characteristics. Signals were perceived with frequencies of 3304 and 1707 cm^{-1}, showing O-H and C=O stretching vibrations. More analysis exposed vibrational absorptions at 1151 & 1069 cm^{-1}, formed through C-O and C-C groups present in the molecular structure. The spectral range between 3000-2800 cm^{-1} shows signals depicting asymmetric and symmetric stretching vibrations for CH_2/ CH_3 groups, suggesting the occurrence of KT in KL1.

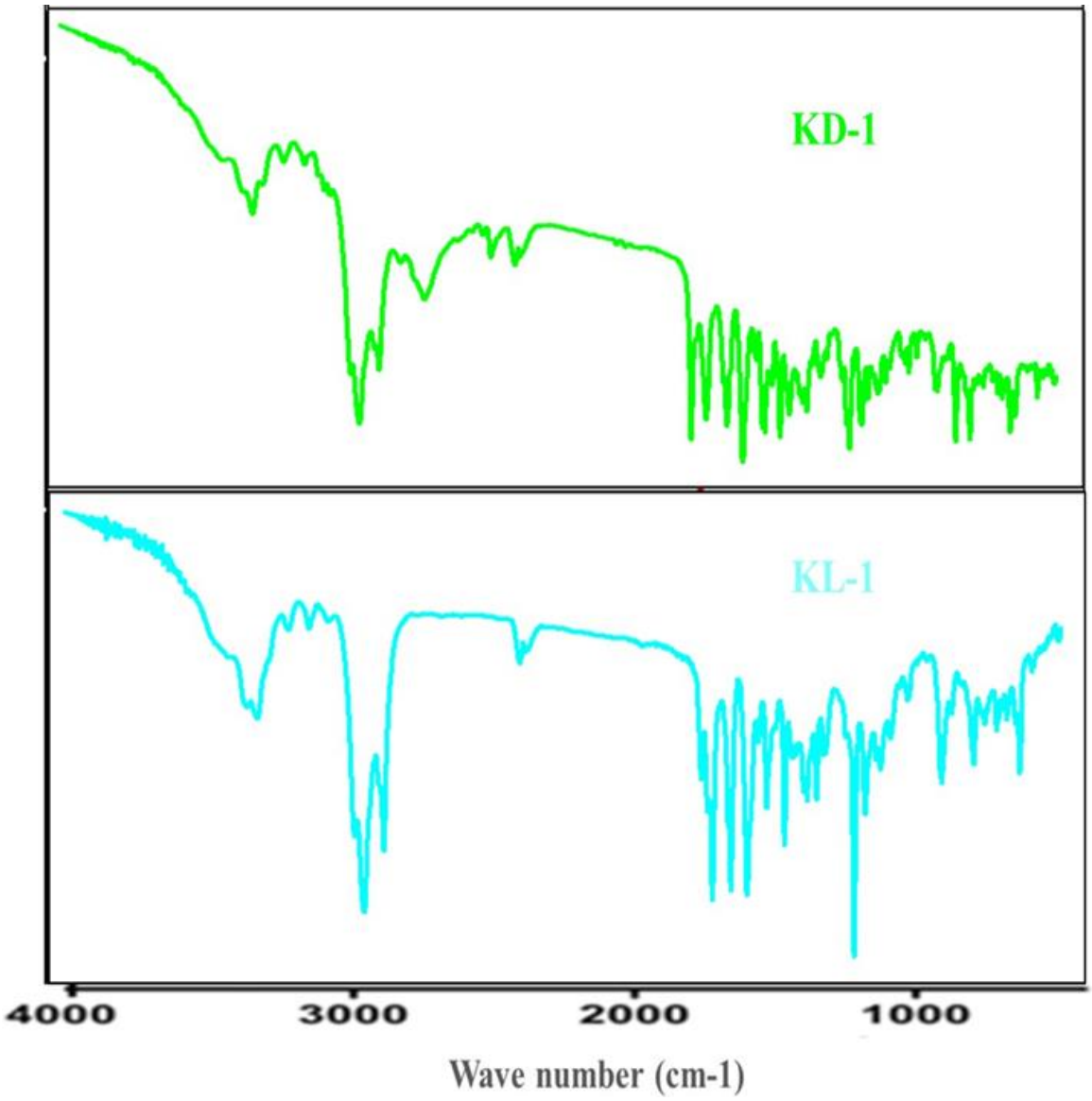


Fig. 4: FTIR spectra showing peaks in KD1 and KL1.

Anxiolytic Effects in the KD1 formulation of KT for the Rat Model

EPM

EPM check was employed as a cherished device to assess anxiety-like behavior through analyzing 2 important factors: potential for entering the closed arm and time spent in the open arm [23]. EPM data provided remarkable outcomes, showing important influences of diverse actions on both expectancy for entering the closed arm [$F_{(4,19)}$ = 3.941; $P<0.05$] and time spent in the open arm [$F_{(4,19)}$=12.40; $P<0.01$].

