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Original Article

EVALUATION OF LIPOSOMAL GOSSYPIN IN ANIMAL MODELS OF EPILEPSY

DHEERAJ NAGPAL[a], NIDHI AGARWAL[b*], DEEPSHIKHA KATARE[c*]

[a]Amity Institute of Pharmacy, Amity University, Noida Uttar Pradesh, India, [b]Jamia Hamdard, Hamdard University, Centre for Translational and Clinical Research, Faculty of Science, New Delhi, India, [c]Amity Institute of Biotechnology, Amity University, Noida, Uttar Pradesh, India
Email: dpkatare@amity.edu

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ABSTRACT

Objective: Epilepsy is a chronic neurological disorder affecting 1 % of the population worldwide. A number of studies have reported neuroprotective, anticonvulsant and anti-oxidant activity of gossipin a bioflavonoid isolated from *Hibiscus vitifolius.* The present study was carried out to evaluate the acute effects of liposome entrapped gossypin on Increasing Current electroshock seizures (ICES) test; Pentylenetetrazole (PTZ) induced seizures and status epilepticus in mice.

Methods: Gossypin liposomes were prepared by film hydration technique, and the effect of liposomal Gossypin formulations was studied in two doses i.e. 2.5 mg/kg and 5 mg/kg given per oral on ICES test and PTZ induced seizures in mice. Same doses of the formulation were administered by intravenous route during PTZ induces status epilepticus in mice.

Results: The results indicated that liposome entrapped Gossypin in doses 2.5 mg/Kg and 5 mg/Kg demonstrated significant increase in seizure threshold and latency to generalized seizures in ICES test and PTZ induced seizures respectively. Oxidative stress parameters like malondialdehyde (MDA) and glutathione were estimated in brain tissues in mice. Increased levels of MDA and glutathione were reduced and liposomal Gossypin suppressed the progression of kindling in mice. These results suggest that liposomal Gossypin appears to possess protective activity against kindling in mice.

Conclusion: To conclude, the study supports that liposomal Gossypin offers protection against PTZ kindling in mice. Liposomal Gossypin administration significantly reduced the progression of kindling in mice therefore it could be a promising candidate to control both development of seizures and oxidative stress during epilepsy.

Keywords: Epilepsy, Gossypin, Liposomes, ICES and PTZ induced seizures, Status epilepticus

INTRODUCTION

Epilepsy is a neurological disorder that affects a wide range of people throughout the world. It is a disorder of the brain characterized by the unpredictable and periodic occurrence of a transient alteration of behavior due to the disordered, synchronous and rhythmic firing of populations of brain neurons [1].

In the early 1990s, many new antiepileptic drugs (AEDs) have entered the market. Despite, the therapeutic arsenal of old and new AEDs, approximately 30 % of patients with epilepsy still suffer from seizures.

As the majority of antiepileptic drugs are consumed life long, concomitant administration of other drugs predisposes to the risk of drug interaction [1].

Adverse effects of AEDs are common and can have a considerable impact on quality of life and contribute to treatment failure in up to 40 % of patients. The most common adverse effects are dose dependent and reversible. Idiosyncratic effects, such as skin rashes, and chronic effects, such as weight gain, can lead to high rates of treatment discontinuation and complicate clinical management. Nearly all conventional AEDs increase the risk of congenital malformations when taken during pregnancy, with valproate posing a potentially greater risk.

Data from clinical and experimental reports suggest the involvement of oxidative stress in the pathophysiology of epilepsy [2].

Excessive oxidative stress contributes to neuronal degeneration through lipid peroxidation and decreased glutathione concentrations in the epileptic focus [3].

Thus, it is necessary to investigate for an antiepileptic agent that is highly efficacious as well as safe in terms of drug related toxicity.

Ongoing research on certain plant products has paved the way towards the development of the newer category of AEDs therapy [4].

Flavonoids are polyphenolic compounds that occur ubiquitously in foods of plant origin. Over 4000 different naturally occurring flavonoids have been described. The flavonoids show various biological activities including antioxidant, anti-inflammatory activity, cardioprotective and antitumor activity [5].

