

Pharmacopuncture of *Bauhinia variegata* Nanoemulsion Formulation against Diabetic Peripheral Neuropathic Pain

Pushpraj S Gupta¹, Sunil K Singh², Abhishek K Tripathi^{1*}

¹ Department of Pharmaceutical Sciences, Sam Higginbottom University of Agriculture, India

² Department of Pharmaceutical Sciences, United Institute of Pharmacy, Naini, India

Key Words

Neuropathic pain, plantar test, Von Frey filament stimulation, *Bauhinia variegata*, diabetes, nanoemulsion

Abstract

Objectives: The objective of the study was to prepare *Bauhinia variegata* loaded nanoemulsion (formulation and determine the efficacy of herbal drug formulation against diabetic peripheral neuropathic pain through acupuncture technique.

Methods: Nine different batches of nanoemulsion (NE1-NE9) of BVN was prepared by varying the S_{mix} ratio and the concentration of oil. BVN was characterized to determine particle size, shape, zeta potential, polydispersity index, optical transmittance, drug release profile and storage stability.

The optimized formulation was subjected to plantar test, behavioral tests of neuropathic pain and Von Frey filament stimulation test. Diabetes was induced by intraperitoneal injection of freshly prepared solution of Streptozotocin (60 mg/kg) to the experimental rats. Animals were made diabetic divided into four groups, Group I was untreated normal control group, Group II was diabetic control group, Group III was *Bauhinia variegata* extract (treated group (100 mg/kg/day, p.o) and

Group IV was BVN treated groups (100 mg/kg/day, p.o) acute and chronically.

Results: The prepared *B. variegata* loaded nanoemulsion was nanosized (124 nm), spherical, uniform and stable over the period of 180 days with no change in physicochemical properties. The blood glucose and body weight of animals was normalizing after four weeks of treatment that was significant with BVN in comparison to diabetic control group. The chronic administration of BVN significantly ($P < 0.001$) decreased hind paw withdrawal latency and attenuated mechanical allodynia as compared with diabetic rats.

Conclusion: Thus, BVN may be an effective drug formulation against diabetic peripheral neuropathic pain.

1. Introduction

Diabetes mellitus (DM) is a metabolic syndrome that have been affected people life around the globe, it is due to the body's inability to either produce or use insulin [1]. In DM, chronic micro and macro vascular effects such as neuropathy, cardiovascular disease and stroke due to constant hyperglycemia [2, 3]. Neuropathic pain is most crucial complications of diabetes mellitus, it is due to severe muscle aches and a continuous burning or tingling symptoms mostly in lower extremities.

Allodynia and hyperalgesia may be appeared in patients having diabetic peripheral neuropathic pain [4].

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Conventional drug delivery systems for treatment of various metabolic disorder often face some limitations, lack of efficacy or availability of drug in body due to improper or ineffective dosage, as well as reduced potency due to first pass metabolism. Nanotechnology has been diverged class of science and its application in several fields with nanometric range of objects to overcome trouble related to drug administration system. Application of nanocarriers, nanoemulsion and other nanotechnological approaches leads to enhance activity against the threatening diseases, fewer side effects, proper drug accumulation and adsorption in target. Nanoemulsions are the advanced nanocarriers for targeted drug delivery of several oral hypoglycemic drugs in comparison to other conventional oral hypoglycemic drugs. Now a days these modern approaches are using frequently to control hyperglycemia and becoming the most promising technology. Lipid nanoparticles and nano emulsion are an effective and novel colloidal delivery system for drug targeting, cosmetic and pharmaceutical products [5].

Bauhinia variegata belongs to family Leguminosae, it belongs to genus Bauhinia that comprises more than 300 species of plants all over the world [6] *Bauhinia* is also known as Cow's hoof because of its leaves shape. This species of Bauhinia is found in throughout the tropical zones, especially in Asian, African and South American countries. *Bauhinia* is used as a folk medicine in some parts of these zones by local people [7] Biological studies of *Bauhinia* extracts have affirmed the free radical reducing and blood lowering activities by its polyphenol and flavonoid [8] Several glycosylated and aglycone flavonoids have been also reported for *Bauhinia variegata* [9] The present study was designed to investigate the effect of *Bauhinia* based nanoemulsion for treatment of diabetic peripheral neuropathic pain.

