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Development of Morin loaded nanocarrier for the management of Alzheimer's disease

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Abstract

Background: Morin is a bioactive polyphenol derived from plants. However, its inadequate biopharmaceutical properties impede its therapeutic effectiveness. Hence this study focused on developing a nanoparticle (NP) formulation of morin to improve its oral bioavailability and bioactivity.

Materials and methods: MoR was encapsulated in lipid-cored poly(lactide-co-glycolide) (PLGA) nanoparticles by using the emulsification-solvent evaporation process. The formulated NP was subjected to physiochemical characterization, TEM analysis, in-vitro drug release, and in-vivo behavioral study.

Results: The formulated nanoparticles (NPs) had a loading efficiency of 80%, a size of around 211 nm, and an essentially neutral surface charge. In 24 hours of duration, the NPs showed a continuous release of MoR. When oral administration of either free or MoR-PLGA NPs was used, a pharmacokinetic analysis of the plasma and brain accumulation revealed that, while the initial MoRconcentrations were identical in both situations, the long-term (5-24 hours) concentrations were about 5 times greater with MoR-PLGA NPs. In-vivo analysis showed that MoR-PLGA NPssignificantly enhanced memory and spatial learning.

Conclusion: The study's findings provide a potentially effective NP formulation for MoR that may improve its oral bioavailability and bioactivity in the context of treating Alzheimer's disease.

Keywords: Alzheimer's disease, Bioavailability, Bioactive, Morin, Nanoparticle

1. Introduction

Disorders of the central nervous system impact around 1.5 billion people worldwide [1]. Because of the gradual death of central nervous system neurons, neurodegenerative illnesses result in persistent impairment of motor, sensory, behavioural, and cognitive functioning [2]. The majority of types are linked to advanced age and are probably caused by neuroinflammation, misfolded protein aggregations, and oxidative stress. The primary factor behind the development of neurodegenerative diseases is neuroinflammation, which is characterised by the glial cells' altered morphology, the invasion of leukocytes that cause significant tissue damage, and the breaching of the Blood-Brain Barrier (BBB). The inflammatory cascade is started by lymphocytes and myeloid cells expressing more cytokines. Then, ROS, cytokines, and secondary messengers (prostaglandins and nitric oxide) mediate it. Neuronal damage and death are caused by the overproduction of the inflammatory mediators mentioned above [3].

The current therapeutic approaches for Alzheimer's disease (AD) can be divided into different strategies based on their mechanisms. These strategies include targeting tau (therapies focused on tau post-translational modifications, using tau stabilisers and aggregation inhibitors, and anti-tau immunotherapy), targeting amyloid (stimulating Aβ clearance, suppressing Aβ production, and preventing Aβ aggregation), using neuroprotective therapies (such as therapies focused on oxidative stress and neuroinflammation, neurotrophins and their receptor-based therapies), andtargeting apolipoprotein-E (ApoE) function. Furthermore, alternative methods in the treatment of AD include cognitive enhancers that target symptoms, as well as therapies and treatments aimed at preventing AD (both secondary prevention interventions and primary prevention) [4]. AD preventive studies have investigated lifestyle adjustments and risk factor management, which include non-pharmacological therapies. In addition, the effectiveness of phytochemicals in treating neurodegenerative disorders such as AD and Parkinson's disease (PD) has been extensively studied. Several research have examined the potential effectiveness of phytochemicals in treating the most prevalent neurodegenerative disorders, such as AD and PD.

Plant polyphenols are a heterogeneous set of chemicals with varying chemical structures that are present in several herbs, fruits, and vegetables. These chemicals have attracted significant attention over the years due to their remarkable characteristics in combating cancer, reducing inflammation, treating diabetes, protecting the heart, and modulating the immune system [5,6] [7,8]. Morin (MoR), is a significant flavonoid that has been extracted from the Moraceae family. It has high solubility in organic solvents but has lower solubility in water. Its oral bioavailability is less than 1% [9]. Poorly water-soluble medications have limitations in their absorption due to their low dissolution rate, which in turn affects their capacity to be absorbed into the bloodstream when taken orally. Although MR has low solubility, it has several health advantages including antioxidant, anti-inflammatory, anticancer, free radical scavenging, antidiabetic, antibacterial, and neuroprotective properties [10-14].

