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Incorporating Design of Experiment (Doe) Principles To Niosomal Cerebroprotective Phytoformulation Development

Shilpa.P.Chaudhari*[1],Jeetendra V Bangar[2]
1. Padmashree Dr.D.Y.Patil College of Pharmacy, Akurdi ,Pune-44 ,Maharashtra, India
2. Marathwada Mitra Mandal's College of Pharmacy,Thergaon ,Pune-33, Maharashtra, India

ABSTRACT

An cerebroprotective phytoconstituent loaded niosomes for nose to brain delivery was formulated. The main objective of this study was firstly to enhance the solubility and bioavailability of poorly soluble phytoconstituents Rutin and Quercetin, secondly to ease the administration through nasal route and thirdly to investigate the niosome encapsulated phytoconstituent for its cerebroprotective activity. Method: Niosomal formulations were optimized using design of experiment by altering the proportions of range of non-ionic surfactants(Tween 80, Pluronic L81, PluronicP123, span80,captex 200P,Capmul PG NF8,Labrasol), Ratio of cholesterol to surfactant, RPM and sonication. The formulations were prepared by ether injection method. The formulation was then evaluated for morphological characterization, encapsulation efficiency, and viscosity. Cerebroprotective activity of optimized niosomal formulation against bilateral carotid artery occlusion (BCAO) induced stroke in rats was studied. Phytoconstituent niosomes formulated using Tween 80 was found to entrap high amounts of drug, and show sufficient quantity of in vitro release. Consequences showed that the niosomal formulation of Rutin and Quercetin gives the cerebroprotective action at the dose of 3mg/kg of each drug. Based on the results of histopathology study, it can be concluded that test formulation may have potential to attenuate histopathological alterations caused due to ischemia and can be considered as a promising approach for the cerebroprotective Phytoconstituents.

Keywords: Cerebroprotective,Quecetin, Rutin,noisome,phytoconstituent

*Corresponding Author Email: shilpapchauhdari78@yahoo.com
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INTRODUCTION

After the heart disease Stroke is the second largest cause of mortality worldwide. A term Brain attack is being used to describe the acute presentation of stroke, and to emphasize the need for urgent remedy. Deaths due to stroke are 9.5% of all deaths. More than 700,000 new cases of strokes occur annually In the United States. It is also the leading cause of disability with a stroke survivors living with stroke related deficits. More than 70% of stroke survivors remain vocationally impaired, more than 30 % require help for activities of daily living, and more than 20% walk only with assistance. In India the information on stroke is minimal and therefore there is a need to initiate steps towards control measures for the stroke[1]. For the treatment of stroke various agents have been developed, however only a few drugs have shown limited efficacy in the clinical trials. Antioxidant agents like melatonin, adenosine, resveratrol, α-tocopherol and also the combination of antioxidant like melatonin and meloxicam give the protective effect in experimental model of middle cerebral artery occlusion in rats. In the treatment of cerebral ischemia an agent, who can improve the blood flow and also beneficial effect on the biochemical cascades will be more useful. Also the agents, which relieve the spasm of the nutrient artery and having an antioxidant property, could be an additional important approach to prevent the further damage immediately after the spasm of the affected artery in the brain [5]. In stroke attack oxygen free radicals are generated are causative agents of neuronal damage. A balance is maintained between oxidative attack and antioxidant defence system prevailing in different tissues. But the brain has a low level of antioxidative defence .This Increased level of oxidative stress seen in cerebral tissue is thus the major contributing factor for the development of neurodegenerative disease like cerebral ischemia reperfusion. Due to the Anti-oxidant property of flavonoids, they act as cerebroprotective agents. Flavonoids can interfere directly with different free radical producing systems or can also increase the function of the endogenous antioxidant. Rutin and Quercetin are flavonoids show the antioxidant action so it's used to formulate into niosomal formulation. With optimistic approach the formulation of niosomal formulation for cerebroprotective activity was considered. They are widely distributed in the plant kingdom.

Quercetin and Rutin are members of the class of flavonoids termed as flavonols. They are widely distributed in the plant kingdom. Quercetin is found abundantly in red wine, green tea, onions, berries, citrus fruits, parsley, apples, and garlic[6]. Rutin is abundantly found in black wheat and also in apple peels, garlic, tomatoes, and black tea. Living organisms have developed antioxidant line of defense systems include enzymatic and nonenzymatic antioxidants that keep in check

ROS/RNS(reactive Oxygen species/Reactive nitrogen species) level and repair oxidative cellular damage. The major enzymes, constituting the first line of defence, directly involved in the neutralization of ROS/RNS are: superoxide dismutase (SOD), catalase (CAT) and glutathioneperoxidase (GPx).

