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Exploring the protective effect exhibited by curcumin-loaded coconut oil microemulsion in the experimental models of neurodegeneration: an insight of formulation development, in vitro and in vivo study

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Abstract

Background: Neurodegenerative diseases are a major health concern which requires promising drugs with appropriate drug delivery systems. The aim of the present study was development and characterization of curcumin-loaded coconut oil microemulsion (Cur-ME) and to improve the pharmacokinetic and pharmacodynamics performance. Initially, solubility study and emulsification study were performed for preliminary screening of the components. Pseudoternary phase diagram was constructed using selected components, and composition of Cur-ME was finalized. Furthermore, in vitro drug release in vivo pharmacokinetics and pharmacodynamic was performed.

Results: The final formulation exhibited globule size less than 20 nm with PDI and zeta potential as 0.24 and -17 mV, respectively. The formulation showed more than 90% drug content with no signs of precipitation upon dilution and centrifugation. In vitro drug release revealed 2.12-fold improvement in dissolution. In vivo plasma pharmacokinetics of Cur-ME revealed twofolds and 2.48-fold improvement in AUC and C_{max}, respectively, than that of Cur-Sol. In vivo pharmacokinetics in adult zebrafish revealed significant enhancement ($p < 0.01$) in curcumin delivery to the brain with 1.96-fold and 1.92-fold improvement in C_{max} and AUC, respectively. Furthermore, the pharmacodynamics of the formulation was evaluated using trimethyl tin (TMT)-induced neurodegeneration in wistar rats. The results revealed that Cur-ME treated group significantly decreased the escape latency and pathlength as compared to the neurodegeneration control group. The observed effects were also markedly significant than Cur-Sol treated group. Further, the brain malondialdehyde (MDA) and glutathione (GSH) levels were found to be increased significantly as compared to Cur-Sol treated group.

Conclusion: The encouraging results exhibited by Cur-ME can be regarded as a mark of an effective formulation that can be used in neurodegeneration. Overall, these findings indicate that an orally delivered microemulsion has enormous potential for drug delivery to the brain.

Keywords: Neurodegeneration, Virgin coconut oil, Curcumin, Microemulsion, *Danio rerio*

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Background

Neurodegenerative disorders have become a major public health issue around the world. Parkinson's disease (PD), Alzheimer's disease (AD), frontotemporal dementia, spinocerebellar ataxias, Huntington's disease, and

amyotrophic lateral sclerosis are examples of neurodegenerative diseases [1, 2]. One of the most common neurodegenerative disorders is Alzheimer's disease (AD), caused by genetic and environmental factors [3, 4]. AD is a progressive neurodegenerative disorder which involves multiple mechanisms of disease progression like neuroinflammation, cholinergic depletion, extracellular amyloid plaques and intracellular neurofibrillary tangles. The drugs currently in treatment of AD includes acetylcholinesterase (AChE) inhibitors and memantine, which fail in addressing the disease progression.

The dietary polyphenols present in the turmeric, preferably curcumin, has already been investigated for its wide biological activity against many diseases and disorders including neurodegenerative disorders [5]. It exhibits potent anti-inflammatory and antioxidant activity by modulating specific signaling pathways [6]. Furthermore, many preclinical studies in experimental models of neurodegeneration of curcumin revealed significant neuroprotective action against AD and PD [7]. However, biological action of pure curcumin is limited due to poor solubility and rapid metabolism dependent poor bioavailability [8].

The brain is the most complex and important organ of the body, which regulates physiological processes [9]. The brain is guarded by various protective barriers, such as the blood–brain barrier (BBB) and the cerebrospinal fluid barrier (CSFB), which prevent foreign particles from entering the brain and protect it from mechanical damage, harmful toxins, and infectious agents, while also maintaining brain homeostasis. Environmental changes and illness, as well as age, can induce changes in the structure and physiological function of the brain, which can lead to neurological disorders [10, 11]. The drug need to be delivered to the brain by evading the BBB [9]. As medical science still lacks promising curative medicines to target-specific brain diseases or cross the blood–brain barrier, a new nanotechnology-based approach that help in target-specific drug delivery to the brain is desperately needed [12].

There are number of strategies adapted for effective brain targeting [13]. Nano-sized carriers ranging from 10 to 100 nm improve and facilitate drug transport across the blood–brain barrier because of their smaller particle size and target-specific ligand. The nano-sized particles can easily be absorbed and delivered across the barriers. These nano-formulations include liposomes, solid lipid nanoparticles, microemulsion, dendrimers, polymeric nanoparticles, etc., which are used for target-specific drug delivery [14].

Microemulsion (ME) is a transparent and homogenous mixture which is thermodynamically stable. Major goals of microemulsions are to improve drug solubilization

capacity for hydrophilic and lipophilic drugs, small globule size which can be advantageous to enhance permeability, long shelf life, and improvement in bioavailability [15]. The primary components of microemulsions are oils, surfactants, and co-surfactants which can also act as a carrier and can modulate the drug uptake in brain [14]. This modulation can be attributed to multiple features of components either singly or in synergy which could further help in traversing BBB. Additionally, submicron-sized swollen micelles could bypass reticuloendothelial system (RES) that could help in achieving improved concentration in the brain [16].

Virgin coconut oil (VCO) is an unrefined oil extracted from wet processing of fresh coconut kernel. The bioactive components (polyphenols) and nutrients (vitamin E) exhibit several health benefits through its hypolipidemic, antioxidant, anti-inflammatory, and analgesic properties [17]. A comparative study examining the beneficial effects of VCO over other oils showed that VCO reduced lipogenesis and enhanced fatty acid degradation that could lead to the prevention of oxidative stress because of the polyphenols and tocopherols in the VCO [17]. Also, a recent study showed improvement in memory and learning in CD1 mice upon consumption of VCO rich diet [18]. Furthermore, a pilot study revealed improved cognitive functions in AD patients when treated with coconut oil enriched diet [19].

The main objective of this study was to develop VCO-loaded Cur-ME and evaluate its pharmacokinetic property and pharmacodynamic performance in experimentally induced neurodegeneration. The components for the microemulsion were selected based on solubility and emulsification study; further, the final composition was decided based on microemulsion region in pseudoternary phase diagram. The final formulation was thereafter characterized and evaluated for plasma as well as brain pharmacokinetic and pharmacodynamic performance in suitable laboratory animal.

Materials and methods

Materials

Curcumin was procured as a gift sample from Arjuna Naturals, India. Virgin coconut oil was purchased from Tamil Nadu Test House Pvt. Ltd, Tamil Nadu. Capmul MCM and Captex 300 EP/NF were generous gift samples received from Abitec Corporation, India. Donepezil hydrochloride was purchased from Alkem Laboratories Ltd., India. Kolliphor ELP and RH40 were gifted by BASF India. Ellman's reagent (DTNB) was purchased from Loba Chemie Pvt. Ltd, Mumbai, India. Tween 80, Tween 20, polyethylene glycol 400 (PEG 400) and propylene glycol were purchased from Loba Chemie, India. Acetonitrile, methanol, ethanol and glacial acetic acid were

purchased from Merck, India. All other chemicals were analytical grade.