Currently, Nanomeds are being used in clinical practice to enhance standard treatment for several illnesses, including allergies, cancer, and cardiovascular problems. Multiple clinical investigations have been conducted on the utilisation of gold, liposomal, and polymeric nanoparticles [15-17]. Furthermore, oncologists are now using a few of items that are based on nanotechnology. Nanomedicines transport and administer drugs to specific locations,

ensuring controlled release and protection against enzymatic degradation. They enhance drug availability, and offer valuable insights at the cellular levels for tailoring personalised therapeutic approaches in different diseases [18]. As previously stated, morin provides hopeful, secure, and cost-effective preventative measures for neurodegenerative disorders, namely Alzheimer's disease (AD), due to its effects on several molecular elements of these conditions [19].

We created a morin loaded polylactic acid (MoR-PLGA) nanoparticle system with the purpose of determining whether or not the nanoparticles are suitable for use as delivery vehicles for hydrophilic medicines. Additionally, we investigated the release kinetics of the nanoparticles in vitro. An in-vivo investigation was conducted to assess the biological activity and neuroprotective impact of encapsulated MoR.

2. Materials and methods

PLGA and MoR (purity 95%) were acquired from Sigma-Aldrich. All analytical grade solvents and chemicals were used. Throughout the investigation, deionized and double-distilled water was used.

2.1 Preparation of MoR loaded PLGA nanoparticles

PLGA nanoparticles loaded with drug (MoR) were created using a significantly altered emulsification-solvent evaporation method [20]. In summary, 3 ml of DCM was used to dissolve the medication 2.5 and 5 mg as well as 100 mg of PLGA while being constantly stirred. This combination was added to a 20 ml solution of 12 mM sodium deoxycholate, and using a microtip probe sonicator D-12207, the mixture was probe sonicated for 10 minutesat 20% power. Once the emulsion was made, it was slowly stirred in a fume hood at room temperature until all of the organic phase was evaporated. After centrifuging the NPs for 10 minutes at 12,000 rpm to remove excess emulsifier and release free drug, they were twice cleaned with new water. A fine powder of the MoR-PLGA NPs was obtained by freeze-drying the suspension of nanoparticles. Following centrifugation, a spectrophotometer with a wavelength of 520 nm was used to measure the amount of morin in the supernatant. The PLGA nanoparticles' drug encapsulation effectiveness was tested in accordance with earlier findings [21]. The single emulsion approach was used to create drug-free nanoparticles utilising the same methodology. Rhodamine-123 (0.5 mg/ml) was added to the inner aqueous phase for the creation of fluorescence-labeled NPs, and NPs were made via a single emulsion–solvent evaporation technique.

2.2 Physicochemical Characterization of MoR-PLGA NP

Zetasizer Nano ZS was used in the dynamic light scattering method to estimate the Zeta potential, particle size, and Polydispersity index. Prior to measurement, the isolated NPs were reconstituted in 15 millilitres of deionized water and sonicated for ten minutes. Every sample was measured intriplicate, and the mean ± SD was used to reflect the findings.

2.3 Determination of Encapsulation efficiency(EE)

The percentage of EE for the formulated NPs was determined by separating the NPs from the solution by centrifugation at high speed and low temperature. The obtained supernatant was collected, filtered through a 0.45 μmMillipore membrane filter, and then used to determine

the concentration of un-entrapped MoR using a UV spectrophotometer at a wavelength of 342 nm. The EE was determined using the below equation:

$$EE\% = \frac{\text{total drug} - \text{free drug}}{\text{total drug}} * 100$$

2.4 Transmission electron microscopy (TEM) imaging

The morphology of the NPs was determined by visualising them using TEM. A volume of 10µL of newly prepared NPs was applied onto the grids of TEM and allowed to sit for a duration of 1 minute. The surplus liquid was absorbed using filter paper, and then the grids were captured using the same method as previously explained [22].