Following a post-hoc examination, fascinating configurations appeared. In particular, the Anx group exhibited a considerable reduction in expectancy and duration in the open arm in comparison to control rats (with $P<0.05$). This scrutiny showed discriminating anxiety-like performance in the Anx group. Yet, the exposure with KD1 at a dose of 50 mg/kg produced fascinating outcomes. Unambiguously, rats exposed to this action showed a noteworthy rise in expectancies for entering the closed arm and interval spent in the open arm compared to the Anx group ($P<0.05$). This distinguishing change describes that KD1 applied anxiolytic influences on the participants (Fig. 5).

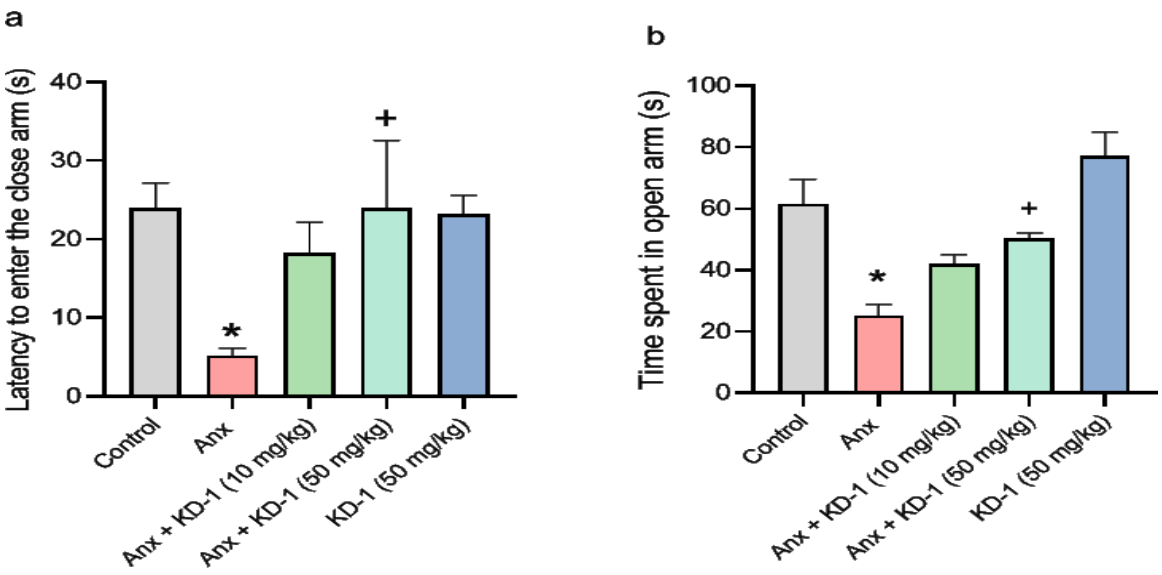


Fig. 5: Effects of KD1 action on the function of the EPM test in the rat model for anxiety. Values show average ±SEM. Noteworthy findings were found through one-way ANOVA with **P*<0.05 in comparison to control; +*P*<0.05 as related to the Anx group.

Conclusively, the EPM experiment offered insightful information about anxiety-like effects for a variety of applications. The change in latency upon entering the closed arm and the duration in the open arm showed the animals' anxiety levels. Particularly, the Anx group exhibited improved anxiety-like behavior, whereas cure with KD1 at a dosage of 50 mg/kg verified anxiolytic activity, efficiently reducing the practical anxiety-like performance. These results demonstrate the prospective therapeutic efficiency of KD1 to address anxiety-related situations.

OFT

The OFT analysis revealed notable properties of usage on 3 different factors: latency to travel to the corner, number of squares crossing, and stretch appear attitude. Statistics of OFT data showed substantial therapeutic effects on these variables: latency to travel towards the corner [$F(4,19)=6.61$; $P<0.01$], crossed squares [$F(4,19)=8.51$; $P<0.01$], and stretch-based posture [$F(4,19)=9.38$; $P<0.01$].

Subsequent analysis using a post-hoc study revealed significant differences across groups. The Anx group displayed a notable reduction in potential to travel towards the corner ($P<0.05$) and squares crossed ($P<0.01$) as compared to the control. Remarkably, this set exhibited sharp intensities of anxiety-like performance, shown by reduced assessment and travel in the open field. Furthermore, the Anx group revealed considerable growth in stretch postures ($P<0.01$), suggestive of amplified vigilance.