Determination of saturation solubility

Saturation solubility of curcumin was determined by adding curcumin in surplus amount to the selected oils [Virgin coconut oil (VCO), Capmul MCM (CPM), Captex 300 EP/NF (CPT)], surfactants, and co-surfactants. Samples were taken in Eppendorf tubes. This mixture was vortexed and sonicated for 15 min, respectively. The samples were kept in a water shaker bath (Classic Scientific, India) at 37 °C for 48 h for attaining equilibrium and facilitating the solubilization of curcumin. The samples were centrifuged at 15,000 rpm for 15 min to remove the undissolved curcumin. The supernatant was taken and subsequent dilutions were done. The curcumin content in samples was quantified using UV spectrophotometer (Jasco V-630, Easton USA) at 421 nm as detection wavelength.

Determination of emulsification potential of surfactant

Emulsification study was performed for the selection of suitable surfactant and co-surfactant. The mixtures of oil and each surfactant in the ratio of (1:1) was taken in Eppendorf tubes, and samples were vortexed for 15 min followed by sonication for 10 min. The samples were further allowed to stabilize overnight and observed on next day for phase separation. The samples were further diluted 1000 times in distilled water and percentage transmittance was recorded using UV visible spectrophotometer (Jasco V-630, Easton USA) at 650 nm against distilled water as reference.

Construction of pseudo-ternary phase diagram

Pseudo-ternary phase diagrams were plotted to determine the microemulsion region corresponding to the specific composition of the mixture. Tween 80 was used as a surfactant and PEG 400 was used as a co-surfactant. The mixture of VCO and CPM in the ratio of 1:1 w/w was used as an oil phase. The mixture of surfactant and co-surfactant in three different ratios (1:1, 2:1, 3:1 w/w) was used as Smix. The oil phase and Smix phase was blended in a different ratio ranging from (1:9 to 9:1 v/v). Distilled water was added as aliquots. Change in appearance from clear to turbid was considered as the endpoint. The readings were recorded, and pseudo-ternary phase diagrams were generated using TriDraw software (version 2.6).

Effect of oil and Smix concentration on globule size

The effect of oil and Smix concentration in microemulsion was assessed by changing one factor at a time and keeping other factor constant. The effect of Smix concentration was assessed by keeping oil concentration

constant at 10% and varying the Smix concentration from 60 to 70%. Similarly, the effect of oil concentration was determined by changing oil concentration from 5 to 25% with Smix concentration constant at 70%. Globule size measurement was determined by using a nanoparticle analyzer (Horiba SZ-100 V2 series).

Preparation of curcumin-loaded microemulsion

The Cur-ME was prepared by simple mixing of the components. Briefly, oil (20%) was mixed with Smix (60%) using a vortex mixer. Curcumin was added and dissolved at a concentration of 10 mg/mL considering the final volume of the formulation. Water (20%) was finally added to the previous mixture and mixed for 15 min followed by sonication for 15 min. The final formulation was kept overnight for stabilization.

In vitro characterization

Appearance

The microemulsion was observed for appearance like color, drug precipitation and phase separation.

Determination of globule size, polydispersity index, and zeta potential

Globule size, polydispersity index (PDI), and zeta potential of microemulsion were determined by the dynamic light scattering method. The microemulsion was diluted suitably with distilled water, and abovementioned parameters were determined by using particle size analyzer Horiba nanopartica Scientific (SZ-100 V2 series), India. The study was performed in triplicates ($n=3$).

Determination of pH, dilution potential, and centrifugation

The pH of the microemulsion formulation was assessed by using a digital pH meter (Toshniwal Process Instruments Pvt. Ltd, India). The microemulsion was diluted 100 times with distilled water, vortexed for 5 min and kept for 48 h to determine the dilution potential. After 48 h, the samples were observed for the precipitation of drug and phase separation. The microemulsion was centrifuged at 6000 rpm for 15 min for centrifugation study and observed for phase separation or drug precipitation. All the measurements were done in triplicate ($n=3$).

Drug content by HPLC

The drug content of microemulsion was determined by using the reported RP-HPLC method [20]. The final formulation was suitably diluted with methanol. The samples were further centrifuged at 10,000 rpm for 15 min, and the supernatant was injected into HPLC (Jasco UV-1575, PU1580). The samples were quantified at 421 nm as detection wavelength.

In vitro drug release

In vitro release of curcumin from formulations was determined by using the dialysis bag method [21]. The dialysis bags were activated in phosphate buffer pH 7.4 before 24 h. Curcumin solution (Cur-Sol) and Cur-ME (equivalent to 5 mg curcumin) were transferred to respective dialysis bags in which the other end was tied tightly using a thread. These bags were transferred to dissolution vessels containing 200 mL media maintained at 37 °C at 50 rpm. In vitro drug release study was performed using dissolution apparatus (Electrolab USP II) and was quantified using UV Visible spectrophotometer (Jasco V-630, Easton USA).

In vivo Plasma Pharmacokinetics in Wistar rats

The protocol was approved by Institutional Animal Ethical Committee (IAEC) of Poona college of Pharmacy, Bharati Vidyapeeth (Deemed to be University), Pune, MH, India (IAEC/PCP/PCL29/2020–2021) and in vivo plasma pharmacokinetic study was performed. The animals were fasted overnight before the experiment. The animals were divided into two groups ($n=18$); Cur-Sol and Cur-ME. The Cur-Sol and Cur-ME were administered orally at a dose of 150 mg/kg to the respective groups. The blood samples were collected from the animals at 0.083, 0.25, 0.5, 1, 2, 3, 6, 9, and 12 h, respectively. The blood samples were centrifuged at 3500 rpm for separating the plasma. Plasma was stored at -80 °C until analysis. Plasma was mixed with methanol for precipitating the proteins. Samples were further centrifuged at 10,000 rpm for 15 min, and the supernatant was collected and injected into RP-HPLC (Jasco UV-1575, PU1580). The pharmacokinetic parameters were assessed by using PK Solver, MS-Excel add-in Program [22].