2. Material and Methods

2.1. Drugs, chemicals and solvents

Streptozotocin, neem oil, coconut oil, cinnamon oil, black cumin seed oil, soyabean oil, span 80, tween 80, tween 20, ethanol, acetone and methanol were procured from Sigma Aldrich Co., USA.

2.2. Extraction of *Bauhinia variegata*

The flowers of *Bauhinia variegata* were collected from the local surroundings of Allahabad, Uttar Pradesh. The air dried and powdered flowers (1.2 kg) of *B. variegata* were extracted with petroleum ether to remove fatty substances. The mark was further extracted with 95% ethanol by hot percolation method. The extract was filtered and concentrated under vacuum at 40 and stored in desiccator. The percentage yield of ethanolic extract (BVE) was found to be 20.8% w/w.

2.3. Compatibility studies of the extract

2.3.1. Solubility study

Selection of components (surfactants and co surfactants) is very importance for preparing a stable nanoemulsion system. It was selected by determining the solubility of *B. variegata* extract in different oils like neem oil, coconut oil, cinnamon oil, black cumin seed oil, soyabean oil; surfactants like span 80, tween 80, tween 20 and co surfactants like ethanol, acetone and methanol.

The physical stability of the excipient and the plant extract was evaluated by dissolving 1mg of the extract in 1ml of the above mentioned excipients at room temperature $25 \pm 1^\circ\text{C}$ and accelerated temperatures $40 \pm 1^\circ\text{C}$ for 8 weeks and visually observed with eyes [10].

2.3.2. Preparation of *B. variegata* loaded nanoemulsion

Ultrasonic emulsification method was used for preparation of nanoemulsion. Based on the solubility study, it was found that *B. variegata* extract was completely soluble in cinnamon oil, ethanol and Tween 80. Briefly, accurately weighed quantity of *B. variegata* dried extract was dissolved in measured quantity of ethanol (co surfactant) followed by addition of measured amount of Tween 80 (surfactant). S_{mix} ratios were prepared by addition of varying concentration of surfactant and co surfactant. The mixture was homogenized with the help of a magnetic stirrer.

Cinnamon oil was added to this mixture followed by addition of distilled water and allowed to get a uniform, homogenized emulsion. The prepared emulsion was sonicated for 30 min to get nanoemulsion.

Nine different batches (NE1 - NE9) of *B. variegata* nano

Table 1 Formulation of different batches of *B. variegata* nanoemulsion

Ingredients	NE1	NE2	NE3	NE4	NE5	NE6	NE7	NE8	NE9
BVE*	100	100	100	100	100	100	100	100	100
Tween 80	40	40	40	40	52.5	52.5	52.5	64	64
Ethanol	20	20	20	20	17.5	17.5	17.5	16	16
Cinnamon oil	20	15	10	5	15	10	5	10	5
Distilled water	20	25	30	35	15	20	25	10	15

* Marked values are in milligrams. All other values are in ml. BVE; *B. variegata* extract.

emulsion was prepared by varying the S_{mix} ratio and the concentration of oil as shown in Table 1 [11].

2.4. Characterization of prepared nanoemulsion

2.4.1. Particle size and particle morphology study

Particle size of BVN was determined using optical microscope. The size distribution analysis was performed using particle size analyzer. The microstructure of BVN was characterized using TEM Hitachi (H-7500) at room temperature. The photo micrographs of BVN were obtained by drying on a microscopic carbon coated grid and viewed under microscope after staining at suitable magnification.

2.4.2. Zeta potential evaluation

Zeta potential of BVN was determined using zeta sizer version 6.2. Sample (1 ml) was placed in a disposable zeta cell. Analysis was performed by inserting palladium electrode in distilled water in the cuvette at 25°C. All values were determined in triplicate.