Gossypin a bioflavonoid (gossypin-8-0 glucoside; 3, 5, 7, 3, 4-pentahydroxy-8-0-glucosylflavone), is naturally occurring in various plants belonging to the family of *Malvaceae* [6].

Gossypin has been found to possess anti-carcinogenic, anti-allergic, anti-diabetic, anti-inflammatory and neuroprotective effects [5, 7-10]

Gossypin (10 and 20 mg/kg, per oral p. o) was reported to have anticonvulsant activity against pentyelene tetrazole, strychnine and maximal electroshock-induced seizures in mice [11]. Data from the study suggested that gossypin showed neuroprotection/seizure protection probably through GABA (gamma amino butyric acid) aminergic and glycine inhibitory mechanism. Although Gossypin possesses anticonvulsant activity but the same as a formulation is still not available. Thus, in view of the above liposomal formulations of gossypin were formulated and tested against ICES test, PTZ induced seizures and status epilepticus in mice.

Further MDH and glutathione levels were also measured to determine if oxidative stress was involved in epilepsy.

MATERIALS AND METHODS

Animals

Healthy Swiss albino inbred mice weighing 24-30 g was used. The animals were acquired from the animal house facility from the central animal house Amity University.

Animals were housed in a group of six mice per cage with free access to pellet diet and water. Care of animals was taken as per the guidelines of committee for the purpose of central and supervision

of experiments on animals, and the study was approved by Institutional Animal Ethics Committee, Amity Institute of Pharmacy, Amity University (CPCSEA/AIP/2013/12/04).

Drugs and dosing schedule

Gossypin, PTZ, Cholesterol, Dipalmitoyl phosphatidylcholine, Distearoyl phosphatidylcholine & Egg phosphatidylcholine were purchased from M/S Sigma-Aldrich USA.

In order to perform experiments, liposomal Gossypin was prepared by Film hydration technique. Briefly, the desired amounts of lipid and cholesterol were weighed (including drug) into 50 ml pear-shaped flask, and 4 mL of chloroform and methanol mixture (1:1) was added to dissolve lipids. The organic solvent mixture was then removed under vacuum using rotary evaporator at 60 °C for 30 min, after which the flask was kept under vacuum overnight to remove completely any residual solvent.

Encapsulation of Gossypin into liposomes was accomplished by the addition of pH 7.4 phosphate buffer saline (PBS) containing 2 % Poloxamer P188. Dry lipids were hydrated with PBS [12].

The prepared liposomes were characterized for particle size (measured by photon correlation spectroscopy), particle shape through optical microscopy and TEM [12, 13].

Gosssypin containing liposomes were prepared using different phospholipids viz. dipalmitoyl phosphatidylcholine (PPCL), distearoyl phosphatidylcholine (SPCL), Egg phosphatidylcholine (EPCL), 1-2-dimyristoyl-sn-glycero-3-phosphocholine (MPCL) and were evaluated for entrapment efficiency and *in-vitro* drug release. Formulation EPCL (table 1) containing egg phosphatidylcholine showed highest entrapment efficiency of 89.29 % and an *in-vitro* drug release of 83.441 %. EPCL was selected as the final optimized formulation.

The effect of liposomal gossypin formulation was studied in two doses 2.5 mg/Kg & 5 mg/Kg given per oral on ICES test and PTZ induced seizures in mice.

Same doses of the formulation were administered by intravenous route during PTZ induced status epilepticus in mice. For ICES and PTZ induced seizure test control animals received (placebo) formulation orally.

However, in status epilepticus model the animals received the liposome (placebo) formulation intravenously (i/v).

Placebo formulations (without drug) were given in order to assess that the anti-epileptic effect was only due to Gossypin and not of any other excipient present in the formulation.

Evaluation of liposomal formulations in animal models

ICES test

The ICES test as proposed by Kitano [14] and modified by Marwah [15] *was* used to evaluate the anti convulsogenic effect of formulations.

Starting with a current of 2 milliamperes (mA) electroshock was delivered to each mouse via ear electrode as a single train of pulses (for 0.2 s).