The second line of defence is represented by radical scavenging antioxidants such as vitamin C, vitamin A and plant phytochemicals that inhibit the oxidation chain initiation and prevent chain propagation .This may also include the termination of a chain by the reaction of two radicals. Quercetin and Rutin prevents free radical induced tissue injury by various ways. By scavenging free radicals, Flavonoid; particularly Quercetin can inhibit Low density Lipids (LDL) oxidation in vitro. This action protects against atherosclerosis.

The present research focuses on the development of the Rutin and Quercetin (Each 150mg) niosomal formulation for the cerebroprotective activity. Formulation given by the nasal route as it targeted to the brain for its activity with high percent drug entrapment, maximum drug release, good stability and alternative route of administration being intra venous (i.v.) is developed. Intranasal absorption avoids the gastrointestinal and hepatic presystematic metabolism, enhancing drug bioavailability in comparison with that obtained after gastrointestinal absorption[10]. On the other hand, intranasal administration also offers several practical advantages either from view point of patients (non-invasiveness, essentially painless, ease drug delivery and favourable tolerability profile) or pharmaceutical industry[3]. It is unknown how different process and product variables impact on product quality and performance. Therefore, an investigation of the application of design of experiment concepts to niosomes containing Rutin and Quercetin will provide statistical significance for optimization of formulation parameters like effect of type of surfactant, surfactant concentration and lipid concentration and process variables like effect of sonication, rpm, and temperature on drug encapsulation efficiency and vesicle size.

MATERIALS AND METHOD

Materials

Rutin was purchased from Otto Chemie Pvt. Ltd (Mumbai, Maharashtra), Quercetin (dihydrate) purchased from sigma Aldrich Pvt. Ltd. All the chemicals and reagents were of analytical grade and were purchased from S.D fine, Mumbai.

Experimental methods

Preparation of niosomes

Niosomes containing Rutin and Quercetin were prepared by single method studying the effect of

variables on this method i.e., modified ether injection technique using nonionic surfactants. Cholesterol and surfactants were dissolved in 6ml diethyl ether mixed with 2ml methanol containing weighed quantity of Rutin and Quercetin. The resulting solution was slowly injected using micro syringe (14 gauge needle) at a rate of 1ml/min into 15 ml of aqueous phase maintained at 60^0C. The solution was stirred continuously on magnetic stirrer and temperature was maintained at 60-65ºC. Then the formulations were sonicated three times at50 Hz in a bath-sonicator (Ralsonics model RP 120, Mumbai, India) for 15 min with 5-min interval between successive times[7, 11].

Design for Experimental for niosomal formulation:

A IV Optimal Factorial design (response surface methodology) using quadratic model was applied to the formulation of niosomes with 43 runs. The levels of these variables were determined from the preliminary studies(Table:1).

Table 1.Formulation variables and factors

Variables	Factors	Levels	
		+1	-1
Formulation variables	Type of surfactant	Tween 80, Pluronic L81, PluronicP123, span80, Captex 200P,Capmul PG NF8, Labrasol	
	Ratio of surfactant to cholesterol	1:1	3:1
Process Variables	Revolutions per minute (RPM)	300	600
	Effect of sonication	With	Without
Dependent variables	Vesicle size	Y1	
	% Entrapment	Y2	

Determination of Vesicle Diameter:

The size, shape, and lamellar nature of vesicles in sonicated formulations were observed by optical microscopy at 10x magnification using a Motic microscope.

Determination of Entrapment Efficiency:

Niosomal formulations were centrifuged at 15,000 RPM for 90 min at -4°C using a cooling microcentrifuge to separate niosomes from non-entrapped drug. Concentration of the free drug in the supernatant was determined by measuring absorbance at respective nm with a UV spectrophotometer (Shimadzu, UV 1800). The percentage of drug entrapment in niosomes was calculated. This process was repeated thrice to ensure that free drug was completely removed.