2.5. In vitro release study

In vitro release study of BVN was evaluated using Franz diffusion cell an artificial cellulose acetate membrane. Franz diffusion cell was maintained at $37 \pm 1^\circ\text{C}$. The membrane was equilibrated before application of the nanoemulsion equivalent to 10 mg of drug onto the donor side.

Aliquots of 1.0 ml were removed at suitable time interval and observed for drug content using UV visible spectrophotometer after appropriate dilutions [12].

2.6. Stability studies

Stability of BVN was determined for a period of 6 months. The formulation was placed at room temperature and studied for physical stability such as creaming, phase separation, or flocculation, accelerated centrifugation cycle and chemical stability such as particle size, drug content, and zeta potential determinations after 6 months of storage.

2.7. Biological evaluation of *B. variegata* nano-emulsion (BVN)

2.7.1. Animals used

Male albino Wistar rats weighing 220 - 280 g were obtained from inbred animal house of C.D.R.I., Lucknow and maintained at standard environmental conditions such as temperature $25 \pm 2^\circ\text{C}$ and relative humidity 45 - 55%. Animals were free access to standard pelleted diet and water ad libitum. The study was approved by IAEC (UIP/IAEC/April 2015/03).

2.7.2. Induction and assessment of diabetes

Stability of Streptozotocin (STZ) was given in the dose of

60 mg/kg, i/p to induce diabetes in animals. Hyperglycemia was produced within 2 days of STZ injection. The animals were included in the study showing blood glucose level more than 250 mg/dL (Fox *et al.*, 1999)

2.7.3. Drug treatment

Animals were selected randomly and divided into six groups each containing 10 animals after assessment of neuropathic pain at 7th day of diabetes induction. Group I was untreated normal control group, Group II was diabetic control group, Group III was *Bauhinia variegata* extract (BVE) treated group (100 mg/kg/day, p.o) and Group IV was BVN treated groups (100 mg/kg/day, p.o); acute and chronically. Group V and VI received naloxone (1 mg/kg, i.p.) 30 min before BVN administration. The treatment schedule was single dose of BVN 30 min before pain assessment for acute condition; however, for chronic treatment administration of BVN was started from 7th day till 21st day injected once a day.

2.7.4. Behavioral tests of neuropathic pain

Thermal stimulus and mechanical stimuli causing hyperalgesia and allodynia, respectively was measured by radiant heat plantar and von Frey test, respectively. The behavioral tests were performed during day time after cage exploration and major grooming activities ceased. The measurement of neuropathic pain, plasma glucose level and body weight was performed before the experiment and on 7, 14 and 21 day after induction of diabetes [13].

2.7.5. Plantar test

Rats were placed in a plexiglass enclosure with transparent glass floor. Infrared beam was passed as a heat source at the hind paw surface. Thermal withdrawal latency was determined to calculate the latency between heat stimulus and withdrawal of paw. It was measured in seconds and paw latency was measured in every 5 min. Tissue damage was avoided with a cut off time of 22 sec. Hyperalgesia was scored by measuring mean latency of the withdrawal responses [14].

2.7.6. Von Frey filament stimulation (VFFS)

The response of hind paw withdrawal was measured using von Frey filament to quantify mechanical allodynia. VFFS was studied with the bending forces ranging from 2 to 60 g (Stolting Inc. Wood Dale, IL). The animals were allowed for 15 min to adapt or to cease exploratory behavior by placing on mesh floor enclosed with plastic chamber. The stimulation was provided at the plantar surface of paw and it was continued till the rat removed its paw. The withdrawal threshold was determined by change in filament size in three consecutive applications [15].

2.8. Statistical analysis

Data are represented as mean SEM. Statistical analysis

was performed using MS excel and one way ANOVA by Bonferroni statistical hypothesis using Graph Pad Prism 8.0.2, statistically different at * $P < 0.05$, $P < 0.01$, $P < 0.001$ in comparison to diabetic control group.