2.5 In vitro release of MoRfrom MoR-PLGA NPs

MoR release from MoR-PLGA NPs was assessed in two different environments: 0.1 N HCl, which replicated the stomach environment, and PBS pH 7.4, which mimicked physiological settings. Fresh MoR NPs were aliquoted one ml in a dialysis membrane (12–14 kDa MWCO) in triplicate, and to maintain sink conditions, the membrane was submerged in 30 mL of either 0.1 N PBS or HCl mixed with 0.5% w/v Tween 80 [23]. For comparison, free MoR (1 mL, 0.5 mg/mL in DMSO) was also examined. An orbital shaking incubator (100 rpm, 37 °C) was used to hold the samples. At particular times, samples were taken out and replaced with an equal amount of new release medium until the cycle was complete. Plotting the release profile versus time (h) included calculating the percentage ratio between the total drug in the NPs and the cumulative drug released at different time interval. The drug release was measured using UV-Vis.

2.6 Stability studies

The stability of MoR-loaded PLGA NPs was assessed using zeta-potential, mean particle size, PDI, and drug leakage. To be more precise, formulations were put in glass vials right away after preparation and kept at 4 °C for five days. As previously mentioned, aliquots of the samples were obtained at predetermined intervals, and the mean particle size, entrapment efficiency, and zeta-potential were examined.

2.7 In-vivo study

2.7.1 Experimental Animals

The present research involved a total of 24 adult male Swiss mice, aged 8 weeks and weighing between 20-25 grammes. These mice were chosen for their superior spatial learning abilities [24]. Upon their arrival, the subjects were placed in conventional metal enclosures at a relative humidity of 65% and a temperature of 21 ± 1°C, with 10 hours of light cycle followed by 14 hours of darkness. Furthermore, the animals were allowed unrestricted access to regular chow and water. Additionally, a period of 2 weeks was allotted for the subjects to acclimatise to their surroundings before the commencement of the experiment. The animals were housed in the animal facility of the .... Approval from the ethics committee was obtained and the animals were handled in accordance with ethical principles. Efforts have also been taken to reduce animal distress in line with the globally recognised guidelines for the use and treatment of animals in laboratories, as outlined in the European Community's

EEC Directive of 1986; 86/609/EEC. The MoR treatments were administered daily by oral ingestion in the drinking water at a dosage of 40 mg/kg.

2.7.2 Experimental design

The AD model was induced using stereotaxic surgery, which included the intracerebroventricular (i.c.v.) injection of streptozotocin (STZ). In the beginning, the animals were put under inhalational anaesthesia containing 2% isoflurane by the application of continuous flow anaesthesia equipment. Following the administration of anaesthesia, the subjects were positioned in the stereotaxic apparatus. The scalp was then numbed using a solution containing phenylephrine (2 mg kg−1) and 2% lidocaine hydrochloride by intradermal injection. Next, a single-sided cut was performed to expose the skull, followed by the creation of two tiny holes on both sides of the brain's chambers using a slow-speed drill. The holes were produced at the following positions: 0.1 mm front to back, 1 mm to the side, and 3 mm top to bottom. Ultimately, i.c.v. injection of STZ (3 mg kg^{-1}) was carried out using a 10 µL Hamilton micro syringe. Following surgery, animals were monitored until they fully regained their health.

The animals were categorised into four distinct groups consisting of 6 mice each: The control group consisted of healthy subjects. Group 2 consisted of AD induced untreated mice. In Group 3, the AD-induced mice were administered free MoR at a dosage of 40 mg per kg of body weight. In Group 4, the AD-induced mice were administered MoR/PLGA NPs at the same dosage.

2.7.3 Pharmacokinetic assay

Mice were orally treated with 40 mg/kg of MoR-PLGA NPs and free MoR by oral gavage. In order to assess the pharmacokinetic profile of the NPs, mice were rendered unconscious using isoflurane and then euthanized by puncturing their hearts at various time intervals. Both brain and blood samples were taken in order to quantify the concentration of MoR using LC-MS-MS. The processing of samples was carried out in accordance with the approach that Chen et al. first disclosed, but with a few adjustments [25]. At a temperature of 4 degrees Celsius and a force of 4,000 grammes, blood samples were centrifuged for a period of four minutes. The plasma component was separated, treated with ethyl acetate twice for extraction, and then reconstituted in a solution containing 20% AA and 10% acetonitrile. The brains were measured in weight, then crushed in a solution containing 0.5 mg/ml Na2EDTA, 20% AA, and PBS 0.4 M. The mixture was then spun at 4 °Cat a speed of 16,000 g for 10 minutes. Subsequently, the supernatant was removed using the same procedure used for the plasma samples. The measurements were done on three separate occasions.