Percent drug entrapment = (Total drug- Drug in supernatant/ Total drug) x 100

Determination of Viscosity:

Viscosity of the formulations was determined using Brookfield's Viscometer at room temperature. (64 spindle, 50 RPM)

***In vitro* drug diffusion studies:**

The horizontal diffusion chamber was used for the present study using goat nasal mucosa. Phosphate buffer solution of pH 6.4 was used in the receptor chamber. Before starting the study, the mucosa was pre-incubated with phosphate buffer solution of pH 6.4 so as to saturate the mucosa so that there will not be any change in permeability. The niosomal formulation solution was taken into the donor compartment. The quantity of the niosomal formulation was taken in such a way that the formulation contained about 10 mg of the each drug (approximately about 1 ml of formulation). The speed of the magnet was adjusted at an optimum speed. Sampling was done at regular intervals, i.e. for 0, 5, 10, 15, 30, 45, 60, 75, 90, 120 and 150 min. The sink condition was maintained with phosphate buffer solution. The samples were diluted with methanol and further measurements were carried out on the UV spectrophotometer at 257 nm and 372nm.[2,11].

In Vivo Study

In vivo study was carried out for evaluation of cerebroprotective activity rutin and quercetin niosomal formulation against bilateral carotid artery occlusion (BCAO) induced stroke in rats.

The animals were pre-treated with niosomal formulation for a period of 1week (nasal drop 3mg/kg of each drug). The animals were anaesthetized with thiopentone sodium (45mg/kg) and stroke was induced by Bilateral Carotid Artery Occlusion (BCAO) for defined period with aneurism clamps placed on both arteries and later (10 minutes) clamps were removed to allow reperfusion and animals were then returned to their cages. After 24 hours of reperfusion, the animal behaviors were evaluated by various methods such as open field behavioral parameters, locomotors activity, rotar rod test, in grouped animals.[8, 9, 12]

Histopathological examination

At the end of behavioral testing, the animals were deeply anaesthetized with thiopentone sodium. Following decapitation, the brains were taken out and after fixation in 10% buffered formalin the tissues were rinsed in running water and were processed for dehydration with alcohol and embedded in paraffin. Multiple, coronal sections of 4-5 μm were taken from each brain passing through striatum to caudal hippocampus and stained with hematoxylin–eosin. Sections were examined under light microscope.

RESULTS AND DISCUSSION

Preliminary study was done to select syringe diameter, with large diameter vesicle size was more with small diameter vesicle size decreases. Whereas decrease in % entrapment was within 5%.

DSC of optimized formulation showed the peak at 190^0C which is melting point of pure Rutin, and small peak at 210^0C is for the pure Quercetin hydrate. Two peaks showed that there was an entrapment of drugs within vesicles and they did not interact to form any additional chemical entity.

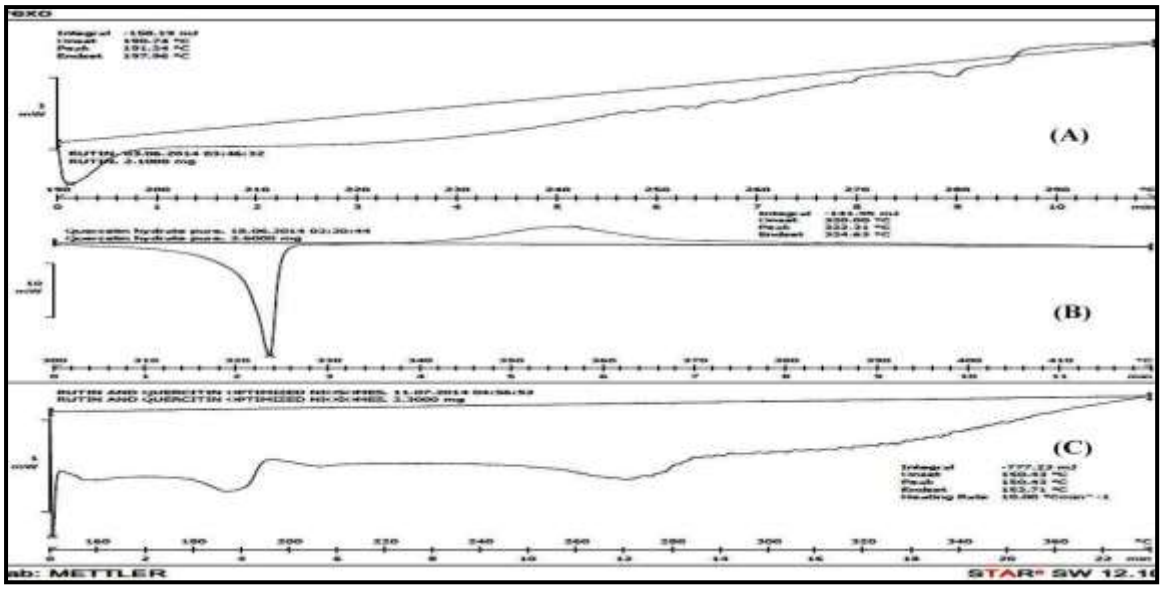


Fig 1: DSC graph of (A) Rutin, (B) Quercetin and(C) optimized formulation

Effect on vesicle size:

From the graph (figure.2) we can observe that as the stirring rpm and ratio of Cholesterol to Surfactant increases, vesicle size also increases. On increasing amount of cholesterol decreases the vesicle size might be due to decrease in crystallinity of the bilayers and thus is expected to increase the repulsive forces between vesicles. In second graph (figure.3) we observe effect of type of surfactant & effect of sonication on Vesicle size, Tween 80 gives less vesicle size and Labrasol gives a highest size vesicles. Sonication show the effect on vesicle size, formulation with sonication gives a less vesicle size as compared to without sonication. Sonication generates cavitation bubble in liquid which oscillates nonlinearly & collapse eventually this results in slight increasing temperature and pressure of the system and decreases particle size. Vesicles with no or small amount of cholesterol that is high concentration of surfactant show a tendency to a grow at elevated temperature[4].

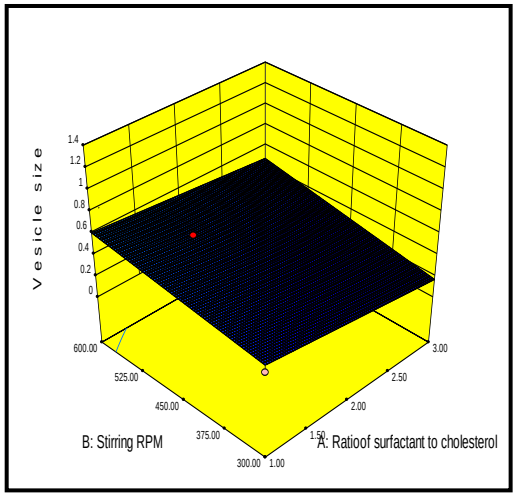


Figure. 2: Response surface plot for effect of Ratio of Surfactant to cholesterol & stirring RPM on Vesicle size

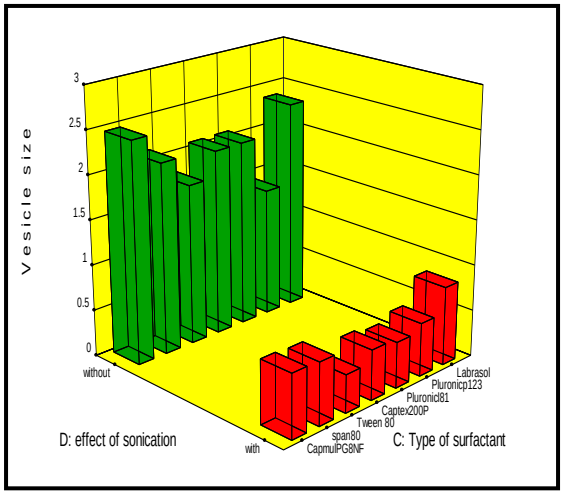


Figure.3: Response surface plot for effect of type of surfactant & effect of sonication on Vesicle size

Effect on % entrapment

From above graph (figure.4 and6) we can observe that as the stirring rpm and ratio of Cholesterol to surfactant increases, % Entrapment of Rutin and Quercetin also increases. As cholesterol increases the micro viscosity of the formulation hence increase of cholesterol, increases the viscosity of the formulation indicating more rigidity of bilayer membrane .It was observed that niosomes prepare without cholesterol form the gel and only on addition of cholesterol homogeneous niosomes dispersion was obtain .