3. Results

3.1. Characterization of prepared nanoemulsion

The characterization of prepared nine nanoemulsion formulations of *B. variegata* is shown in Table 2. The globule size was decreasing with increase in surfactant concentration upto certain extent i.e. NE1 - NE6 (311.00 - 124.33 nm); after that, increase in globule size was observed. The nanoemulsion preparations NE5, NE6, NE7 and NE8 were showing small globule size, in which globule size of NE6 (124.33 nm) was smallest among all formulations. NE6 was also suitable with PDI, zeta potential and transmit-

tance. Thus, NE6 was selected for further evaluation due to its suitability with other physicochemical characters. Initial observation of developed nanoemulsion under optical microscope showed uniform small size vesicles. It was also confirmed by TEM that reveals unilamellar and spherical structure (Fig. 1). The formulation NE6 has 0.252 PDI that was indication of uniformity of globule size (Table 2). The zeta potential is a key indicator of the stability of the nanoemulsion formulation. The higher range of zeta potential showed nanosize globules and conferred stability. The zeta potential of NE6 was more negative compared to the other nanoemulsions. The percentage transmittance of 99.66 for NE6 indicated clear dispersion (Table 2). The batch NE6 was selected as the optimized batch as it displayed optimum response variables of 99.66% optical transparency, low globule size (124.33 nm), polydispersity of 0.252 and zeta potential of 34.48 mV. Hence, NE6 was selected for further processing.

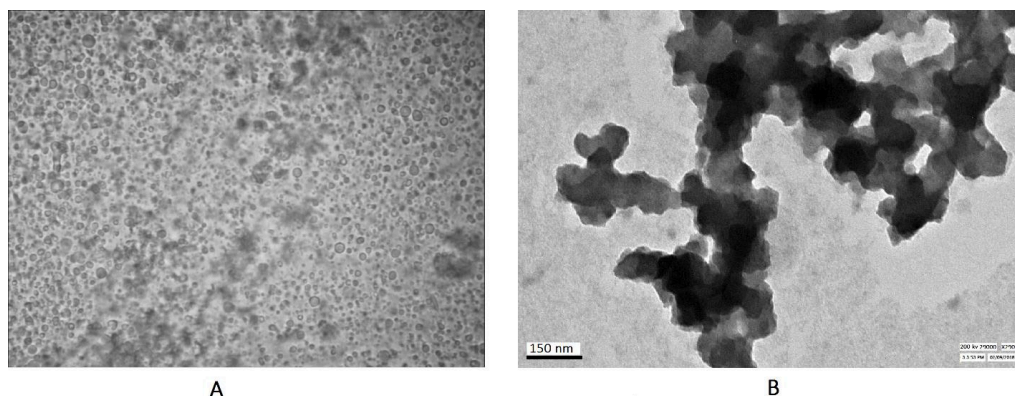


Figure 1 Globule size of *B. variegata* nanoemulsion (A) (A) Optical microscopy image (B) TEM image

Table 2 Characterization of *B. variegata* nanoemulsion

Formulations	Globule size (nm)	PDI (%)	Zeta potential (mV)	Transmittance (%)
NE1	311.00 ± 3.3	0.311 ± 0.019	-25.87 ± 2.43	95.44
NE2	287.42 ± 3.7	0.244 ± 0.111	-32.09 ± 3.22	96.86
NE3	184.37 ± 2.8	0.236 ± 0.013	-26.56 ± 3.45	97.12
NE4	179.74 ± 1.8	0.228 ± 0.027	-31.15 ± 0.78	98.34
NE5	142.17 ± 2.3	0.198 ± 0.019	-32.27 ± 0.15	99.28
NE6	124.33 ± 1.2	0.252 ± 0.029	-34.48 ± 1.54	99.66
NE7	145.86 ± 1.4	0.174 ± 0.016	-28.44 ± 0.12	99.12
NE8	156.86 ± 1.0	0.243 ± 0.001	-26.45 ± 1.12	99.69
NE9	167.32 ± 1.1	0.286 ± 0.004	-27.10 ± 1.09	99.78

Data are represented as mean ± SD (n=3).