2.7.4 Behavioral Test

- **Morris water maze (MWM) test**

The MWM test was carried out in a circular tank with a platform and filled with opaque white water. The platform remained stationary during the whole experiment and was positioned 1 cm under the water. The light intensity and temperature were maintained at a constant level of 25 ± 2 °C and 30 lx, respectively, for the whole duration. The protocol consisted of six training sessions, with each session lasting for one minute each trial, followed by one test session. During the test, the platform was taken away and the micewere

put in the tank in the quadrant that is opposite to the previous location of the platform for a duration of 1 minute. The SMART V3.0 video tracking system was used to capture and evaluate the behavioural data [26].

- **Novel object recognition (NOR)**

The NOR test was employedto measure the cognitive impairment. During the first habituation phase (days 1-3), mice were introduced to an empty open area to get used to their surroundings. On the fourth day, two similar items were positioned at equal distances from each other, and the duration of investigation for each object was evaluated. Throughout the test, the same procedure was replicated by substituting one of the familiar things with a new one. Each trial lasted for a length of 10 minutes. The test results were measured as below given equation:

$$\text{Exploration time } (\%) = \frac{\text{Exploration time of new object (s)}}{\text{Total exploration time (s)}} \cdot 100$$

2.8 Statistical analysis

"SPSS 26.0" was used to perform all statistical analyses.The results were shown as the mean±S.D. of three separate tests. The data was analysed using one-way ANOVA for comparisons between multiple groups. When the p-value <0.05, it was deemed to be statistically significant.

3. Results and discussion

The emulsification-solvent evaporation approach was used to encapsulate MoR in PLGA NPs. This technique was chosen for its ease and simplicity. The conventional approach results in the creation of nanoparticles, in which the medication is confined inside a uniform polymer matrix, as the organic phase is replaced by the aqueous phase. Prior research has shown that this method enhances the drug loading capacity of polymeric nanoparticles in comparison to the traditional matrix-type nanoparticles [27-29].

3.1 Physicochemical characterization of synthesised MoR NPs

The NPs were mainly characterised based on their polydispersity, particle size, and EE%. According to the findings shown in Table 1, the addition of MoR at a concentration of 2.5 mg (which corresponds to 10% of the weight of the polymer) resulted in F1 achieving a high EE of 80%. Nevertheless, in F2 (5 mg drug) the efficiency of EE dropped to 54%, indicating that the nanoparticles had reached their maximum capacity for loading. The two NP formulations exhibited similarities in terms of PDI, particle size, and zeta potential. The size of F2 NPs was around 270 nm and it exhibited more wider distribution, while F1 were somewhat smaller, measuring around 211 nm. The PDI of both formulations was around 0.33, which suggested a modest degree of polydispersity. The zeta potential assessed the colloidal stability of MoR NPs by measuring the surface charge. It plays a crucial role in their interaction with the cell membrane, which often has a negative charge. The zeta potential of MoR NPs exhibited a nearly neutral value, which may be attributed to the presence of nonionic surfactants (Tween 80 and Span 80) in close proximity to the NP surface. These surfactants are known to enhance the colloidal stability of the NPs, consistent with findings

from earlier studies [30]. After demonstrating a higher EE%, F1 NPs were chosen for further studies.

Table 1. Physicochemical characteristics of formulated NPs

Formulations	PDI	Particle size	EE%	Zeta potential
F1	0.36±0.01	211±51	80±7	-1.3±0.9
F2	0.30±0.20	270±99	54±3	-0.8±1.4

3.2 TEM results

The transmission electron microscopy (TEM) picture of MoR nanoparticles (F1) is shown in Figure 1. The nanoparticles are seen as black spheres with a lighter rim, which is indicative of their core/shell structure.