The current at which tonic hind limb extension was observed was recorded as seizure threshold current. If no tonic hind limb extension was observed by a current of 30 mA, electroshock was terminated.

PTZ induced seizure model

PTZ [16] seizures were induced with a dose of 60 mg/kg, intraperitoneally (i. p) this being the dose that produced myoclonic jerks and generalized seizures in all animals without mortality. The formulation was administered to the animals 30 min prior to the administration of PTZ. The animals were observed for 30 min by placing them in a separate cage. The latency to generalized seizures was recorded. In the absence of seizures within 30 min, the latency time was taken as 1800 s.

Status epilepticus

The method as proposed by Raines [17] was used to induce status epilepticus. Phenytoin sodium (40 mg/Kg) dissolved in alkaline saline was administered i. p. in a volume of 0.1 ml/10 g body weight, to prevent the terminal tonic hind limb extension produced by PTZ which was administered 2 h later, in a dose of 80 mg/Kg through subcutaneous(s/c) route. The time needed for the development of unequivocal sustained clonic seizure activity involving the limbs was noted. Seizure Free State of 1 h was taken as protection. The formulation was administered i/v and the observation was made 30 min after administration.

Measurement of oxidative stress parameters

Tissue preparation

At the end of the study period, the animals were sacrificed under deep anesthesia; the brain was quickly dissected out, washed with ice-cold sodium phosphate buffer, weighed and stored over ice. The brains wee further processed within half an hour of dissection, and the oxidative stress parameters were conducted on the same working day. Brain tissues were homogenized in a volume 10 times weight/Volume (w/v) sodium phosphate buffer (7.4 pH, ice cold, a mixture of potassium dihydrogen phosphate and disodium hydrogen phosphate). The homogenate was centrifuged at 3000 rpm for 15 min. The supernatant was collected for estimation of various oxidative stress parameters.

Measurement of lipid peroxidation

MDA, which is a measure of lipid peroxidation, was measured spectrophotometrically by the method described by Okhawa [18].

Reduced glutathione was estimated spectrophotometrically by the method described by Ellman [19].

The data were expressed as mean±standard error means (SEM), and the results were analyzed by ANOVA followed by *Tukey's* test. *p*-value less than equal to 0.05 was considered significant.

RESULTS AND DISCUSSION

Characterization of liposome's

Vesicle size

The size of the drug loaded liposomes was carried out using Master sizer 2000. Optimized formulation showed a vesicle size of 104.35 nm.

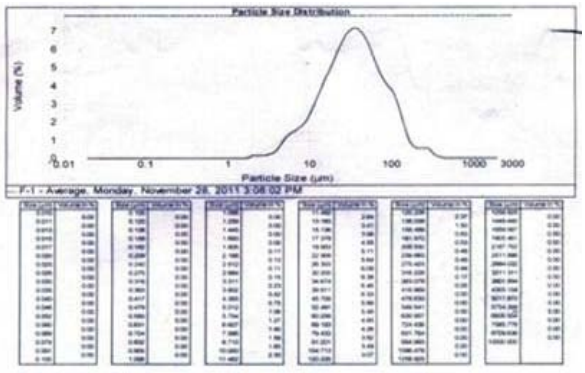

Fig. 1: Vesicle size for optimized formulation

Vesicle shape

Vesicle Shape of the liposomes was analyzed by optical microscopy. Pictures of liposomes (fig. 2) were taken at a magnification of above 44 times by Nikon camera.

The liposomes were spherical and bilamellar. The TEM of optimized formulation also showed the presence of spherical particles (fig. 3).