In vivo brain pharmacokinetics in adult zebrafish

The in vivo brain pharmacokinetic study was performed as per the reported method [20]. The protocol for the study was approved (IAEC/PCP/PCL28/2020–2021) by Institutional Animal Ethical Committee (IAEC) of Poona college of Pharmacy, Bharati Vidyapeeth (Deemed to be University), Pune, MH, India, with CPCSEA (1703/PO/Re/S/01). Adult zebra fish (*Danio rerio*) (2.5 ± 0.5 cm length and 250 ± 50 mg weight) were fasted overnight before the experiment. The zebrafish were divided into two groups as Cur-Sol and Cur-ME with 25 fish each. The fishes were partially anesthetized using cold anesthesia during dose administration. The Cur-ME and Cur-Sol were orally administered at a dose of 25 mg/kg using a micropipette by holding them in upright position. The fishes were sacrificed using cold anesthesia at each time points (5, 15, 30, 60, and 120 min). The skull roof was opened by surgical procedure and the brain was

removed and immediately transferred into the Eppendorf tube containing methanol. The extraction of drug from the brain was carried out by the homogenization process. Further, the samples were centrifuged at 10,000 rpm for 15 min. The supernatant was collected and injected into RP-HPLC (Jasco UV-1575, PU1580). The pharmacokinetic parameters were assessed by using PK Solver, MS-Excel add-in Program.

In vivo pharmacodynamics in TMT induced neurodegeneration in rats

The protocol was approved by Institutional Animal Ethical Committee (IAEC) of Poona college of Pharmacy, Bharati Vidyapeeth (Deemed to be University), Pune, MH, India (IAEC/PCP/PCL29/2020–2021). Male wistar rats weighing 200–230 g were used for the study. The animals were divided into five groups ($n=8$) as follows: group 1—control, group 2—neurodegeneration control, group 3—standard (donepezil 1 mg/kg), group 4—Cur-Sol and group 5—Cur-ME. Morris water maze is a maze of 150 cm diameter filled with water at 25 °C till 40 cm. A hidden platform was placed 2 cm below the water level. The animals were trained in the Morris Water Maze for seven days to reach the platform from four different cues. The time taken to reach the platform (escape latency) and distance traveled (path length) were measured. On day 8, all group of animals except control group were injected with of trimethyltin chloride (TMT) at the dose of 7 mg/kg, intraperitoneally (i.p.) for induction of neurodegeneration [23]. Respective treatment was administered from 15th day to 28th day. The Morris water maze test was performed on 0th, 7th, 14th, 21st, and 28th days of the study to evaluate spatial memory. The animals were sacrificed on 28th day and brains isolated for evaluating biochemical parameters and histopathology.

Biochemical parameters

The brain homogenization was done as per the reported method [24] in Tris-HCl buffer (pH=7.4). The brain glutathione (GSH) [25] and malondialdehyde (MDA) [26] were determined as per the reported studies.

Histopathological evaluation

The sample of the brain was isolated and preserved in a collection tube containing neutral buffered formalin. The sample was stained with Hematoxylin and Eosin (H&E) for histopathological study. The changes occurred in the rat brain was compared with the vehicle control group at $10 \times$ magnification.

HPLC method

The HPLC method used in this study was according to earlier reported methods [20]. The Pearless C18

Chromatopak, India, column was used. The mobile phase composition was 2% acetic acid pH 3 and acetonitrile at a ratio of (40:60). The pump was operated at 1.0 mL/min flow rate and the elution was monitored at 425 nm wavelength using a UV detector (Jasco UV 1575). The retention time of curcumin was observed at 9 min. The raw data were assessed by Bowin HPLC software (Jasco Easton, USA).

Statistical analysis

All the experimental data was expressed as mean ± SD. The statistical analyses were carried out with help of GraphPad Prism demo v8 using one-way ANOVA followed by Dunnet’s ‘t’ test and two-way ANOVA followed by Bonferroni’s multiple comparison test.

Results

Solubility study

Maintaining the drug in solubilized form is the most important task which depends on the saturated solubility of the drug in the system components. Curcumin solubility in various components like oils, surfactants, and co-surfactants was determined and is shown in Fig. 1. It was found that curcumin had good solubility in Capmul MCM (78.48 ± 11.87 mg/mL), while virgin coconut oil (17.75 ± 2.40 mg/mL) and Captex 300 EP (54.04 ± 8.96 mg/mL) showed comparatively less solubility. Tween 80 (80.67 ± 9.51 mg/mL) caused the highest curcumin solubility as compared to Tween 20 (31.56 ± 2.37 mg/mL), Kolliphor RH40 (76.01 ± 8.57 mg/mL), and Kolliphor ELP (64.66 ± 5.63 mg/mL). Among

co-surfactant, PEG 400 (288.48 ± 53.37 mg/mL) showed higher curcumin solubility followed by ethanol (131.56 ± 16.16 mg/mL). The component selected for this study were based on the literature of their ability to improve permeation. The components were further selected based on the combined results of solubility and emulsification study.

Emulsification study

An emulsification study was performed to screen the surfactant and co-surfactant based on its ability to emulsify the virgin coconut oil. Higher the emulsification potential lower the globule size. Emulsification studies were performed using surfactants as Tween 20, Tween 80, Kolliphor RH40, Kolliphor ELP, and co-surfactant