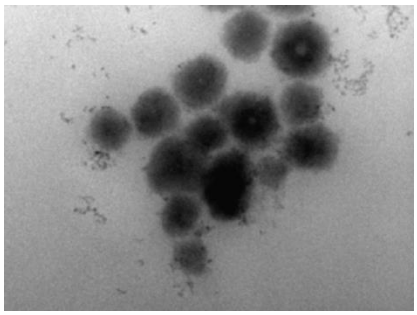


Figure 1. TEM image of formulated MoR-PLGA NP

3.3 In-vitro release of drug

The drug release was investigated in 0.1 N HCl and in PBS (pH 7.4) for a duration of 24 hoursat 37 °C. At different time intervals, samples were collected to evaluate the total quantity of MoR released throughout time (Fig. 2). The release of Free MoR from the dialysis membrane occurred rapidly with 100% at 24 hours, irrespective of the pH of the release media. In contrast, the drug release pattern of MoR NPs in both mediums exhibited two distinct phases, characterised by a gradual and continuous increase in the total amount of medication released during the first 8 hours. The release in PBS was faster as compared to 0.1 N HCl. This percentage increased to 20% and 36% after 4 and 8 hours, respectively. Regarding the release at a pH level that is neither acidic nor basic, around 18% of the MoR was released after 1 hour of being incubated. This percentage subsequently climbed to 58% and 84% after 4 and 8 hours, respectively. At the 24-hour mark, the MoR reached a cumulative release rate of 92%.

The drug release may be due to the diffusion of the drug through the oil core, facilitated by the polymer shell, and the simultaneous erosion of the polymer [31,32]. The results were consistent with prior research, which demonstrated that hydrophobic medicines like thymoquinone and baicalin were encased in nanoparticles with a polymeric shell and an oil-filled core. These pharmaceuticals also showed comparable release kinetics [33].

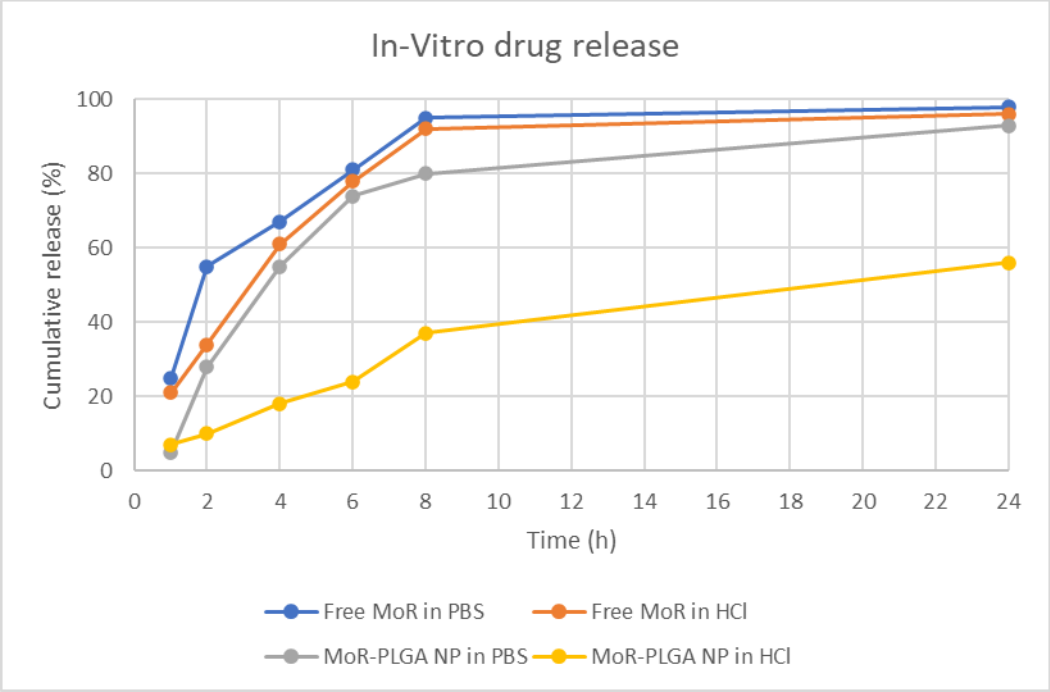


Figure2. In vitro drug release of MoR from NPs in 0.1 N HCl and PBS pH 7.4 for 24 hours.