Fig. 2: Microscopic picture of the optimized formulation

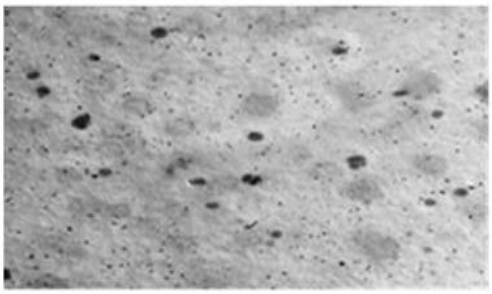

Fig. 3: TEM picture of the optimized formulation

Table 1: *In-vitro* drug release and entrapment efficiency

Formulation	Drug (mg)	Phospholipid (mg)	Cholesterol (mg)	Surfactant concentration (% w/v)	% Entrapment efficiency	*In-vitro* drug release
EPCL	10	41.80	17.40	0.2	88.81±0.30	83.41±0.52
MPCL	10	37.38	1740	0.2	43.19±0.54	54.37±0.25
PPCL	10	40.37	17.40	0.2	98.40±0.39	64.80±0.38
SPCL	10	43.45	17.40	0.2	82.45±0.26	60.00±0.20
SLL	10	37.28	17.40	0.2	98.68±0.28	66.96±0.34

Number of Samples used 03, Data expressed as mean±SD

Animal studies

The results indicated that liposome entrapped gossypin was able to cross the blood-brain barrier as indicated by the mass spectrophotometric analysis of brain homogenate solutions (fig. 4) of formulation treated mice. Liposome entrapped gossypin increased seizure threshold current as compared to vehicle treated and placebo treated animals in ICES test.

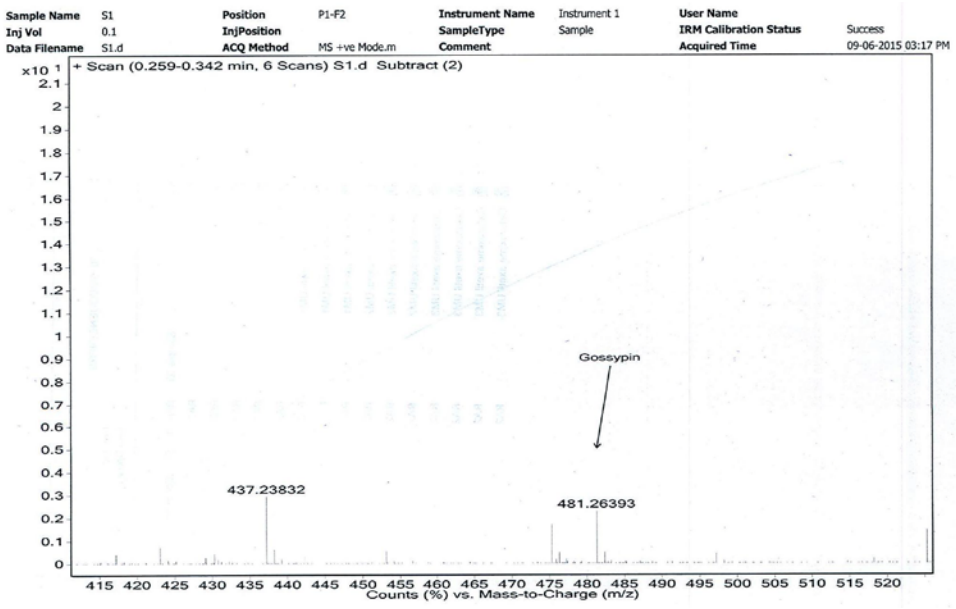

Fig. 4: Mass spectrophotometric analysis of brain homogenate

Table 2: Effect of liposomal formulation (F1 & F2) in ICES test in mice

Groups	Treatment	Dose	ICES seizure threshold (mA)
I	Vehicle	10 ml/Kg	15.33±1.63
II	Placebo	10 mL/Kg	14.38±0.67
III	F1	2.5 mg/Kg	26.66±3.01
IV	F2	5 mg/Kg	27.16±2.22*

A number of animals used 6, Data expressed as mean±SEM ANNOVA followed by Tukey's Test P<0.05 was considered statistically significant, F1 and F2 are liposomal gossypin formulations, *p<0.05 Vehicle vs F2**,** Liposome-entrapped Gossypin also increased the latency to PTZ induced generalized seizure as compared to vehicle and placebo.