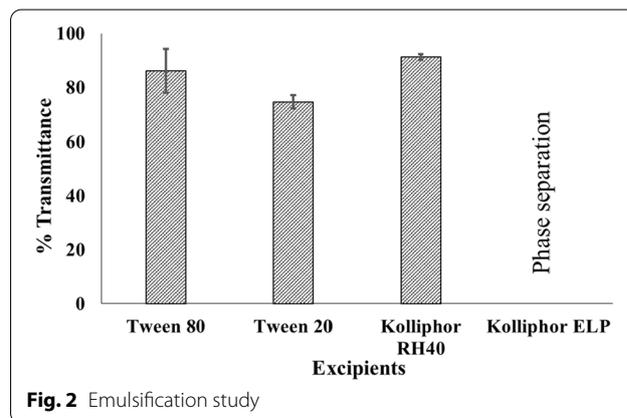


Fig. 2 Emulsification study

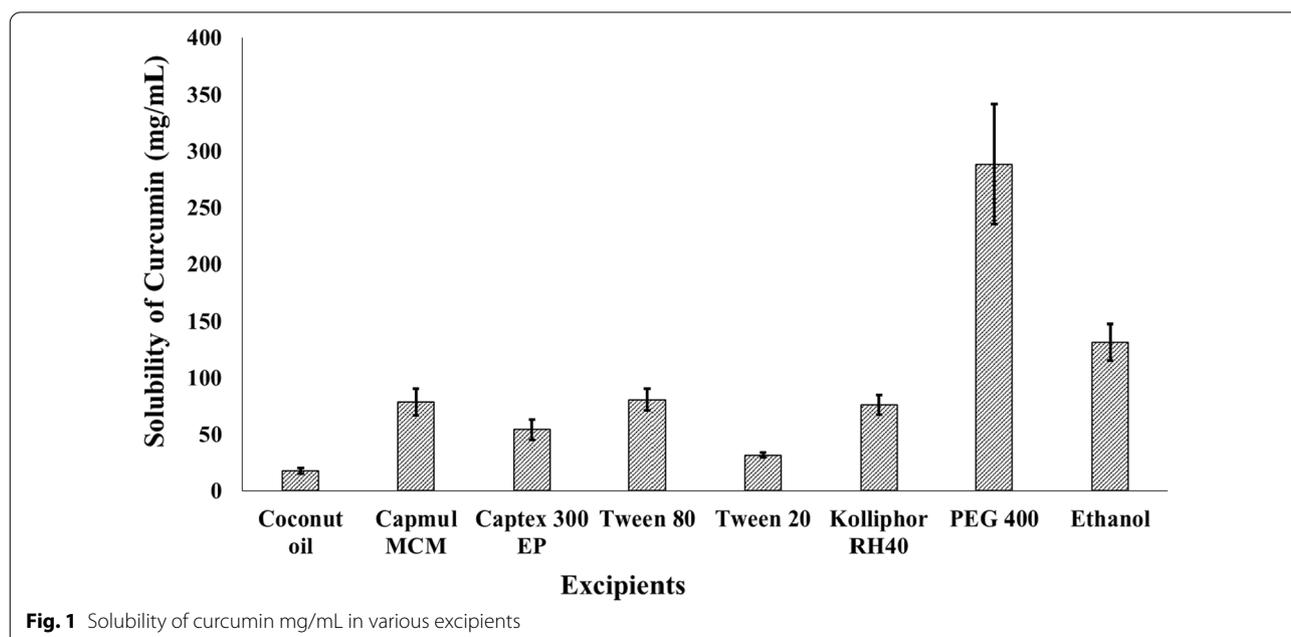


Fig. 1 Solubility of curcumin mg/mL in various excipients

like PEG 400 and ethanol as shown in Fig. 2. Initially, it was observed that virgin coconut oil was very difficult to emulsify. Hence, VCO was mixed with Capmul MCM at a ratio of 1:1 v/v and checked for miscibility. As this mixture was found completely miscible and no signs of phase separation after 24 h, this mixture was used for further studies. The results revealed improved emulsification of virgin coconut oil upon mixing it with Capmul MCM at 1:1 v/v ratio. The results demonstrated that all the surfactants exhibited higher emulsification except Kolliphor ELP which resulted in phase separation. Tween 80 was chosen over Kolliphor RH40 based on the less number of inversions observed for forming homogeneous and high drug solubility than Kolliphor RH40. PEG 400 was chosen as a co-surfactant based upon higher solubility of curcumin. Further studies were carried out using Tween 80 as a surfactant and PEG 400 as a co-surfactant.

Construction of pseudo-ternary phase diagram

Pseudo ternary phase diagrams were constructed to determine the specific concentration range of surfactant to co-surfactant that can result in the larger area of microemulsion region, using TriDraw software (version 2.6). The mixture composed of oils with various proportion of surfactant and co-surfactant were prepared and titrated with water. An increasing ME region was observed from Smix ratio 1:1 w/w to 3:1 w/w, i.e., increase in the surfactant proportion revealed a greater microemulsion region. Hence, a higher ratio, i.e., 3:1 w/w was selected for further studies.

Effect of oil and Smix concentration on globule size

The effect of oil and Smix concentration on globule size was determined by the varying one factor at a time (OFAT) model. Directly proportional relationship was observed between oil concentration and globule size, whereas an inversely proportional relationship was

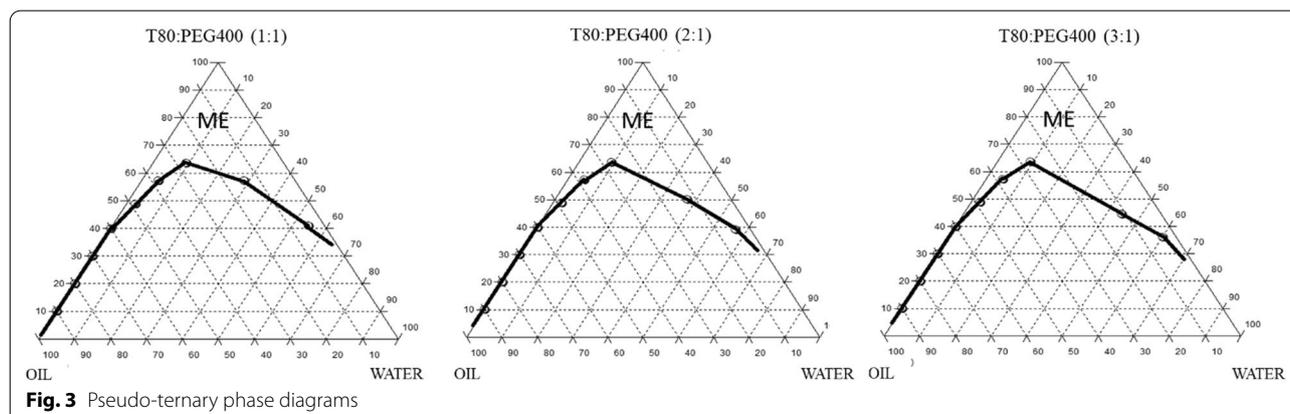
observed between Smix concentration and globule size. However, this change in globule size upon changing the concentration of both oil and Smix was not significant and profound. Hence, the final formulation comprising of maximum oil loading was finalized to evaluate the pharmacokinetic and pharmacodynamic effect of oil in further studies. Hence, the final composition was selected as oil at 20%, Smix at 60%, and water up to 100% (Figs. 3, 4).

In vitro characterization

The final microemulsion was characterized for appearance globule size, PDI, zeta potential, and pH. The Cur-ME appeared clear, transparent, and dark yellow colored. It was found stable upon dilution and centrifugation as no phase separation or sign of drug precipitation was observed. The globule size was below 25 nm with zeta potential of -17 mV. The pH of the final formulation was 6.63. The drug content was determined by RP-HPLC and was found to be greater than 90%. Table 1 depicts results of in vitro characterization.