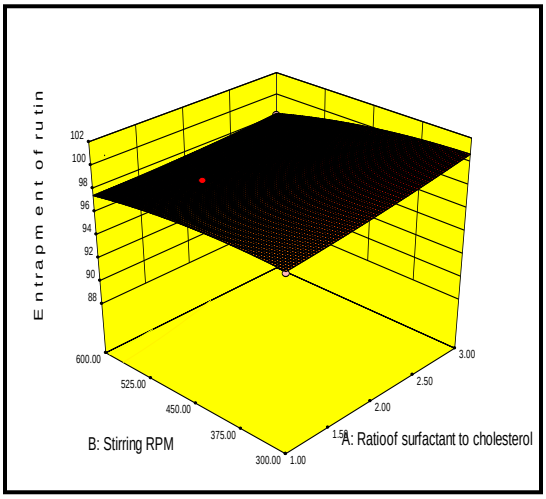


Figure.4: Response surface plot for effect of Ratio of surfactant to Cholesterol & stirring RPM on Entrapment of Rutin

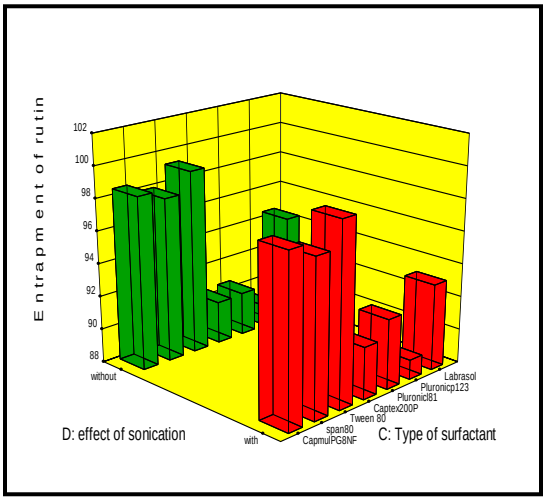


Figure. 5: Response surface plot for effect of type of surfactant & effect of sonication on Entrapment of Rutin

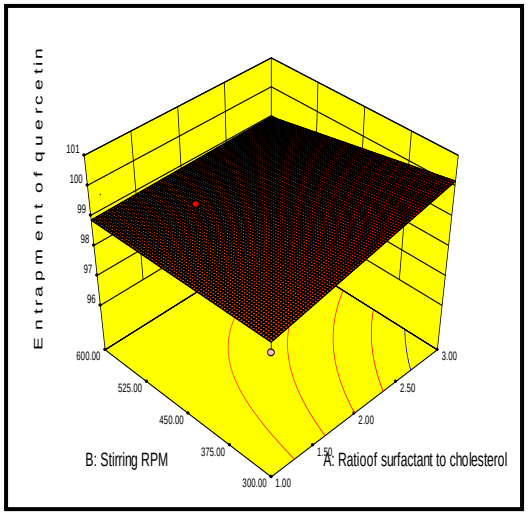


Figure.6: Response surface plot for effect of Ratio of surfactant to cholesterol & stirring RPM on Entrapment of Quercetin

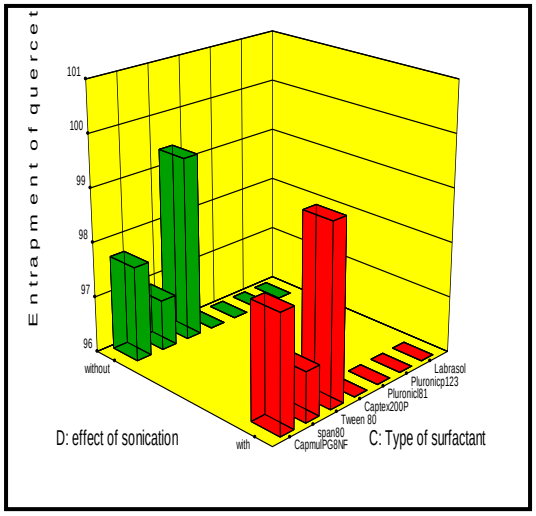


Figure.7: Response surface plot for effect of type of surfactant & effect of sonication on Entrapment of Quercetin

With increase in surfactant concentration less drug entrapment was seen for Rutin might be due to the longer saturated alkyl chain of surfactant tween80.Also the length of alkyl chain influences Hydrophilic lipophilic balance (HLB) value of surfactant and lower the HLB of surfactant ,lower will be the entrapment efficiency[1].

In second graph (figure.5 and 7) we observe effect of type of surfactant & effect of sonication on Entrapment, Tween 80 gives high % entrapment for Rutin 99.98±0.42% and Quercetin 99.87±0.27% while Pluronic P-123 gives a low% Entrapment88.35±0.35% for rutin and 88.93±0.32% for quercetin..Sonication show the less effect on Entrapment, formulation with sonication and without sonication not show much difference in % Entrapment of Rutin and quercetin might be due to syringe diameter.

