IOSR Journal Of Pharmacy *www.iosrphr.org*
(e)-ISSN: 2250-3013, (p)-ISSN: 2319-4219
Volume 6, Issue 11 Version. 1 (Nov. 2016), PP. 30-35

Design, Fabrication and Characterization of Thrombolytic Activity of Bauhinia Racemosa Extract Loaded Nanoemulsion.

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ABSTRACT: Background: Thrombosis is the formation of an unwanted clot within a blood vessel or heart. It is the most common abnormality of hemostasis, but it can turn to larger complication causing disturbance in the normal functioning of many organs.
Aim: To evaluate the blood related properties, acute oral toxicity, thrombolytic activity of *Bauhinia racemosa*, and to convert it into an nanoemulsion formulation.
Material and Methods: The leaves of the plant were collected from Sulur region, Coimbatore, India. They were then shade dried and extracted with ethanol. This extract was then subjected to acute oral toxicity for 14 days. The extract was then studied for thrombolytic activity and finally designed into an nanoemulsion using Tween 80, ethanol, cinnamon oil, distilled water in varying ratios of 2:1, 3:1, 4:1. The prepared nanoemulsion was then evaluated for release study, based on the release the optimized formulation was selected and further studies like particle size distribution, zeta potential, particle morphology, stability study were carried out.
Results: The acute oral toxicity study showed that the extract was safe upto 2000mg/kg of body weight. The thrombolytic activity was carried out by in-vitro method and gave appreciable results. The extract showed activity close to that of the standard. The other evaluation parameters of nanoemulsion like paricle size, zeta potential, particle morphology study was done only for the optimized batch. The particle size and zeta potential reports were good. The stability study shows satisfactory results.
Conclusion: Therefore this study was concluded that the *Bauhinia racemosa* extract loaded nanoemulsion drug delivery is effective for the treatment of thrombosis.

Key words: *Bauhinia racemosa*, nanoemulsion, solublity.
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I. INTRODUCTION

Blood constitutes about 7 % of body weight, this proportion is less in woman and considerably greater in children. Blood in the blood vessel is always in motion. The flow is such that body cells have a fairly constant environment. Hemostasis is the process that maintains the blood within the blood vessels. It is a complicated but efficient mechanism interlocking responses of the blood vessels, the platelets, the coagulation factors and the fibrinolytic mechanism.[(1)]The thrombolytic activity was carried out and the results obtained were significant. The results are given in Table no: 4

3.3 Determination Of Thrombolytic Activity:

Table no: 4. Effect of leaf extract/ drug on *in-vitro* clot lysis.

Extract/Drug	**Percentage of clot lysis (Mean ± S.D)**
Streptokinase	91.13 ± 1.29
Water	2.725 ± 0.98
Ethanolic extract of *B.racemosa* leaves	90.77 ± 1.67
DMSO	1.130 ± 0.25

Thrombogenesis coagulation of blood occurs in blood vessels that have not been injured, resulting in blockage of the concerned blood vessels leading to serious consequences.[(2)]The initial step in making the diagnosis of a blood clot is obtaining a patient history. The blood clot a does not cause any problem but the location of the blood clot and its effect on blood flow that causes symptoms and signs.[(3)]*Bauhinia racemosa* is a small crocked tree. The aerial parts of the plant namely the leaf, flower, bark, fruit and bud are used to extract the active phytochemical constituent and thus used for its medicinal purpose. A number of active constituents are found in *Bauhinia racemosa* like alkaloids, coumarins, apigenin, carbohydrates, flavanoids, quercetin, glycosides, protein, rutin, steroids, tannins. It has a broad range of pharmacological action such as anti-asthmatic, anthelmentic, antibacterial, antiulcer, anti-malarial, astringent, antimicrobial, blood diseases, internal bleeding, anti-tumour, hepatoprotective , anti HIV, antioxidant, anti-hyperglycemic.[(4)]Nanoemulsions are submicron sized, thermodynamicaly stable, isotropic system in which two immiscible liquids (water and oil) are mixed to form a single phase by means of an appropriate surfactant or its mix with a droplet diameter of approximately 5nm-200nm.Because of small size nanoemulsion are transparent. The extract of the plant was designed into an nanoemulsion.(5,6)

II. MATERIALS AND METHODS

2.1 Extraction of leaves [(7)]:
The leaves of the plant were collected, cleaned, shade dried, coarsely powdered and extracted with 500 ml ethanol for 8 hrs by soxhlet apparatus.