In vitro drug release

In vitro drug release was performed to determine the drug release profile. The study was performed in phosphate buffer with pH 7.4 as a dissolution media. In vitro drug release profile (Fig. 5) showed that the drug release from formulation was higher (21.26% at 6 h) than from pure drug (10.26% at 6 h) solution ($p \leq 0.05$). An improvement of 2.12-fold in drug release was exhibited by Cur-ME when compared with the Cur-Sol. The drug release kinetic modeling was done and results revealed that Cur-Sol exhibited Higuchi model, whereas Cur-ME exhibited first-order drug release kinetics. The mathematical model fitting was based on the regression coefficient obtained by plotting parameters required for determining release kinetics. The factors which influence the drug release could be smaller globule size and more surface area for



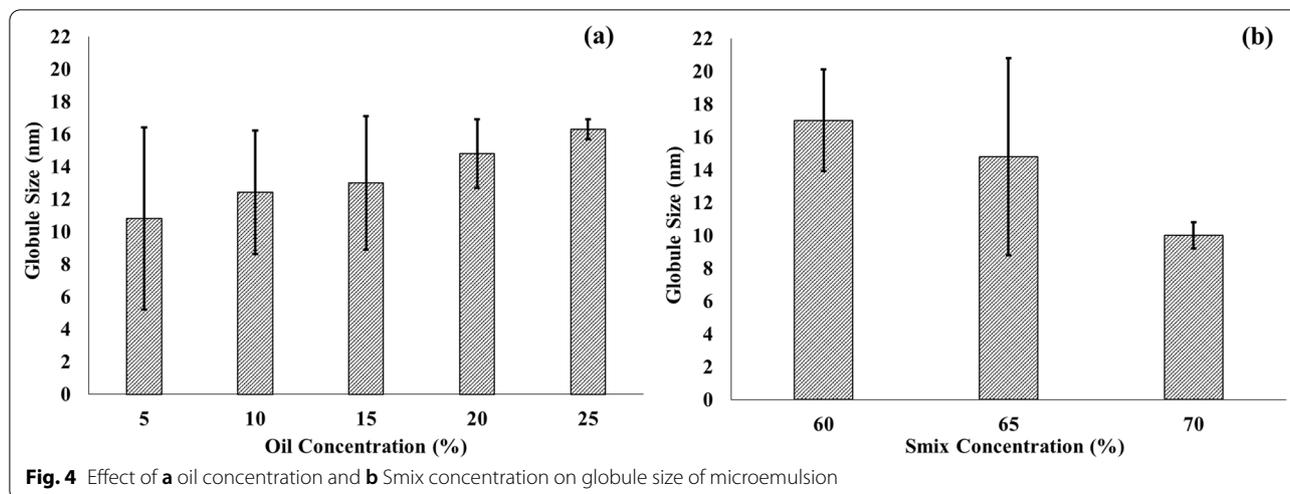
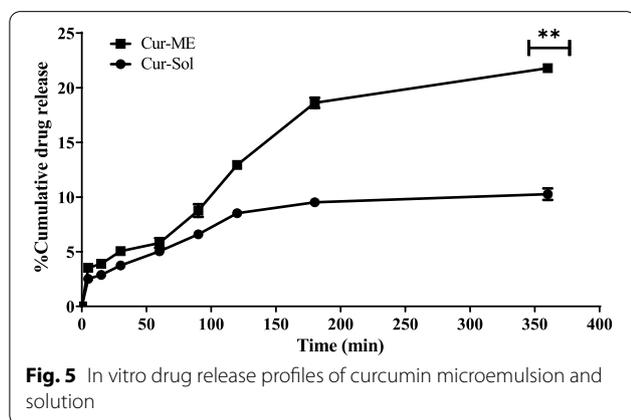


Table 1 In vitro characterization of Cur-ME (n = 3)

Parameters	Value/Inference
Globule size (nm)	17.20 ± 2.02
PDI	0.24 ± 0.06
Zeta potential (mV)	-17 ± 1.24
pH	6.63 ± 0.36
Drug content (%)	94.37 ± 4.97
Dilution potential	No precipitation
Centrifugation potential	No precipitation

Table 2 Plasma pharmacokinetics study of Cur-ME and Cur-Sol in rats (n = 8)

Parameter	Unit	Value	
		Cur-Sol	Cur-ME
t1/2	h	0.767 ± 0.450	0.636 ± 0.056
Tmax	h	1.000	1.000
Cmax	µg/ml	0.085 ± 0.018	0.210 ± 0.014
AUC 0-t	µg/ml*h	0.148 ± 0.009	0.296 ± 0.013
AUC 0-inf_obs	µg/ml*h	0.166 ± 0.023	0.318 ± 0.014
AUMC 0-inf_obs	µg/ml*h^2	0.265 ± 0.133	0.402 ± 0.032
MRT 0-inf_obs	h	1.548 ± 0.554	1.263 ± 0.064



releasing the dissolved form of drug from the system. This study was further substantiated by in vivo studies in animals.

In vivo Plasma Pharmacokinetics study

The plasma pharmacokinetic study was performed in Wistar rats. The pharmacokinetic parameters of Cur-ME

and Cur-Sol were calculated by non-compartmental model analysis and are listed in Table 2. The Tmax was observed at 1 h for both the formulations. The Cmax of Cur-Sol and Cur-ME was 0.085 µg/mL and 0.210 µg/mL, respectively. The significant enhancement (p < 0.05) in AUC (twofolds) and Cmax (2.48-fold) was observed in Cur-ME formulation as compared to Cur-Sol. The mean residence time of Cur-ME was comparable to that of Cur-Sol. Figure 6 depicts the in vivo plasma pharmacokinetic behavior of both formulations.

In vivo brain pharmacokinetics in Zebrafish

In vivo brain pharmacokinetic profile of Cur-Sol and Cur-ME was performed in adult zebrafish. Table 3 summarized the pharmacokinetic parameters of the brain pharmacokinetic study in zebra fish. The Tmax was observed as 5 min for both formulations. Cur-ME showed Cmax as 0.203 µg/brain which was 1.96-fold enhanced than that of Cur-Sol (0.104 µg/brain). Also, AUC shown by Cur-ME (4.622 µg/brain*min) was improved by 1.92-fold when compared to Cur-Sol (2.409 µg/brain*min).

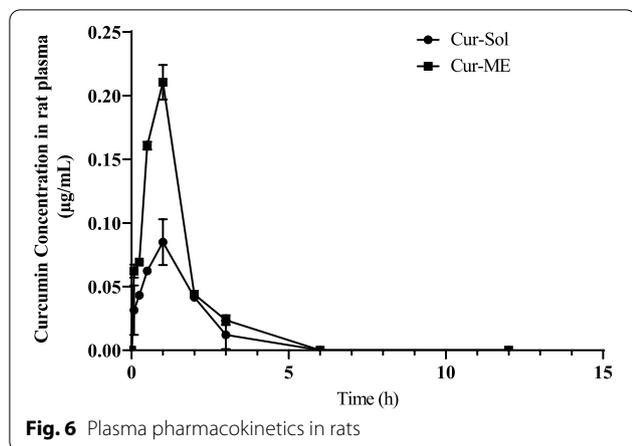


Fig. 6 Plasma pharmacokinetics in rats

Table 3 Brain pharmacokinetics study of Cur-ME and Cur-Sol in adult zebrafish (n = 8)

Parameter	Unit	Value	
		Cur-Sol	Cur-ME
t1/2	Min	21.088 ± 3.276	35.627 ± 6.519
Tmax	Min	5.000	5.000
Cmax	µg/brain	0.104 ± 0.013	0.203 ± 0.061
AUC 0-t	µg/brain*min	2.409 ± 0.218	4.622 ± 0.443
AUC 0-inf_obs	µg/brain*min	2.455 ± 0.224	5.045 ± 0.459
AUMC 0-inf_obs	µg/brain*min ²	65.325 ± 13.160	219.294 ± 45.875
MRT 0-inf_obs	Min	26.441 ± 3.720	43.513 ± 8.302

The results revealed the significant ($p < 0.01$) improvement in Cmax and AUC as shown in Fig. 7.