Increase in cholesterol concentration increase drug encapsulation and improve drug retention inside the niosomes while reducing layer fluidity and hence permeability.

Results obtained for Tween 80 (HLB15) indicate that higher the HLB value higher the entrapment compared to Labrasol (HLB14), capmulPG8NF and other surfactants. Also the Tween 80 has long alkyl chain as compared to the span and other surfactant. It may play role to increase the entrapment of drug.

As mentioned earlier noisome vesicle size and drug entrapment efficiency are very critical product parameters and an understanding and awareness of the potential risk is very important. Form the analysis for the ether injection method the variables affecting niosomal encapsulation and vesicle size were type of surfactant, ratio of surfactant to cholesterol concentration, rpm. This information

can be used by scientist in formulation and process screening studies to identify the most significant variables for noisome formulation and process optimization.

Effect on % Phyto constituent Release:

From above graph (fig.8 and 10) we can observe that as the stirring rpm and ratio of surfactant to cholesterol increases, % Release of Rutin and Quercetin also increases. Cholesterol is reported to act as membrane stabilizing agent and to sustain drug release. The higher surfactant concentration tween 80 shows higher drug release might be due to the unsaturation in chain of tween 80 increases the chain fluidity and permeability[7]. In second graph (fig.9 and 11) we observe effect of

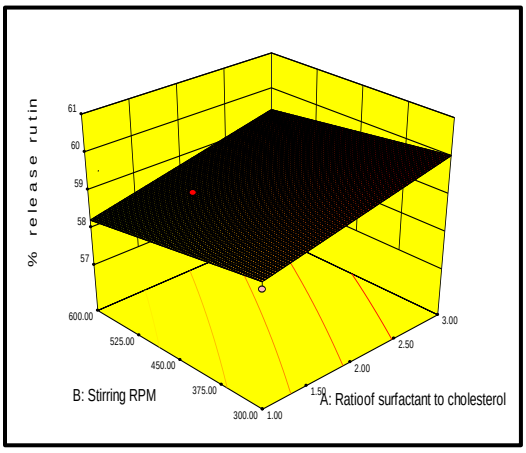


Figure 8: Response surface plot for Effect of Ratio of surfactant to cholesterol & stirring RPM on % Release of Rutin

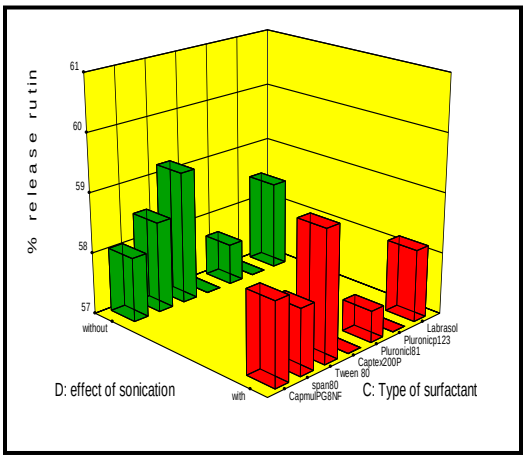


Figure.9: Response surface plot for effect of type of surfactant & effect of sonication on %Release of Rutin

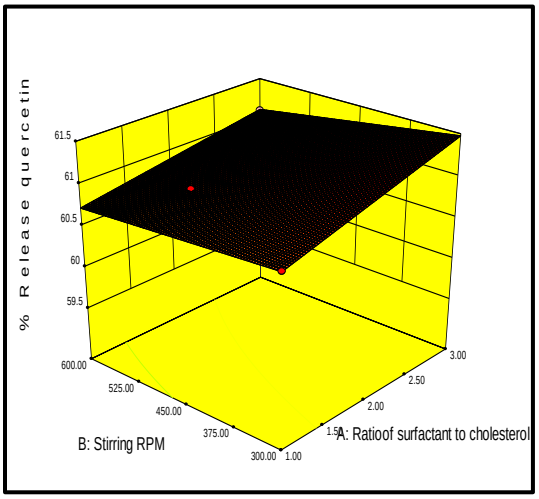


Figure.10: Response surface plot for Effect of Ratio of surfactant to cholesterol & stirring RPM on % Release of Quercetin