Treatment with KD1 at a dosage of 10 mg/kg demonstrated considerable development merely in the number of crossed squares ($P<0.01$), despite the fact that therapy with KD1 at varying doses provided intriguing results. This advocates that the amount of KD1 demonstrated a positive influence on investigative behavior, improving the subjects' readiness to cross an open field. Extraordinarily, the delivery of KD1 with 50 mg/kg provided multidimensional effects. Initially, it resulted in a noteworthy rise in potential to travel towards the corner and the number of crossed squares ($P<0.01$) in comparison to the Anx group. This scrutiny proposed that a greater amount of KD1 prolonged the time to go into a corner and also amplified the complete locomotion across an open field, representing a decline in anxiety-like trends. Additionally, the occurrence of stretch attend posture reduced ominously ($P<0.05$) in the Anx+KD1 (50 mg/kg) group in comparison to the Anx group, proving a significant decrease in attentiveness and anxiety-like performance. These outcomes jointly emphasize elaborate interaction between usages and activities in the open field site. Anx group demonstrated sharp anxiety-like performance with reduced investigation and improved vigilance. Remarkably, KD1 involvement at diverse doses established different effects: KD1 with 10 mg/kg boosted activity, while KD1 (50 mg/kg) diminished anxiety-like behaviors (Fig. 6).

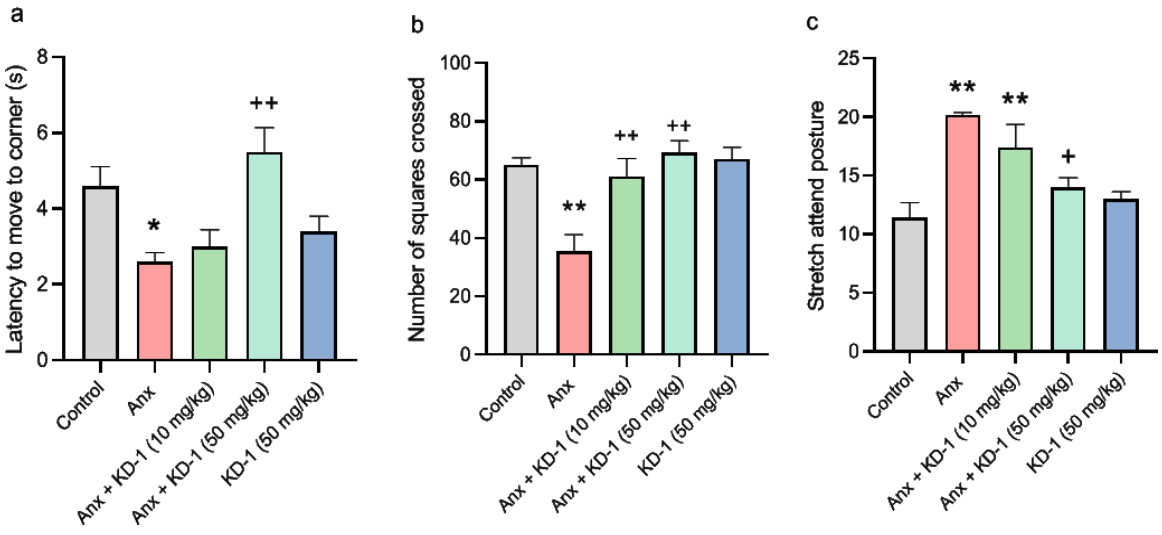


Fig. 6:Influences of KD1 treatment on the activity of the OFT for the anxiety rat model. Values show average ± SEM. Substantial outcomes were observed through one-way ANOVA. *P<0.05, **P<0.01 in comparison to control; +P<0.05, ++P<0.01 related to Anx group.

The OFT thus revealed the complicated influences of treatments for several behavioral conditions. Changes in latency to travel to the corner, number of crossed squares, and elasticity attend posture assist as pointers for anxiety-like performance and investigative tendencies. The anxiolytic capability of KD1, mainly observed at greater amounts, promises therapeutic usefulness to address anxiety-related symptoms.

SIT

The SIT was used to evaluate the social performance of rats based on various exposures. The data revealed noteworthy outcomes of treatment on 2 vital parameters: interaction time [$F_{(4,19)}$ =25.43; P<0.01] and interaction number [$F_{(4,19)}$ = 11.79; P<0.01]. Successive post-hoc studies delivered profounder understandings of the perceived effects. Extraordinarily, rats exposed to foot-shock only showed a considerable reduction in time and number in comparison to control animals (P<0.01). This result specified that treatment with foot-shock caused diminished social conduct, demonstrating the influence of exterior stressors upon the rats' social contacts. Yet the action of KD1 via a 50 mg/kg dosage presented fascinating observations. This usage showed the ability to reduce the opposing results of foot- foot-foot-foot-shock-based impaired social performance (P<0.05) in comparison to the Anx group. This suggests that KD1 at such a dose may respond the contrary effect of strain on social communications to such a level. Though it's notable that while the treatment via 50 mg/kg KD1 revealed promise to enhance social performance, the decreased time of social communication was not completely reinstated (P<0.01) in comparison to control animals, which specifies that although KD1 exhibited capacity in alleviating the influence of foot-shock-induced social performance impairment, whole recovery to standard altitudes was not attained. Jointly, the SIT effects emphasize the complicated affiliation between cures and social performance in rats. The perceived changes in time, as well as several connections, served as pointers to rats' social sensitivity. Foot-shock-induced stress ominously troubled social communications, whereas the interference of KD1 with 50 mg/kg slightly lessened these effects, though not entirely reinstating usual social behavior, as shown in Fig. 7.