Pharmacodynamic effect with behavioral, biochemical, and histopathological analysis

Spatial memory in Morris water maze test

The spatial memory of the rats was analyzed by Morris water maze using escape latency and pathlength travelled as evaluating parameters (Table 4).

There was a significant decrease in the escape latency (sec) observed in the Cur-sol ($p < 0.05$) and Cur-ME ($p < 0.05$ and $p < 0.001$) treated animal groups on 21st and 28th day of the study when compared to neurodegeneration control. A significant decrease in escape latency ($p < 0.001$) was observed in Cur-ME when compared with Cur-Sol on day 28 (Fig. 8a). There was a 70-fold decrease in the escape latency in Cur-ME as compared to Cur-Sol, which can be due to the increased lipophilicity of the Cur-ME formulation.

Furthermore, a significant decrease in the pathlength (cm) was observed in the Cur-Sol ($p < 0.01$) and Cur-ME ($p < 0.01$ and $p < 0.001$) treated groups on the day 21st and 28th, respectively, when compared to neurodegeneration control. A significant decrease in pathlength travelled ($p < 0.01$) was observed in Cur-ME (150 mg/kg) when compared with Cur-Sol (150 mg/kg) on day 28 (Fig. 8b). There was a 39-fold decrease in pathlength in Cur-ME as compared to Cur-Sol.

Brain Glutathione level (GSH) and brain Malondialdehyde level (MDA) on TMT induced neurodegeneration

There was a significant ($p < 0.001$) decrease in brain glutathione level in neurodegeneration control group

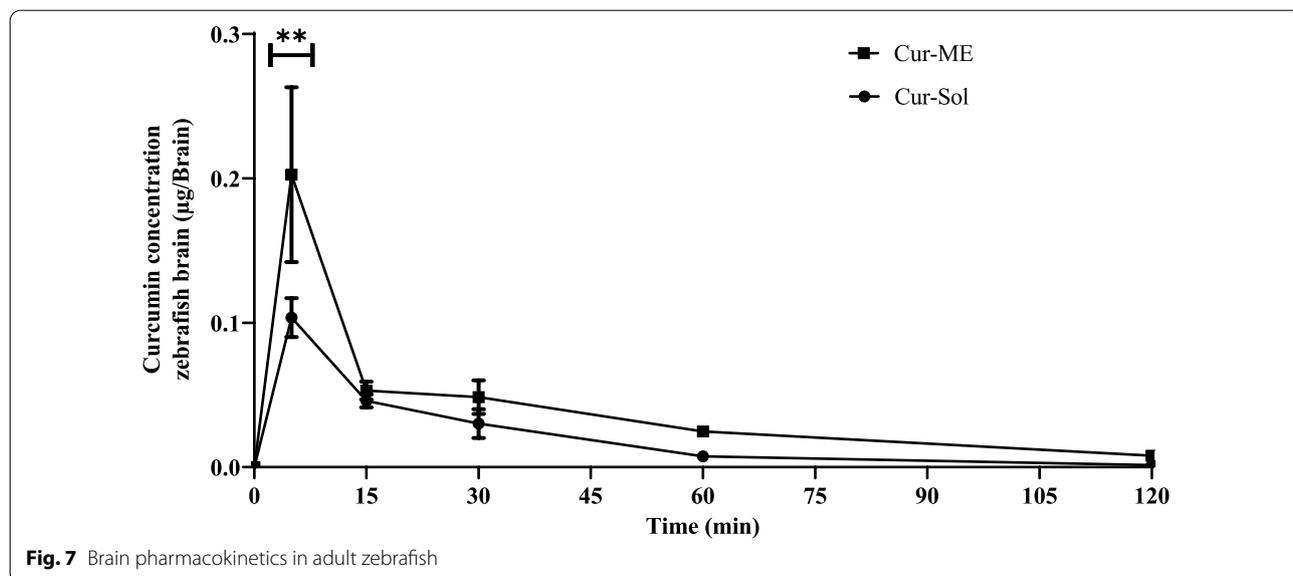


Fig. 7 Brain pharmacokinetics in adult zebrafish

Table 4 Comparative results of Morris Maze water and biochemical analysis ($n=8$)