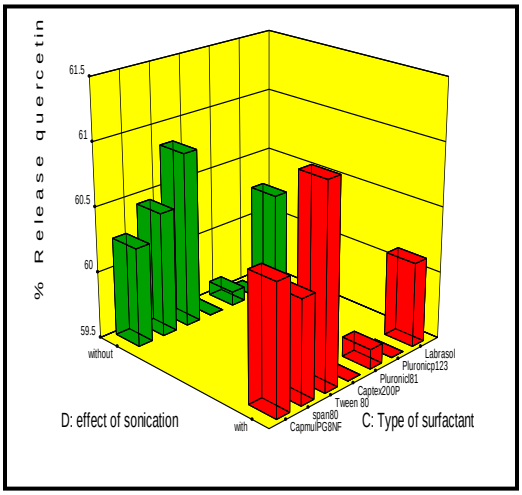


Figure.11: Response surface plot for effect of type of surfactant & effect of sonication on % Release of Quercetin

type of surfactant & effect of sonication on Release, Tween 80 gives high % release of Rutin 59.83±0.31% and Quercetin 61.27±0.19% and Pluronic P-123 gives a low% release54.35±0.45% and 57.93±0.42%. respectively. Sonication show the less effect on % release, formulation with sonication and without sonication not show much difference in % release of both the active constituents. from the above all result we get the Tween 80 fomulation(fb5) is selected as the formulation with best result and used for the further study.

In vivo study:

BCAO for 10 min in rats resulted in selective loss of pyramidal neurons in the CA1 area of hippocampus within 96 h to become apparent morphologically. There was substantial hippocampal neuronal death (80–85%) in ischemic animals as compared with the sham operated animals. Ischemic animals showed hyper locomotion on initial day of reperfusion. This was found to be consistent with the findings stating that on the first day after reperfusion, ischemia induced increase in locomotor activity is prominent, following two days it starts decreasing. (Table:2)

Table 2 Effect of Nasal formulation on open field behavioral parameters in Ischemic rats

Groups	Ambulations (number)	Immobility (Second.)	Rearings (number)	Groomings (number)	Fecal pellets (number)
Sham operated	52.21±3.12	30.98±1.7	00	4.16±1.07	1.16±0.3
Ischemic	25.31±2.94***	46.0±1.86***	00	9.00±1.15*	1.66±0.42
Nasal formulation treated	52.12±4.1###	32.3±1.45#	00ns	8.5±1.25#	00ns

Thus based on this analysis, the group treated with nasal formulation showed the significant ($P<0.01$) improvement in locomotor activity. (Table:3) Global cerebral ischemia causes marked damage to pyramidal neurons in the hippocampal region within days after ischemia in animals and humans. Hippocampal neurons are highly susceptible to ischemia and reperfusion induced injury. Hippocampus is involved in the regulation of short-term memory. Vascular dementia is the second most common type of dementia following Alzheimer's disease-related dementia. Vascular dementia occurs when the blood supply to the brain is reduced by a blocked or diseased vascular system and leads to a progressive decline in memory and cognitive function. Cerebral hypoperfusion can be induced by bilateral occlusion of common carotid arteries (BCAO) in rats, resulting in significant white matter lesions, learning and memory impairment, and hippocampal neuronal damage. Thus, BCAO in rats provides a model useful for understanding the pathophysiology of chronic cerebrovascular hypoperfusion and for screening drugs with potential therapeutic value for stroke.

The present studies suggest that In-vivo behavioral studies such as motor activity, rota rod, and

open field behavioral parameters were carried out. (Table:4)There was a decrease in the motor activity and escape latency in the water maze in stroke induced (negative control) group. The group treated with nasal formulation showed significant (P<0.01) improvement in the motor activity, muscle coordination, and spatial learning. The results of this study confirmed that niosomal formulation of Rutin and Quercetin protects rats from ischemia induced brain injury. This protection was evident from in-vivo behavioral tests. In conclusion, Niosomal formulation of Rutin and Quercetin produced cerebroprotective effects in global cerebral ischemia as evident from reduction in behaviour pattern, hyper locomotion and neuronal damage.