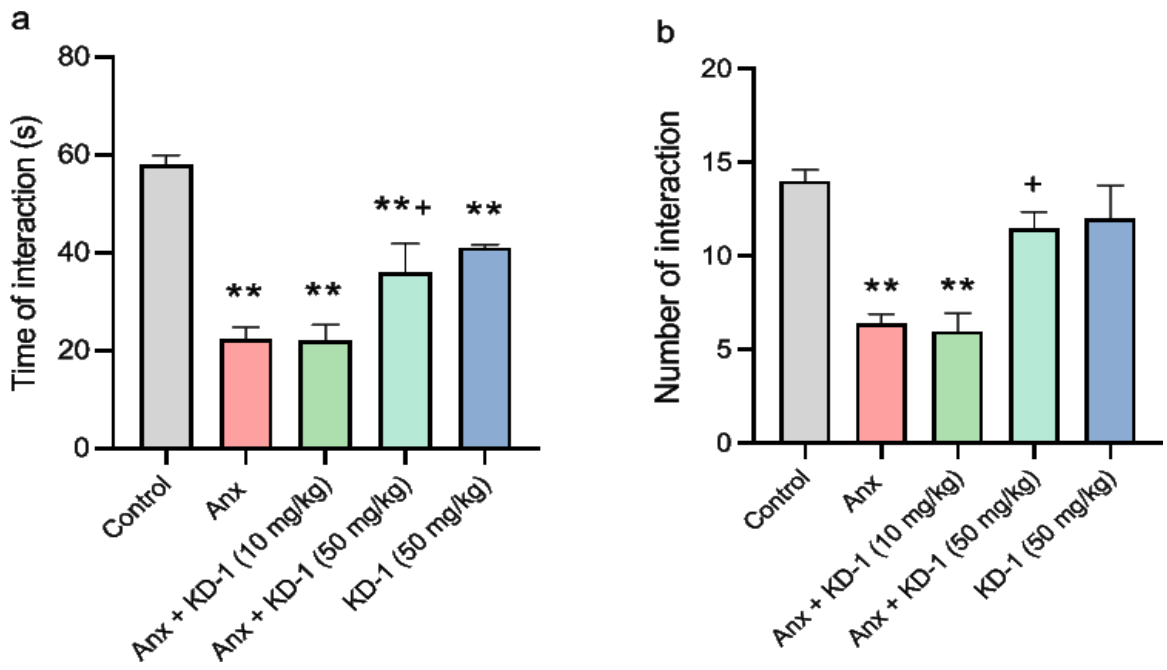


Fig. 7: Influences of KD1 on social interaction check regarding the rat model of anxiety. Values show average ± SEM. Noteworthy outcomes were noticed via one-way ANOVA. *P<0.05, **P<0.01 as compared to control group; +P<0.05, ++P<0.01 as compared to Anx group.

We can say that SIT delivered cherished perceptions of the influence of usage on the social performance of rats. The data emphasized the ability of KD1 with a 50 mg/kg dosage to neutralize the adverse effects of foot-shock on social communications. On the other hand, the findings confirm that more effort may be needed to fully recover from the reduced social performance caused by stressors. The intricacies of social performance regulation and its possible pharmaceutical mediations are thus more understood thanks to this study.

Anxiolytic influence of KL1 regarding the rat model of anxiety

EPM

Anxiolytic activity of KL1 was assessed using EPM data. Based on one-way ANOVA treatment, the study revealed noteworthy effects of usage on 2 essential settings: latency to go in the closed arm [$F_{(4,20)}$ =4.32; P<0.05] and duration passed in the open arm [$F_{(4,20)}$ = 9.26; P<0.01]. Upon additional examination via a Bonferroni post-hoc test, different designs appeared. Unambiguously, rats exposed merely to foot-shock demonstrated a notable reduction in both the potentials to move into the closed arm and the duration spent in the open arm (P<0.01) as compared to the control group. This decay in these parameters advocated sensitive anxiety-like behavior, demonstrating the noticeable influence of the stressor. But, substantial exposure occurred through delivery of KL1 with a quantity of 50 mg/kg in combination with foot-shock exposure. Particularly, this amalgamation resulted in a significant enhancement in latencies to move towards the closed arm and duration passed in the open arm (P<0.05) in the Anx+KD (50 mg/kg) group in comparison to the Anx group. This outcome highlighted KL1's ability to neutralize the anxiety-inducing influences of foot-shock, indicating its anxiolytic capability.