3.4 Stability testing

It is essential to guarantee that the physicochemical properties of the NPs remain unchanged throughout storage, since any alterations in these characteristics indicate changes in the structure of the NPs [34]. Hence, the physical stability of the MoR-PLGA nanoparticles was regularly assessed over a period of 60 days at a temperature of 4°C. There were no noticeable changes in the assessed physical and chemical characteristics of the formulation over the storage time at 4 °C ($p > 0.05$). The formulations did not exhibit any signs of creaming, flocculation, or phase separation. These data suggest that the nanoparticles maintain their stability for a duration of 2 months.

3.5 In vivo pharmacokinetics

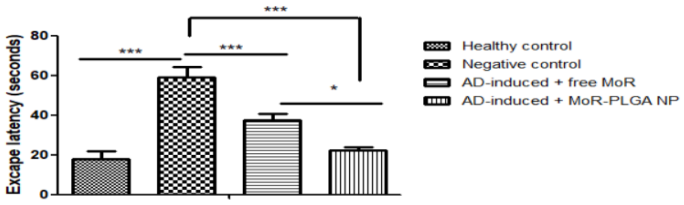
Following oral administration of both free MoR and MoR-PLGA NPs, the pharmacokinetic profiles of MoR were ascertained, as shown in Table 2. After 1 hour, the maximum plasma concentrations of MoR were almost identical in both circumstances (1157.52±140 ng/ml for free MoR vs 854.41±81.3 ng/ml for MoR-PLGA NPs). Following administration of free MoR to mice, there was a significant reduction in plasma MoR concentrationafter 24 h. The analysis of the area under the curve (AUC) of plasma MoR concentrations showed substantial differences. After oral treatment with MoR-PLGA NPs, the AUC was 11980±112.02 h.ng/ml, whereas it was only 5402.95±455 h.ng/ml with free MoR.

The mice's brain had a comparable pharmacokinetic profile. When MoR-PLGA was delivered in the form of NPs, it achieved a maximum concentration of 0.914±0.23 ng/mg, but the concentration was 0.795±0.75 ng/mg when administered in its free form. The concentrations of cerebral MoR remained stable at much greater levels after treatment with MoR-PLGA NPs compared to free MoR.The AUC analysis revealed that the levels of 15.322±1.54 and 6.512±0.75 h.ng/mg were observed after encapsulated and free delivery, respectively. In summary, the findings suggest that nanoparticles (NPs) helped to stabilise the levels of circulating MoR, leading to the accumulation of the medication in the brain.

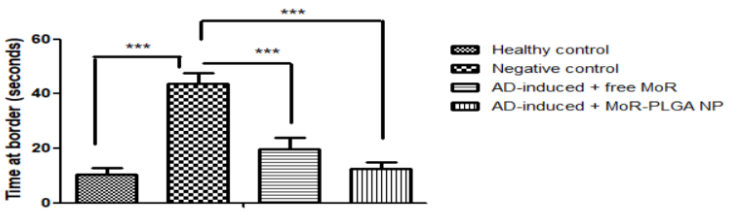
Table 2. Pharmacokinetic profile of free MoR and MoR-PLGA NP in mice				
Parameter	**Brain**		**Plasma**	
	Free MoR	**MoR-PLGA NP**	**MoR-PLGA NP**	**Free MoR**
AUC	6.512±0.75 (h.ng)	15.322±1.54 (h.ng)	11980±112.02 (h.ng/ml)	5402.95±455
Tmax	1.00±0.00	1.00±0.00	1.00±0.00	1.00±0.00
Cmax	0.795±0.75 (ng/n)	0.914±0.23 (ng/n)	854.41±81.3 (ng/ml)	1157.52±140 (ng/ml)
Cl/F	NA	NA	25.78±3.45	220.45±42.10