Table 3 Effect of Nasal formulation on locomotors activity in Ischemic rats

Groups	Locomotor count
Sham operated	101±9.13
Ischemic	35.65±4.46***
Nasal formulation treated	143.79±9.6###

Table 4 Effect of Nasal formulation on neurotoxicity test on rota rod in Ischemic rats

Groups	Time of fall(sec)
Sham operated	69.66 ± 8.81
Ischemic	18.33±1.92*
Nasal formulation treated	44.33±5.80#

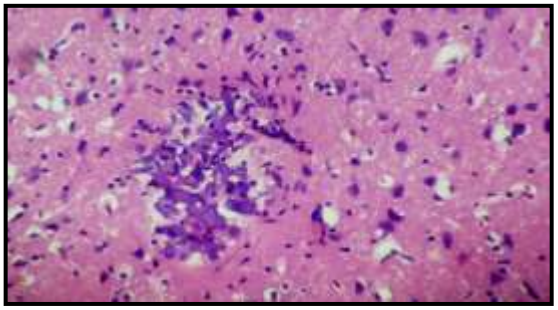
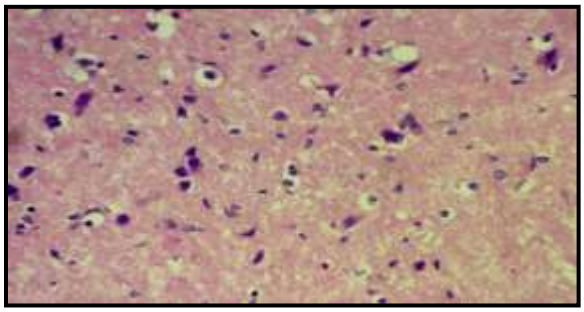
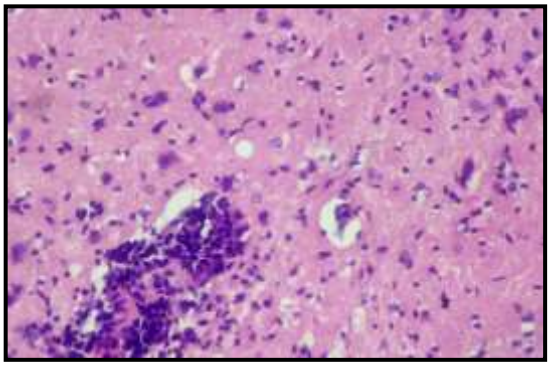
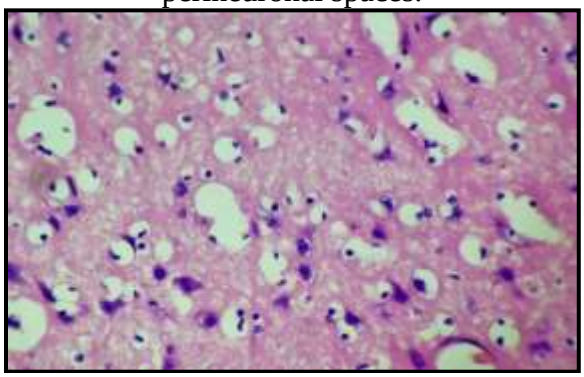
Type	Sections from Brain of Control animal	Sections from Brain of Treated animal
1	 (A) Marked neuronal damage with local calcification.	 (C) Reduced neuronal damage, increased perineuronal spaces.
2	 (B) Marked neuronal damage, microgliosis.	 (D) Moderate neuronal damage with increased peri-neuronal spaces and vacuolations.

Figure12: Histopathological evaluations of sections of Brain of control and treated animals

Histopathology study:

Histopathological observations of the brain from control group (ischemia induced) revealed marked neuronal damage indicated by the neuronal death, dystrophic calcification, microgliosis, increased peri-neuronal spaces and vacuolations, perivascular infiltration of inflammatory cells. (Fig.12 (A and B))

The brains of the rats treated with the test formulation revealed reduction in the neuronal damage, however there was moderate neuronal death, perivascular and perinueronal vacuolations (Fig.12 (C and D)).

The Histopathological evaluations of the test group brains showed attenuation of the changes observed due to ischemia.

CONCLUSION:

The present study suggests that niosomal formulation can provide consistent and release of Rutin and Quercetin from different niosomal formulations. It will lead to sustained action of the entrapped drugs. Moreover, this formulation also provides the ease of administration as it is in the liquid form at non-physiologic conditions and thus helps in increasing patient compliance.

Above all, the present study demonstrated the satisfactory niosomal formulations of Rutin and Quercetin and their evaluation. This information will be useful for a more comprehensive experimental design to better understand the interactions among all the variables and to obtain the appropriate design space. As Quercetin and Rutin formulation has shown reduction in neuronal damage it can also be further evaluated for anticancer and cardioprotective activity.

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