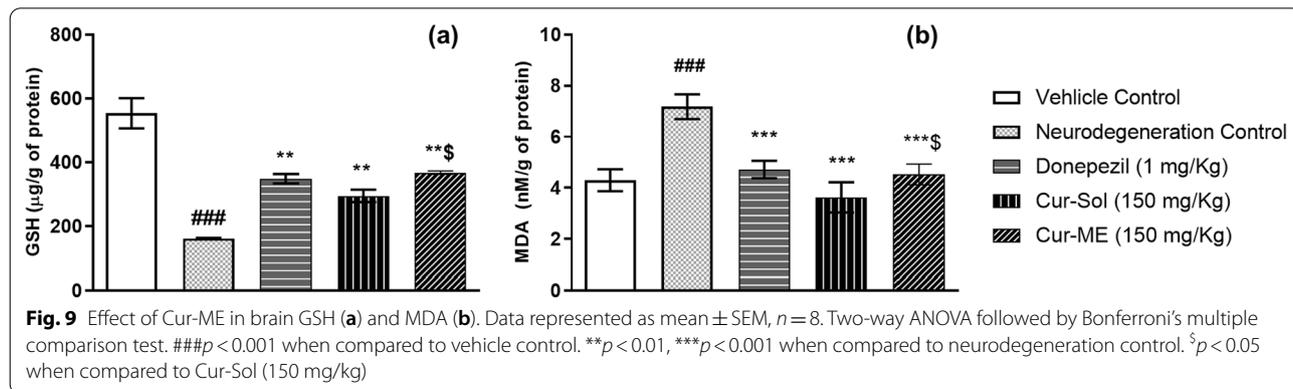
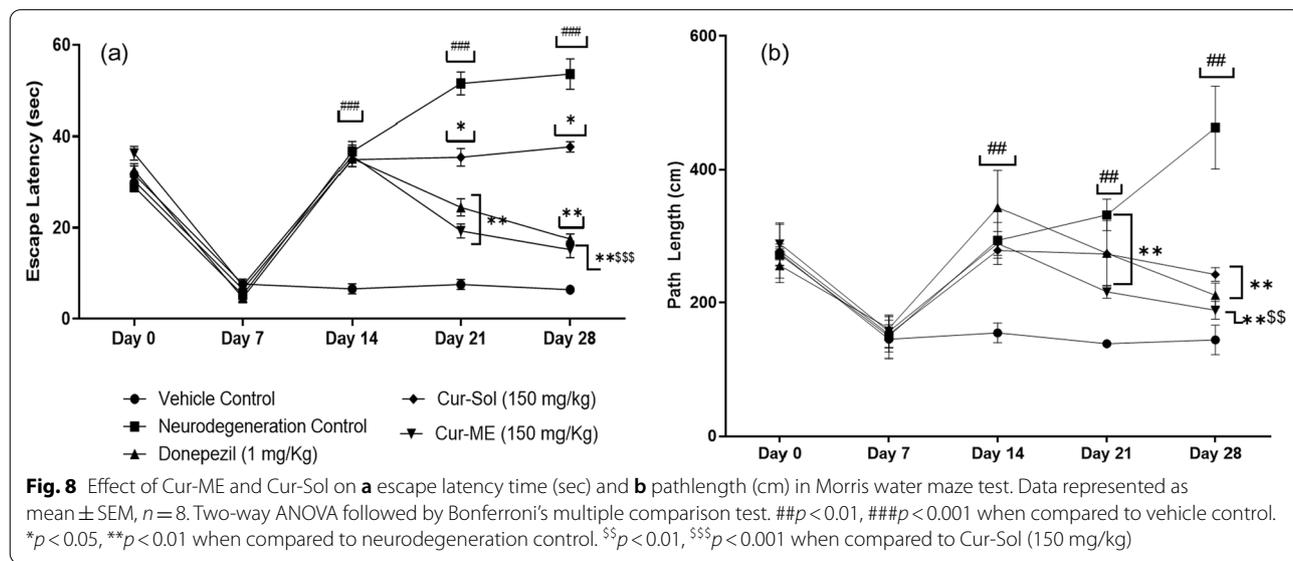
Parameters	Cur-Sol	Cur-ME
Escape latency (sec)	25.70 ± 1.10	15.10 ± 1.80
Path length (cm)	362.19 ± 52.00	220.18 ± 58.90
GSH (µg/g of protein)	249.97 ± 19.42	367.67 ± 5.62
MDA (nM/g of protein)	3.63 ± 0.59	4.53 ± 0.41

when compared to control group. There was a significant ($p < 0.01$) increase in GSH level in Cur-Sol and Cur-ME treated group when compared to neurodegeneration control group. There was a significant ($p < 0.05$) improvement in GSH levels upon treatment with Cur-ME group when compared to Cur-Sol group (Fig. 9a), which confirmed the superior antioxidant effect in Cur-ME.

There was a significant ($p < 0.001$) increase in brain MDA level in neurodegeneration control group when compared to control group. Treatment with Cur-ME and Cur-Sol caused a significant decrease ($p < 0.001$) in brain MDA level as compared to neurodegeneration control rats. The decrease in MDA level with Cur-ME was significant ($p < 0.05$) when compared to the Cur-Sol group (Fig. 9b). The results confirm the improvement in antioxidant defense when treated with Cur-ME.

Histopathology in TMT induced neurodegeneration

There was a significant neurodegeneration observed in the TMT-induced neurodegeneration control group (Fig. 10 b), while the neurons were intact in vehicle control group (Fig. 10 a). The donepezil treated group showed similar destruction of neurons (Fig. 10 c) as that of neurodegeneration group. The Cur-Sol treatment caused minor improvement in the alignment of neurons in the CA-3 region (Fig. 10 d). A significant prevention



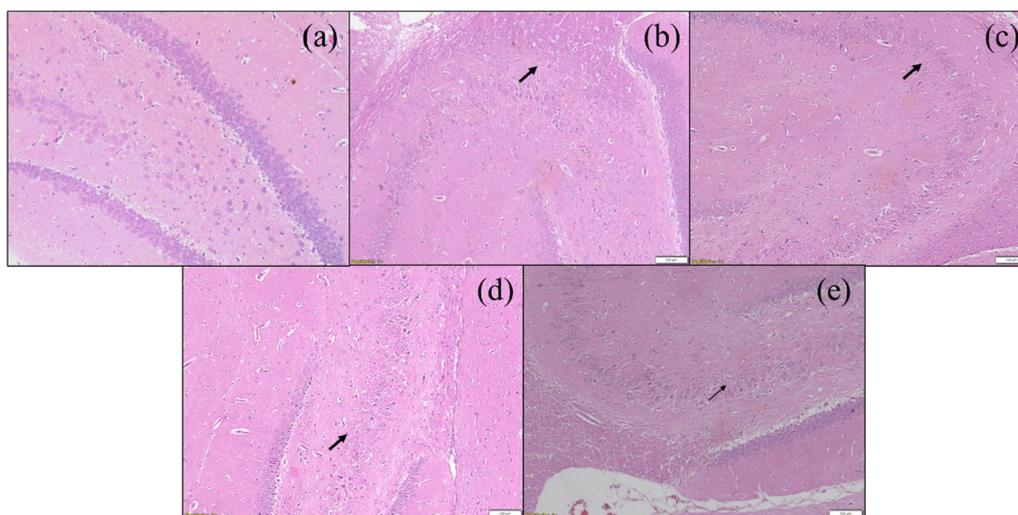


Fig. 10 Effect of Cur-ME and Cur-Sol on brain histology in Trimethyl tin induced neurodegeneration in rats; H&E stain, $\times 10$. **a** Brain histology of Vehicle control group showing intact neurons. **b** Brain histology of Neurodegeneration control group showing significant neurodegeneration (\nearrow). **c** Brain histology of standard donepezil treated group showing significant neurodegeneration (\nearrow). **d** Brain histology of Cur-Sol treated group showing minor prevention of neurodegeneration (\nearrow). **e** Brain histology of Cur-ME treated group showing significant prevention of neurodegeneration (\nearrow)

in the neurodegeneration was observed in the Cur-ME treated group (Fig. 10 e). The Cur-ME showed significant alignment of neurons as compared to Cur-Sol treated group.