Fig. 8 demonstrates the relationship between usage and anxiety-related behaviors ascertained via the EPM. The parameters of latency to travel towards the closed arm and time passed in the open arm assist as measures of rats' anxiety intensities. The unambiguous difference between the foot-shock-only group and the Anx + KD (50 mg/kg) group highlights KL1's capability in upgrading anxiety-like behavior.

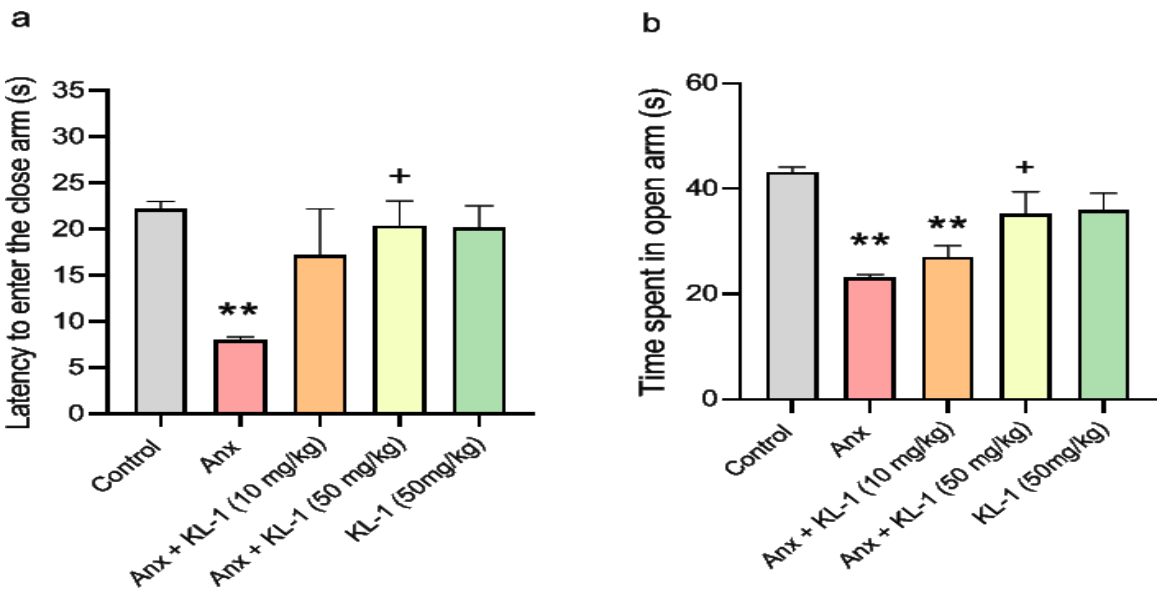


Fig. 8: Influence of KL1 action on activity of the EPM in the rat anxiety model. Values show average ± SEM. Noteworthy effects were attained via one-way ANOVA. **P<0.01 in comparison to control group; +P<0.05 in comparison to Anx group.

We conclude that EPM results provide a treasured preview regarding the sedative impacts of KL1. The noteworthy amendments in settings of latency to move in the closed arm, with duration spent in the open arm, deliver pure signs of anxiety-like performance. The positive effect of KL1, which was mostly confirmed by the Anx+KD (50 mg/kg) group, indicates that it is a highly effective treatment for anxiety-related symptoms brought on by stress. Our knowledge of anxiety-based modulation and possible pharmaceutical involvement to characterize such circumstances is expanded by this work.

OFT

The OFT assisted as a significant stage to evaluate the behavioral consequences of altered treatments. A careful assessment of OFT statistics, via one-way ANOVA test, explicated noteworthy outcomes of dosage on 3 primary settings: latency to travel towards corner [$F_{(4,20)}$ =23.31; P<0.01], number of crossed squares [$F_{(4,20)}$ = 5.40; P<0.01], along with stretch joined posture [$F_{(4,20)}$ = 9.72; P<0.01].