3.6 Effect of MoR-PLGA NPs on spatial memory and learning process

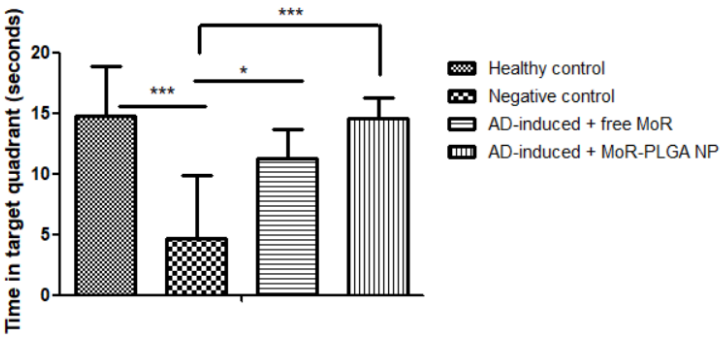
The animal's behaviour was evaluated after being subjected to MoR therapy for a duration of 3 months. The mice underwent a MWM test to evaluate their learning abilities and spatial memory systems [35]. The escape latency time of the mice was substantially greater compared to the control animals. After being treated with either free or nanoparticle-formulated MoR, the mice exhibited a significant reduction in the time it took them to escape during the training phase, as shown in Figure 3A. During the test, animals treated with MoR-PLGA NPs exhibited a considerably shorter time to escape compared to control mice. Administration of MoR treatment led to a significant reduction in the time it took to escape on the test day. However, the duration was remained substantially longer compared to mice treated with MoR-PLGA NPs. The mice also exhibited a considerable reduction in the time spent exploring the target quadrant. The MoR therapy considerably improved exploration time, as seen in Figure 3B. Furthermore, the duration of time spent in the border region was examined as a predictor of the amount of anxiety. The results indicated that both groups that received treatment exhibited a considerable reduction in the time spent exploring the borders, reaching values similar to those of the control group (Figure 3C).



(A)



(B)



(C)

Figure 3. (A) Shows the escape latency of learning process on the test day (B) Shows the time expended at border area (C) Shows the time expended in the target quadrant

3.7 MoR-PLGA NPs improve MoR effect on cognitive process

The NOR test, which utilises the innate inclination of mice to investigate novel items and surroundings, is extensively used for assessing cognitive impairments in Alzheimer's disease, both in the short and long term [36]. The mice were administered with MoR-PLGA NPs or free MoR for a duration of 12 weeks. The NOR test assessment revealed a significant increase in performance for both groups who received treatment. The group treated with MoR-PLGA NPs showed significantly superior outcomes compared to the group treated with the free medication. The NOR scores in the MoR-PLGA NPs group were comparable to those obtained in the control group (Figure 4). Collectively, these findings suggest that MoR-PLGA NPs provide a substantial therapeutic benefit compared to free MoR.

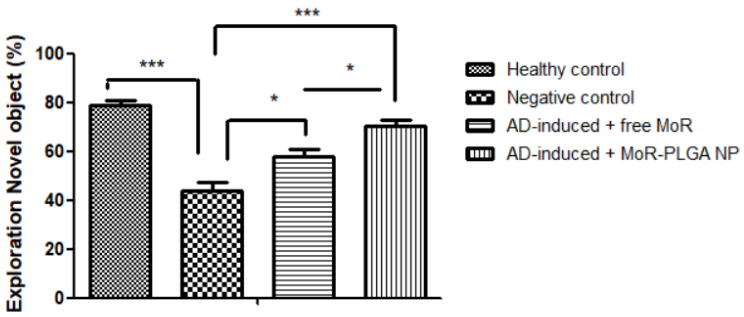


Figure 4. Shows the percentage of exploration time of the novel object in the NOR test.

Conclusion

Overall, this study shows that the stability and efficiency of MoR may be enhanced by loading it into PEGylated PLGA NPs in the presence of an antioxidant environment. The formulated MoR-PLGA NPs had a particle size of 211 nm with an EE of 80%. MoR-PLGA nanoparticles shown the ability to prolong the presence of drugs in the bloodstream and brain tissue, as well as stimulate the formation of synapses and improve memory and learning. According to these data, we suggest using MoR-PLGA NPs as a new, secure, and appropriate therapeutic option for treating AD. In summary, our findings provide a very promising approach using nanotechnology to enhance the effectiveness of MoR and enable its oral delivery.

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