Discussion

Curcumin is explored for enormous biological effects [27, 28]. However, its effect on neurodegenerative disorders are very promising [29]. There is plenty of literature available on various nano-formulations of curcumin for brain ailments especially Alzheimer's disease [5, 30, 31]. However, the biological effects of curcumin are limited due to poor solubility, permeability and extensive metabolism. The current treatments are symptomatically effective on AD and other neurodegenerative disorders and could not fix the molecular cause of the disease. Recently, researchers have explored the ameliorative effects of virgin coconut oil on oxidative stress in rats which can be related to increased oxidative stress in case of neurodegenerative disorders [17]. Also, coconut oil has already been reported as effective in AD conditions [32]. Nevertheless, current literature is devoid of targeted formulation strategies using virgin coconut oil and its effects on experimental animal models. Hence, this study was envisaged to investigate the effects of both curcumin and coconut oil as a microemulsion formulation and its *in vivo* effects on neurodegeneration and pharmacokinetics. Curcumin exhibited good solubility in surfactants. However, the formulation components were selected based on good solubility as well as emulsification potential. The cold pressed

coconut oils was very difficult to emulsify. This may be because of the inability of surfactants to enter into long chains of oil which leads to poor emulsification [33, 34]. Hence, it was mixed with medium chain triglyceride to improve its emulsification [35]. Tween 80 was selected as it exhibited less inversions to form a homogeneous dispersion and higher solubility than other surfactants. However, PEG 400 also exhibited good solubility of curcumin hence selected as co-surfactant/cosolvent and pseudoternary phase diagrams were plotted. The results revealed improved microemulsions region upon increasing the surfactant proportion in Smix. This increase in microemulsion region could be attributed to availability of higher amount of surfactant for emulsification of fixed amount of oil [34]. Surfactant and co-surfactant influences the emulsification of oils, reduce the particle size, and hence, they can maintain a good drug loading capacity in the formulation. After determining the appropriate ME region, a point in ME region was identified and effect of Smix and oil concentration was evaluated. Here, in this study, the directly proportional relationship between oil concentration and globule size was established. This was so observed because of the presence of exceeding amount of oil and availability of fixed amount of surfactant to emulsify it. An inversely proportional relationship was observed with Smix concentration and globule size, wherein increasing amount of Smix was emulsifying the fixed amount of oil hence greater emulsification resulted in reduced size. However, the resultant size of globule remained below 25 nm; hence, proportion

was such chosen so as to have intermediate Smix amount with maximum of oil loading. The final formulation was characterized in vitro, wherein the observed globule size was below 25 nm. Though the variation in globule size was not profound and significant, the final size remained below 50 nm which can enhance brain uptake and bioavailability [15]. The formulation also exhibited negative zeta potential revealing the negatively charged particles dispersed in the suspension. This negative zeta potential could be attributed to the presence of PEG and VCO in formulation which comprises of –OH groups [36]. However, curcumin also shows zeta potential of –15 mV as per the reported literature [37]. Furthermore, microemulsion carriers are found advantageous for brain targeting as the solubility and permeability of the drug could be enhanced by formulating them as MEs which can further improves the drug uptake in the brain by various mechanisms across the blood–brain barrier [13]. An improvement in drug release was observed with ME than the pure drug solution. This could be due to dissolved form of curcumin in microemulsions along with submicron size which can improve solubilization of colloidal system in medium and improve dissolution. Researchers have already reported the improvement in drug release when formulated as microemulsions which may further enhance bioavailability [38, 39]. Hence, in vivo pharmacokinetic profile was established for Cur-ME and Cur-Sol. The short Tmax with enhanced Cmax reflected the rapid absorption of drug in vivo. The Cur-ME exhibited 200% relative bioavailability when compared with Cur-Sol. Nevertheless, this improvement in in vivo absorption could be attributed to nano-sized oil globules containing drugs, improved drug release from ME and functional property of Tween 80 of P-gP efflux inhibition that could result in improved permeation followed by absorption [16, 39, 40]. This enhanced bioavailability could expose the drug to BBB to higher extend. Hence, a separate brain pharmacokinetics was performed in adult zebrafish to assess the brain targeting potential of Cur-ME. The model was chosen based on the genomic resemblance between zebrafish and humans [41] along with similarity in construction of BBB [42]. The results demonstrated almost twofold improvement in brain concentration. Similar results were reported by More et al. when preliminary brain kinetics was established and determined in adult zebrafishes [20]. This improvement in drug concentration in the brain could be the collaborative effects of functions of excipients as well as submicron size and lipid nature which could assist the passive transport across BBB as discussed before. Overall pharmacokinetics results indicated improved absorption and brain targeting potential of Cur-ME. Nevertheless, its pharmacodynamic effect was assessed in experimental animal model for AD. We

had selected TMT induced AD in rat model as it is the most suitable model for spatial learning, memory dysfunction and neurodegeneration [43]. The results indicate an improvement in the spatial memory of rats upon administration of Cur-ME. The neuroprotection was confirmed by histopathological evaluation. The reduction in oxidative stress was also demonstrated by biochemical analysis of rat brains. These results could be attributed to improved drug uptake in brain by using microemulsion carrier loaded with curcumin and coconut oil. Nevertheless, the health benefits and pharmacological effects of coconut oil can lead to improvement in spatial learning and cognitive function [19]. The positive results shown in the behavioral parameters can be regarded as a mark of an effective formulated microemulsion to be used in neurodegeneration.

Conclusion

The formulation development and characterization of a novel curcumin microemulsion was detailed in this study, and its potential as a targeted drug delivery system to the brain was established in a zebrafish model using in vivo brain pharmacokinetics. The coconut oil, Tween 80, and PEG 400 microemulsion had reduced globule size (<50 nm), a negative zeta potential (–17 mV), and an extreme curcumin loading of 49 mg/mL. When compared to Cur-Sol, in vitro drug release showed a 2.12-fold improvement. In vivo plasma pharmacokinetics showed twofold improvement in plasma concentration of curcumin when administered as microemulsion vehicle. In addition, in vivo brain pharmacokinetics in a zebrafish model revealed 1.92 times higher curcumin concentration in the brain when compared to Cur-Sol. The components that block the P-gP efflux pathway may be responsible for this. This innovative microemulsion could be used to develop brain-targeted formulations as a platform technology. Overall, these findings indicate that an orally delivered microemulsion with brain targeting capacity has enormous potential.

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Author contributions

VP and SM has collected the data, compiled it, and prepared a draft. SKM integrated the data along with manuscript editing. SKM and AS have corrected and revised the manuscript. AS and AP has supervised all the work and revised the final draft. All authors read the final draft for approval. All authors read and approved the final manuscript.

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Availability of data and materials

All data and material are available upon request.

Declarations

Ethics approval and consent to participate

The experimental protocol was approved by the Institutional Animal Ethical Committee of Poona College of Pharmacy, Pune, MH, India (Registration No. 1703/PO/c/13/CPCSEA; IAEC protocol numbers: IAEC/PCP/PCL29/2020–2021 & IAEC/PCP/PCL28/2020–2021).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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