A more comprehensive review of the post-hoc evaluation revealed intriguing findings. Importantly, rats in the Anx group displayed a considerable reduction in the ability of moving towards the corner (P<0.01) and the number of crossed squares (P<0.05) in comparison to control animals. Moreover, this group showed a sharp affinity regarding stretch attend postures (P<0.01), demonstrating an enhanced state of attention, significantly revealing intensified anxiety-like behavior. Though the incorporation of KL1 usage, given with doses of 10 mg/kg and 50 mg/kg, produced captivating outcomes. Particularly, both KL1 doses managed to progress in latency time (P<0.01) as well as the number of crossed squares (P<0.05, P<0.01) after comparing with the Anx group. This specified that KL1 has a positive effect on improving exploratory performance and decreasing anxiety-like affinities.

Additionally, KL1 with a dose of 50 mg/kg showed a further impact: it ominously reduced the occurrences of stretch attend postures (P<0.05) in comparison to rats exposed only to foot-shock. This scrutiny proposes that KL1 at such a dose is subsidized with a reduction of sensitive vigilance, further highlighting its probable anxiolytic characteristics.

Fig. 9 provides a complete comprehension of the interaction between treatments and behavioral reactions in the open field framework. The factors of latency to travel towards the corner, the number of crossed squares, and stretch-based postures assist as valued measures for the animals' anxiety intensities and exploratory affinities.

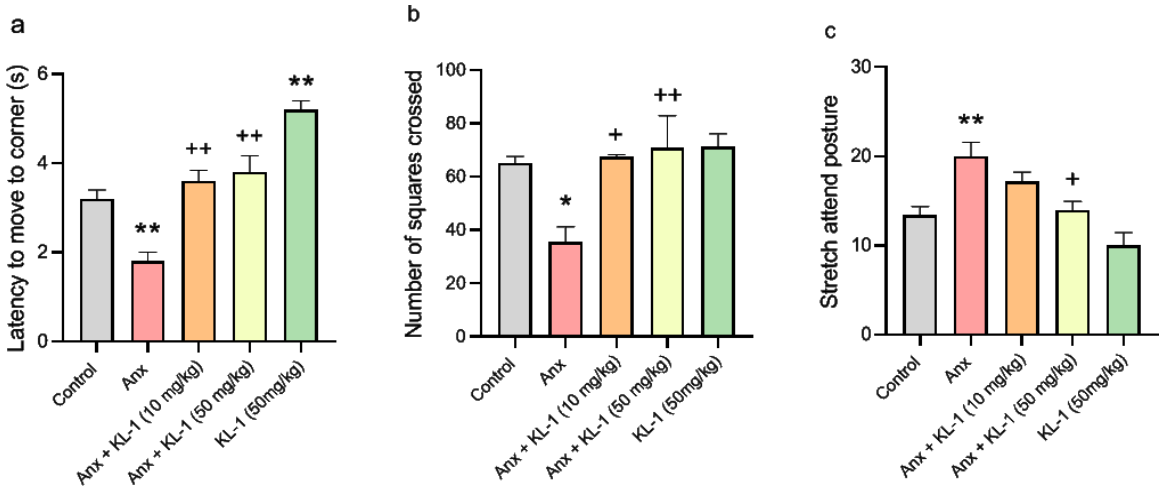


Fig. 9: Influence of KL1 usage on the function of OFT in the rat anxiety model. Values show average ± SEM. Substantial outcomes were acquired via one-way ANOVA. *P<0.05, **P<0.01 in comparison to control group; +P<0.05, ++P<0.01 in comparison to Anx group.

So, OFT data suggest significant potential of KL1. The perceived changes in behavior deliver valued evidence concerning anxiety-like affinities and exploratory performance. The positive influence of KL1, obvious by enhancements of latency time, number of crossed squares, and decreased stretch attend postures, underlines its probable therapeutic usefulness for relieving anxiety-related behaviors, predominantly in the case of stressors. This research work advances our knowledge of anxiety intonation and the important role that pharmaceutical therapies have in changing likely behaviors.

Social Interaction Test (SIT)

Social performance, assessed in relation to time [$F(4,20) = 19.26$; $P<0.01$] and number [$F(4,20) = 7.66$; $P<0.01$] of communications, was ominously prejudiced through administered usages in rats. Strangely, the Anx group established a considerable reduction in the time as well as a number of communications in comparison to control animals ($P<0.01$), emphasizing the intense influence of enhanced anxiety-like behavior on social engagement. Administering KL1 in an amount of 50 mg/kg produced significant enhancements. This action significantly increased time ($P<0.05$) and the number ($P<0.01$) of communications in comparison to the Anx group. This showed the capability of KL1 in alleviating the undesirable outcomes of intensified anxiety on social behavior, offering it as a promising candidate. But, it is notable that the contact period in the Anx+KL1 (50 mg/kg) group persisted ominously reduced ($P<0.05$) compared to that of the control animals, despite KL1 intrusion. This advises that although KL1 demonstrated an affirmative impact on social behavior, thorough rebuilding of social communication to standard points was not achievable.