Table 3: Effect of liposomal formulation (F1 & F2) in PTZ induced seizures

Groups	Treatment	Dose	Latency to generalized seizures (s)
I	Vehicle	10 ml/Kg	90.66±3.93
II	Placebo	10 mL/Kg	98.16±2.07^{c*}
III	F1	2.5 mg/Kg	135.5±8.47^{a*}
IV	F2	5 mg/Kg	212.6±16.69^{b*}

A number of animals used 6, Data expressed as mean±SEM ANNOVA followed by Tukey's Test, P<0.05 was considered statistically significant, F1 and F2 are liposomal gossypin formulations, *a p<0.05 Vehicle Vs. F2, *b p<0.05 Placebo Vs. F2, *c p<0.05 Vehicle Vs. F1

Also, in PTZ induced status epilepticus liposome entrapped Gossypin increased latency and duration of generalized clonic seizures

Table 4: Effect of liposomal formulation (F1 & F2) in PTZ induced status epilepticus

Groups	Treatment	Dose	PTZ induced status Epilepticus	
			Latency (min)	Duration (min)
I	Vehicle	10 ml/Kg	12.83±1.56	32.18±2.10
II	Placebo	10 mL/Kg	19.73±1.93	35.66±2.10
III	F1	2.5 mg/Kg	25.33±1.93^{a**,b*}	21.37±2.05^{d*,}
IV	F2	5 mg/Kg	32.63±2.51^{c*}	15.67±1.06^{e*}

Number of animals used 6, Data expressed as mean±SEM ANNOVA followed by Tukey's Test, P<0.05 was considered statistically significant, F1 and F2 are liposomal gossypin formulations, a** p<0.01 Vehicle Vs. F1, ^{b*}p<0.05 Placebo Vs. F1, ^{c*}p<0.01 Vehicle Vs. F2, ^{d*}p<0.01 Vehicle Vs. F1, ^{e*}p<0.01 vehicle Vs. F2

Free radicals are normal products of aerobic cellular metabolism involved in the development of seizures. Repeated PTZ administration has significantly increased free radical generation as indicated by increased MDA level in the vehicle treated PTZ kindled mice (table 5).

In the present study decreased level of glutathione was observed in the vehicle-treated PTZ kindled mice. Gossypin liposomal formulations demonstrated increased glutathione levels in kindled mice brain tissue (table 5).

Table 5: MDA and Glutathione levels in formulation treated groups measured in n moles/g wet tissue and µg/g wet tissue respectively

S. No.	Treatment/Parameters	MDA levels	Glutathione levels
1.	Control	0.95±0.187	125±17.60
2.	PTZ Treated	1.91±0.160***a	303±16.33***a
3.	PTZ+F1Treated	1.31±0.116***b	203±17.51***b
4	PTZ+F2Treated	1.36±0.19***b	201±21.36***b

Data was expressed as mean±SEM, n=6; statistical analysis was done by ANOVA, ***p<0.001, a Vs control, b Vs PTZ.

Among the novel drug delivery system liposomes can be used to enhance the bioavailability and target the drugs to the brain [20].

This data suggests that oral administration of liposome-entrapped gossypin prevents electron convulsions and PTZ induced seizures. Also intravenous administration of liposome-entrapped gossypin was helpful in status epilepticus.

The enhanced effect could be associated to the fact phospholipids carriers can easily cross Blood-brain barrier. This data is in concurrence with other findings where improved effects have been obtained through the use of liposomes as compared to conventional drug delivery systems [21, 22].

Mechanism of action of gossypin on seizures is not completely established. Some of the suggested mechanism probably may be affecting both GABA aminergic and glycine inhibitory mechanism. However, further biochemical and clinical studies are required for developing a promising formulation as an alternative to the existing anticonvulsant therapy opted during epilepsy.

CONCLUSION

To conclude, the study supports that liposomal Gossypin offers protection against PTZ kindling in mice. Liposomal Gossypin administration significantly reduced the progression of kindling in mice therefore it could be a promising candidate to control both developments of seizures and oxidative stress during epilepsy.

CONFLICT OF INTERESTS

Declare none

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