2.2 Acute Toxicity Study [(8)]:
The acute toxicity study was carried out in both the animals male as well as female wistar albino rats as per Organization Economic Cooperation Development (OECD 423) guidelines. For the evaluation of acute toxicity, the extract 2000mg/kg body weight was used. The animals were divided into four groups with three animals in each group. Prior to dosing, animals were fasted overnight before being weighed and the dose was administered orally by

gavage method. The following parameters were studied gross behavior study [9], body weight analysis[10],hematological study [11], biochemical study [10], gross necropsy and histopathological study [12].

2.3 Determination Of Thrombolytic Activity [13] :

The dry crude extract (1mg) was suspended in 1ml of DMSO. 1ml/tube of blood was added into pre-weighed sterile micro centrifuge tube and incubated at 37°C for 45 mins. After clotnformation, the serum was completely removed without disturbing the clot and each tube having the clot was again weighed to determine the clot weight.To each micro centrifuge tube containing the pre weighed clot, 100µl DMSO solutions of different partitionates along with the crude extract was added separately. As, a positive control100µl of streptokinase (SK) and as a negative non thrombolytic control 100µl of distilled water and 100µl of DMSO were added separately to the different control tubes. All the tubes are then incubated at 37° C for 90 mins and observed for clot lysis. After incubation the released fluid was removed and the tubes were weighed again. The difference in weights taken before and afterclot lysis were expressed as percentage of clot lysis as shown.

Clot weight = weight of clot containing tube – weight of tube alone

% of clot lysis = (weight of released clot/ clot weight)* 100.

2.4 Components screening by solubility determination [14]:

One of the important factors to formulate a stable nanoemulsion system is choosing components of nanoemulsion which are oil, surfactants and co-surfactants. The components were selected by determining the solubility of the extract in different oils like coconut oil, neem oil, black cumin seed oil, cinnamon oil, soyabean oil; surfactants like tween 80, tween 20, span 80; co-surfactants like acetone, ethanol, methanol.1mg of the extract was dissolved in 1ml of the above mentioned exipients and the solubility was determined by visual observation with eyes.

2.5 Preparation of nanoemulsion of the extract [15]:

Nanoemulsion was prepared by the ultrasonic emulsification method. Briefly, accurately weighed quantity of drug was dissolved in measured quantity of ethanol (co-surfactant). This was followed with the addition of measured amount of Tween 80 (surfactant). This mixture was called as Smix. By varying the concentration of surfactant and the co- surfactant various combinations of Smix ratios were prepared. The mixture was allowed to get homogenized properly by the help of an magnetic stirrer. To this cinnamon oil was added followed by required amount of distilled water and allowed to get a uniform, homogenized emulsion. This formed emulsion was then sonicated for 30 mins to get the nano emulsion. By, varying the Smix ratio as 2:1, 3:1, 4:1and the concentration of oil and water 9 batches of nanoemulsion were formulated. The prepared nanoemulsion was then evaluated for release study, based on the release the optimized formulation was selected and further studies like particle size distribution, zeta potential, particle morphology, stability study were carried out

III. RESULTS

3.1 Extraction of leaves:

The extract was collected, concentrated and the yield was found to be 15.95%.

3.2 Acute Toxicity Study:

During the study period of 14 days, there was no mortality or morbidity observed in the experimental animals followed by single oral administration of extract.

3.2.1 Gross Behavior Study:

The results of the study revealed no adverse change in cage side observation in animals. All the animals in the control and treated groups were found healthy as well as active.

3.2.2 Body Weight Analysis:

No considerable changes were observed in the body weight between the groups and the data are in Table no: 1.

Table no: 1. Effect of leaf extract on body weight.

Groups	Body weight (gm) on day 0 (n=3)	Body weight (gm) on day 14(n=3)
Control	178.66 ± 6.02	176.00 ± 3.60
Group – 1 (minimum dose)	174.66 ± 4.51	173.33 ± 1.53
Group – 2 (medium dose)	206.33 ± 6.02	201.66 ± 8.08
Group – 3(maximum dose)	238.00 ± 7.54	236.00 ± 3.60

3.2.3 Hematological Study:

All hematological parameters of test group was compared to that of the control group. There were no significant changes in the tested hematological parameters. The results are shown in Table no: 2.

Table no: 2. Effect of leaf extract on hematological parameters.

Parameters	Control group (n=3)	Group – 1 (minimum dose). (n=3)	Group -2 (medium dose). (n=3)	Group-3 (maximum dose). (n=3)
Hb (g %)	13.76 ± 0.44	13.36 ± 0.61	13.03 ± 03	14.23 ± 0.30
WBC (10^3/ cubic millimeter)	12.3 ± 0.4	13.2 ± 0.3	11.7 ± 0.65	12.23 ± 0.45
RBC (10^6/cu mm)	5.71 ± 0.07	5.44 ± 0.187	5.37 ± 0.40	5.73 ± 0.04

3.2.4 Biochemical Study:

There were was significant changes in the treated group when compared to the control group. The biochemical parameters of the control and treated group are presented in Table no: 3.