Table 3 Stability study of *B. variegata* nanoemulsion on storage

Time (days)	Globule size (nm)	Polydispersity index	Zeta potential (mV)
0	124.61 ± 1.04	0.252 ± 0.01	-34.45 ± 2.4
30	129.11 ± 0.15	0.251 ± 0.06	-33.13 ± 3.2
60	131.32 ± 1.13	0.252 ± 0.02	-33.12 ± 3.1
90	134.22 ± 0.73	0.251 ± 0.05	-32.01 ± 2.3
120	134.02 ± 1.05	0.251 ± 0.02	-32.14 ± 1.2
150	136.43 ± 0.94	0.252 ± 0.03	-32.08 ± 2.6
180	136.71 ± 1.03	0.250 ± 0.08	-31.05 ± 3.3

Data are represented as mean ± SD (n = 3).

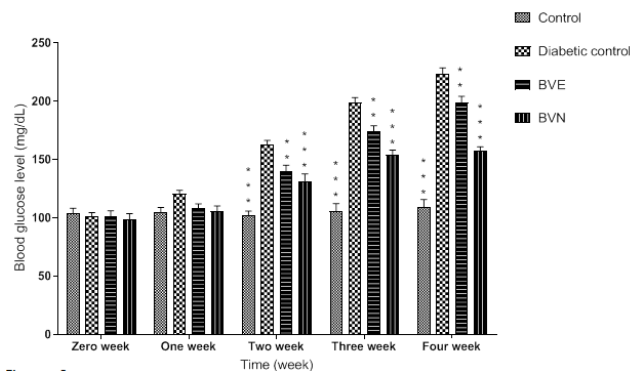


Figure 2 Effects of BVN on blood glucose level of STZ induced diabetic rats All data are represented as mean SEM. Statistically different at P<0.05, P<0.01, P<0.001. BVE - *B. variegata* extract, BVN *B. variegata* nanoemulsion.

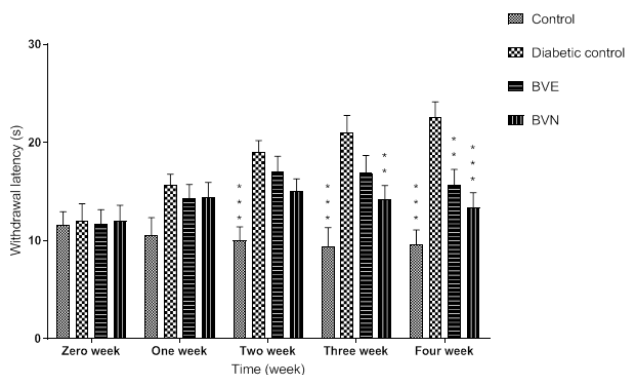


Figure 3 Effects of BVN on the heat hyperalgesia of STZ induced diabetic rats All data are represented as mean SEM. Statistically different at P<0.05, P<0.01, P<0.001. BVE *B. variegata* extract, BVN *B. variegata* nanoemulsion.

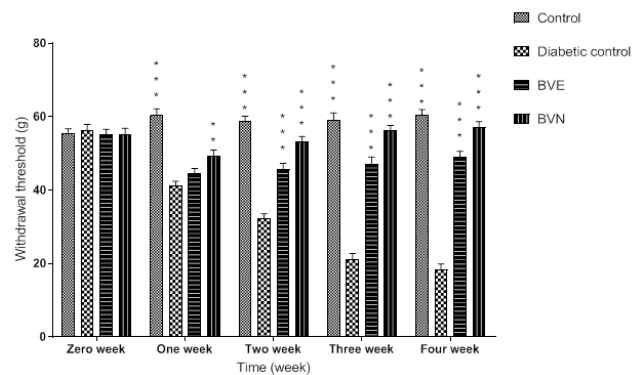


Figure 4 Effects of BVN on the mechanical allodynia of STZ induced diabetic rats All data are represented as mean SEM. Statistically different at P<0.05, P<0.01, P<0.001. BVE *B. variegata* extract, BVN *B. variegata* nanoemulsion.

3.2. *In vitro* drug release

It was observed that maximum drug release from nano-emulsion was achieved within 8h(92.19%). Tween 80 as a surfactant also contributed to release maximum percentage of drug from NE. It was observed that percent drug release was respective with time.