On the other hand, treatment via KL1 with a dose of 10 mg/kg could not show substantial results compared to the Anx group. This scrutiny highlights the probable dose-dependent aspects of KL1 on social performance in case of anxiety-like trends. The respective results displayed in Fig. 10 deliver inclusive insights into complicated dynamics between usages and social performance. The time and interaction number are key measures of rats' social responsiveness, demonstrating their anxiety-induced changes.

This study emphasizes the potential of KL1 to rectify anxiety-induced variations in social behavior. Considerable fluctuations in time, together with various interactions, emphasize its influence on social awareness. Although full return to baseline levels was not attained, KL1 administered at 50 mg/kg may alleviate the adverse influences of heightened anxiety on social behavior. The outcomes enrich our comprehension of anxiety management and outstanding pharmaceutical therapies in recognizing anxiety-induced fluctuations in social commitment.

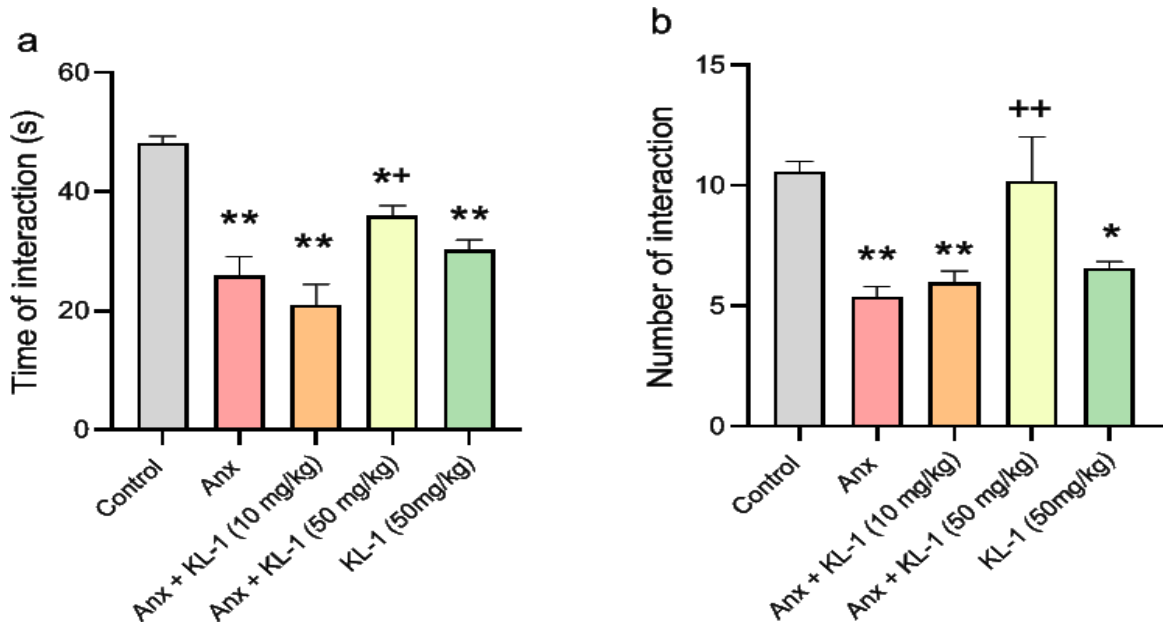


Fig. 10: Influence of KL1 usage on social communication test for rat model of anxiety. Values show average ± SEM. Substantial findings were acquired via one-way ANOVA. *P<0.05, **P<0.01 when compared to control group; +P<0.05, ++P<0.01 when compared to Anx group.

Comparison between KD1 and KL1

Here, inclusive valuations of anxiety-like indications were studied through behavioral tests comprising EPM, OFT, and SIT. The findings revealed substantial introduction of anxiogenic impacts regarding rats subjected to only electric shock. Such anxiety model rats showed decreased investigation of the open arm in EPM and the open field in the OFT, in addition to shrank social communication with new cage-mates for SIT [24]. Though a notable reversal was perceived after the delivery of KT-NSMs, especially KD1 and KL1. Both cures showed a considerable reduction of detected symptoms. The diminished exploration affinities witnessed in EPM and OFT were remarkably improved, and social communication trends in SIT were enhanced. This finding implies that KD1 and KL1 are effective in reducing stress-related anxiety-like behaviors.

The findings collectively highlight the promising potential of KD1 and KL1 nanoformulations in the treatment of anxiety disorders. These nanoformulations present significant therapeutic potential by effectively diminishing perceived anxiety-related symptoms. Their ability to tackle various anxiety-related symptoms is confirmed by the consistent positive outcomes observed across numerous behavioral metrics. The findings of behavioral assessments offer persuasive proof of the efficacy of KD1 and KL1 nanoformulations in alleviating anxiety-like symptoms generated by stresses. This initiative facilitates further research and advancement in this significant therapeutic domain by offering valuable insights into potential pharmaceutical treatments for anxiety disorders.