Table no: 3. Effect of leaf extract on bio-chemical parameters.

Parameters	Control group. (n=3)	Group – 1 (minim dose). (n= 3)	Group – 2 (medium dose). (n=3)	Group – 3 (maximum dose). (n=3)
SGPT (IU/L) (International unit/ liter)	103.4 ± 0.45	103.56 ± 1.76	104.73 ± 1.78	102.33 ± 0.47
SGOT (IU/L)	277.3 ± 0.6	272.3 ± 2.55	279.73 ± 4.65	270.23 ± 0.7
Total cholesterol (mg/dl)	67.66 ± 0.54	60.63 ± 1.52	73.46 ± 0.90	66.23 ± 0.41
Triglycerides (mg/dl)	81.16 ± 0.44	79.86 ± 1.07	83.4 ±0.55	79.13 ± 0.60
Protein (mg/dl)	7.46 ± 0.15	7.26 ± 0.40	7.2 ± 0.35	7.13 ± 0.2
Creatinine (mg/dl)	0.68 ± 0.03	0.62 ± 0.02	0.64± 0.03	0.66 ± 0.03

3.2.5 Gross Necropsy and Histopathological Studies:

Morphological observation of abdominal cavities, their content, organs like liver, heart and kidney were examined and found that there was no sign of inflammation or toxicityin all the groups.The results of acute toxicity studies clearly demonstrate that the extract treated animals are devoid of any toxic sign and indicates that it is safe at the dose of 2000mg/kg body weight of animal3.4 Components screening by solubility determination:The components of formulation were screened for solubility. The results are given in Table no: 5. Acetone was not selected because of its volatile nature and methanol was not selected because of its toxic nature.

Table no: 5. Screening of components for solubility.

S.NO	COMPONENTS	SOLUBILITY
1	OIL: Coconut oil Neem oil Black cumin seed oil Cinnamon oil Soyabean oil	 Sparingly soluble Insoluble Sparingly soluble Completely soluble Sparingly soluble
2	SURFACTANT: Tween 80 Tween 20 Span80	 Sparingly soluble Insoluble Insoluble
3	CO-SURFACTANT: Ethanol Acetone methanol	 Completely soluble Soluble Soluble

3.5 Preparation of nanoemulsion of the extract:

The naanoemulsion was prepared and the following evaluation test was carried out as follows.

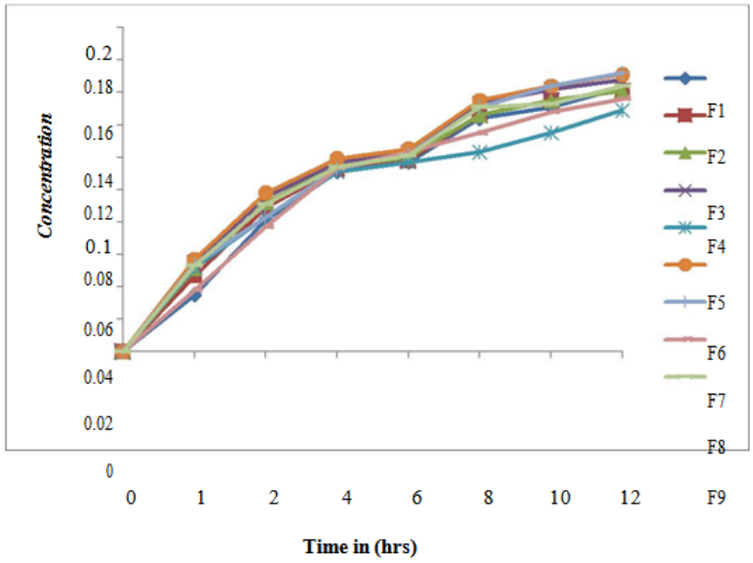
3.5.1 In-vitro release study:

The drug release was slow sustained and dependent upon the drug. Among the nine formulations F6 showed the maximum absorbance value during the 12 hr release study. The results are given in Table no: 6. The release is shown in Fig no: 1.

Table no: 6. Cumulative in-vitro release profile of the prepared nanoemulsion.