3.3. Stability study

In stability studies, the NE6 was devoid of precipitation, phase separation, creaming and flocculation. In addition, the formulation was also stable after centrifugation (2000 rpm for 15min) at ambient temperature. The results of stability testing represented in Table 3, revealed that there were negligible changes in the parameters of CUN after 6 months of storage.

3.4. BVN effect on blood glucose and body weight

Blood glucose level was raised significantly higher with the injection of Streptozotocin (STZ). Treatment with BVE and BVN did not reduce hyperglycemia significantly in diabetic rats at first week (Fig. 2). Chronic administration of BVN from second to fourth week significantly ($P < 0.001$) reduced raised blood glucose level in diabetic rats. However, BVE also decreased blood glucose level but not much significant as BVN. Results showed that there was a significant decrease in the body weight of STZ-induced diabetic rats in comparison to aged matched control animals. Treatment with chronic administration of BVE and BVN caused raised in body weight, which was more significant with BVN.

3.5. BVN effect on heat hyperalgesia

Administration of STZ results into increased hind paw withdrawal latency to the given stimuli in rats after one week as compared with control animals. No withdrawal latency was achieved after acute treatment of BVN. However, chronic administration of BVN significantly ($P < 0.001$) reduced withdrawal latency as compared with diabetic rats. However, BVE also reduced latency but not much as significant as BVN (Fig. 3).

3.6. BVN effect on mechanical allodynia

STZ caused reduction in withdrawal threshold of von Frey filament diabetic rats as shown in Fig. 4. The mechanical allodynia was started at first week due to STZ injection and it was continued for the three weeks. Administration of BVE and BVN significantly ($P < 0.001$) attenuated mechanical allodynia after 7 days but acute dose of BVN did not change mechanical withdrawal threshold in STZ-induced diabetic rats.

4. Discussion

Diabetes is a disorder of carbohydrate, fat and protein

metabolism attributed to diminished production of insulin or mounting resistance to its action [16, 17]. Phytotherapeutics are extracted from medicinal plants and their active constituents used either in whole food form or in the form of standardized extracts and supplements, for healing purposes [18]. Diabetic neuropathy is a common disorder causes peripheral nerve dysfunction commonly in diabetic patient [19]. In addition, there is a sensory loss of pain because of nerve damage [20]. The major risk factor for diabetes neuropathy is hyperglycemia. Painful diabetic neuropathy cause increased blood glucose level condition called Glucojasinogen [21]. Diabetic neuropathy affects specific nerves, it may cause vision problem and nerve damage to the affected individual [22]. Chronic administration (4 weeks) of *B. variegata* loaded nanoemulsion, 100 mg/kg (p.o.) decreased blood glucose level and progression neuropathy in STZ induced diabetic rats. Reduction in blood glucose level due to herbal medicine was in accordance with Kumar *et al.*, 2016 [23]. It has reported that STZ cause mechanical allodynia in rats [24, 25] Diabetic neuropathy caused reduction of antinociceptive effect in diabetes, it is characterized by neurodegeneration; it results in decreased pain perception [26, 27]. In this study the pain threshold was measured by plantar test apparatus, indicated to decrease in the foot with drawal in diabetic rats (Fig. 3). The tactile stimulus is transmitted through myelinated A β fibers [28]. Herbal drug loaded nanotechnology is effective in treatment of diabetic neuropathic pain as reported in earlier studies [29, 30]

5. Conclusion

Nanotechnology has emerged as an attractive field having several applications in the advancement of drugs. Results indicated that BVN was stable nanoemulsion formulation and its chronic treatment result in reduction of blood glucose level and prevention in progression of diabetic neuropathy. Thus, BVN may be an effective formulation for diabetic neuropathy. In conclusion, the present study demonstrates that chronic prevents weight loss and attenuates mechanical allodynia in STZ-induced diabetic rats. Although further studies are required to clarify the exact mechanisms of BVN for therapeutic effect.

Declaration

The authors declare no conflict of interest.

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