Storage Stability

Storage stability (SS) is significant in authenticating the durability of the drug trap inside niosomal formulations. The principal goal of such examination was to determine any possible leakage or weakening of the captured drug for the prescribed storage period. SS studies were precisely conducted for both KD1 as well as KL1 nanoformulations, covering a period of 30 days. The analysis regarding the release of the drug was attained through UV-Vis spectrophotometry at 269 nm, and data are listed in Table 2.

Table-2: % Drug retaining for KD1 and KL1.

Time interval Day	Drug retention (%) KD1	Drug retention (%) KL1
1st	96.4 ± 1.28	97.1 ± 1.08
10th	94.5 ± 1.056	95.3 ± 1.62
20th	91.0 ± 2.01	93.9 ± 1.02
30th	88.04 ± 0.99	89.94 ± 1.11

The principal rule of SS examinations centers on judging the capability of the NSMs for actively retaining the truthfulness of loaded drugs during the required storage timeframe. In case of KD1 nanoformulation, the results revealed an order of

retention percentages determined to be 96.4 ± 1.28%, 94.5 ± 1.056%, 91.0 ± 2.01%, and 88.04 ± 0.99% for the 1st, 10th, 12th, and 13th day of the period, respectively. In case of KL1 nanoformulation, the results interpreted an order of retention percentages as 97.1 ± 1.08, 95.3 ± 1.62, 93.9 ± 1.02, and 89.94 ± 1.11 for the 1st, 10th, 12th, and 13th day, respectively.

The results of the present research underscore the remarkable potential of KD1 and KL1 niosomal formulations for the effective preservation of medication stability over a prolong period. Following a 30-day storage period at 4ºC, these formulations demonstrated negligible symbols of KT escape from nanocarriers. The outstanding stability profile verifies the formulations' durability against degradation during storage, demonstrating their appropriateness for medicinal uses necessitating prolonged storage periods. The analyzed % coefficient of variation, a statistical measure of data stability, remained within a permissible range. This statistical evaluation guarantees the validity and reliability of the outcomes acquired.

In other words, the SS study assists in crucial authentication of continuous drug loading inside niosomal formulations during storage. The findings offered through both KD1 and KL1 preparations highlight their creditable stability, proving their ability to advocate a substantial percentage of encapsulated drug for a 30-day storage intermission. Here, in the first formulation, a non-ionic surfactant (D1) was used as a carrier for KT. A certain type of surfactant has been shown to demonstrate enhanced colloidal stability by lowering interfacial tension. This impedes particle aggregation and improves encapsulation stability. Furthermore, these surfactants increase membrane permeability and bioavailability of the entrapped drug [25]. In the second formulation, lignin was employed as a carrier for KT. The polyphenolic structure of lignin provides significant hydrophobic interactions and hydrogen bonding. These can safeguard the active ingredient against premature breakdown and facilitate regulated release. It has also been found that lignin demonstrates antioxidant and anti-inflammatory features that could support neuroprotection and behavioral effects similar to those of anxiolytics[26]. These attributes of D1 and lignin make them ideal candidates for drug delivery applications.

Current project not only supports the arena of drug delivery and consistency valuation but is equally favorable for the prolonged storage and operational use of such nanoformulations in beneficial solicitations.

Conclusion

Conclusively we synthesized a non-ionic Surfactant (**D1**) and characterized it via NMR and mass spectrometry. Two new formulations, KD1 and KL1, were then prepared from D1 and lignin working as carrier for KT, respectively. The prepared formulations were extensively characterized via several techniques including Zeta potential, DLS, and FTIR spectroscopy. KD1 demonstrated enhanced drug encapsulation efficiency of 75.0%, however KL1 attained even greater EE of 80.1%. These results suggest the capability of the formulations regarding their drug delivery. To evaluate their therapeutic efficacy, behavioral studies like OFT, EPM, and SIT were carried out using rats to investigate anxiety-like signs induced by electric shock experience. The findings demonstrated that rats used openly to experience electric shock alone displayed significant anxiety-like behaviors, which include decreased assessment in the open-arm and open arena tool, and reduced social contact with newly coming cage-mates. Treatment with nanoformulations KD1 and KL1 efficiently decreased these anxiety indicators. This study specifies the encouraging potential of KD1 and KL1 nanoformulations for the treatment of anxiety ailments.

Ethical Approval

All animals were procured from the animal housing facility of the University of Karachi, Karachi, Pakistan, and adhered to the NIH pointers and were conducted with approval from the institutional Ethics and Animal Care Committee (Approval No. IBC KU-340/2023).

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