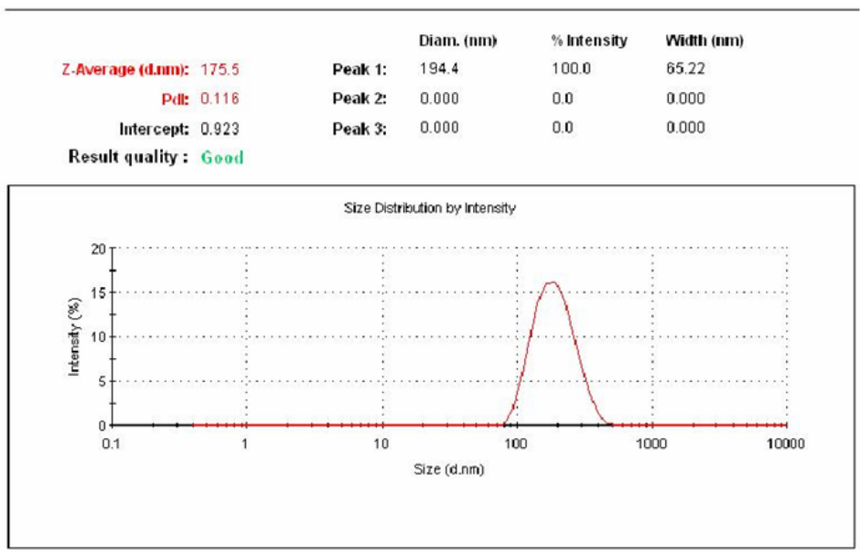
Time (hours)	Concentration.								
	F1	F2	F3	F4	F5	F6	F7	F8	F9
0	0	0	0	0	0	0	0	0	0
1	0.035	0.047	0.051	0.055	0.052	0.057	0.054	0.038	0.053
2	0.081	0.089	0.090	0.095	0.083	0.098	0.080	0.077	0.090
4	0.110	0.113	0.115	0.117	0.111	0.119	0.113	0.112	0.114
6	0.117	0.118	0.120	0.123	0.117	0.125	0.122	0.123	0.121
8	0.144	0.146	0.149	0.153	0.123	0.155	0.150	0.135	0.151
10	0.152	0.155	0.158	0.162	0.135	0.164	0.155	0.148	0.153
12	0.163	0.161	0.165	0.168	0.149	0.170	0.152	0.156	0.164

Fig no: 1. Cumulative in-vitro release profile of the prepared nanoemulsion.



3.5.2 Particle size analysis:
The particle size and size distribution are important for nanoparticles drug delivery. The lower the value the narrow the size distribution or the more uniform of the nanoparticles.
3.5.3 Zeta potential determination:
The zeta potential represents an index for particle stability. This is important in preventing aggregation of particles.

Fig no: 2. Particle size distribution of the prepared nanoemulsion.



3.5.4 Particle morphology:
The prepared nanoemulsion are subjected to morphological analysis by Scanning Electron Microscopy (SEM).

3.5.5 Stability study by mechanical stress method:

The studies revealed that there is no change in the formulationeven after 60 mins centrifugation at 2000 rpm.

Table no: 7. Stability study by mechanical stress method.

Centrifugation time (min)	% phase separated after centrifugation								
	F1	F2	F3	F4	F5	F6	F7	F8	F9
10	-	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-
40	-	-	-	-	-	-	-	-	-
60	-	-	-	-	-	-	-	-	-

3.5.6 Accelerated temperature study:
The studies revealed that there is no change in the physical state of the nanoemulsion on storage.

Table no: 8. Stability study by accelerated temperature method.

Temp/days	% Phase separation								
	F1	F2	F3	F4	F5	F6	F7	F8	F9
4±1°C									
1	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-
30	-	-	-	-	-	-	-	-	-
25±1°C									
1	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-
20	0.5	0.5	0.5	0.5	0.5	1.0	1.0	1.0	1.0
30	1.0	1.0	1.0	1.5	1.5	1.5	2.0	2.0	2.0
40±1°C	BREAKING OF EMULSION								
1									
10									
20									
30									

IV. DISCUSSION AND CONCLUSION

The present study was a satisfactory attempt to formulate the nanoemulsion with a view of improving the oral bioavailability and thus giving a prolonged release of drug.The yield of the extract was found to be 15.95% w/v. From the data of the acute toxicity studies it clearly demonstrates that the extract was safe up to 2000 mg/kg body weight of animal. The thrombolytic activity was carried out by in-vitro method and gave appreciable results. The extract showed activity close to that of the standard.Compatibility study was carried out by screening the components for solubility by visual observations. The nanoemulsion was prepared in 9 batches with varying oil: water ratios like 2:1, 3:1, 4:1. The batch that showed a uniform and continuous high release was selected as the optimized batch. F6 was selected as the optimized batch.The other evaluation parameters of nanoemulsion like paricle size, zeta potential, particle morphology study was done only for the optimized batch. The particle size and zeta potential reports were good. The stability study shows satisfactory results.Therefore this study was concluded that the *Bauhinia racemosa* extract loaded nanoemulsion drug delivery is effective for the treatment of thrombosis.

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