Enhanced Neurobehavioral Effects of Jadwar (Delphinium denudatum) Aqueous Fraction by Implying Nanotechnology Based Approach

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Abstract

Delphinium denudatum Wall. (Ranunculaceae) is an indigenous medicinal herb popularly known as ‘Jadwar’ widely used in traditional Unani system of medicine for the treatment of a variety of human ailments including epilepsy. In order to increase the bioavailability, the nanophytosome of the Delphinium denudatum root aqueous fraction (DNP) was prepared and evaluated.

The phospholipid complex of the obtained aqueous fraction (AF) was prepared with Phospholipon 90H. The size of nanophytosomes was determined by dynamic light scattering. HPTLC fingerprinting of the AF was also performed. PTZ and ICES models were used to evaluate the anticonvulsant activity while Rotarod test, Elevated Plus maze test, and Forced swimming test were used to evaluate other neuropharmacological effects at dose level of 400mg/kg and 800mg/kg p.o.

The particle size of the prepared DNP was around 500 nm. The DNP exerted significant anticonvulsant activity as compared to control, aqueous fraction and placebo treated groups (p<0.001). The DNP significantly increased the threshold current and decreased the mortality percentage against electroshock at all the doses as compared to control, conventional extract and placebo treated groups. However, there was a significant dose dependent reduction in the recovery period following the convulsions (p<0.001). DNP also exhibited anxiolytic and antidepressant activity in a dose dependent manner. Furthermore, DNP did not cause any evidence of neurotoxicity till dose of 2000 mg/kg p.o. It can be hypothesized that DNP may have better access to the brain as evidenced by its improved efficacy than pure aqueous fraction.

Introduction

Delphinium denudatum Wall. (Ranunculaceae) is an indigenous medicinal herb popularly known as ‘Jadwar’ is used widely in traditional Unani system of medicine for the treatment of a variety of human ailments including epilepsy. The aqueous and alcoholic extract of Delphinium denudatum Wall roots have already been reported for its analgesic, anti-inflammatory and other CNS related activities like epilepsy [1]. Earlier reports for the anticonvulsant effect of Delphinium denudatum Wall roots extract reveal significant anticonvulsant activity in aqueous fraction as compared to alcoholic fraction, shows presence of compounds exerting anticonvulsant activity in aqueous extract. However, aqueous soluble phytoconstituent like many flavonoids are poorly absorbed either due to their multiple-ring large size molecules which cannot be absorbed by simple diffusion, or due to their poor miscibility with oils and other lipids, severely limiting their ability to pass across the lipid-rich outer membranes of the enterocytes of the small intestine. Water-soluble phytoconstituent molecules (mainly polyphenoles) can be converted into lipid-compatible molecular complexes. These phospholipid complexes are more bioavailable as compared to simple herbal extracts owing to their enhanced capacity to cross the lipid rich biomembranes and finally reaching the blood [2]. From the literature many studies have reported the beneficial role of phospholipids in enhancing the therapeutic efficacy of some herbal extract/phytoconstituent having poor oral absorption. Silybin is one such phytoconstituent having poor oral bioavailability. It was observed that the Silybin–phospholipids complex has significant upper hand over the pure phytoconstituent in protecting liver and exerting antioxidant activities [3-9]. Recent study with quercetin–phospholipid complex showed that the formulation exerted better therapeutic efficacy than the molecule in rat liver injury induced by carbon tetrachloride [10].

In recent year, the nanonization of traditional Chinese herbal medicines has attracted much attention. Nanoparticles (nanospheres and nanocapsules) are colloidal systems with particles varying in size from 10 nm to 1000 nm. Nanoparticles systems with mean particle size well above the 100 nm standard have also been reported in literature, including nanonized curcuminoids, paclitaxel and praziquantel which have a mean particle size of 450, 147.7, and even higher than 200 nm, respectively. In addition, nanoparticles could also be defined as being submicronic (<1 lm) colloidal systems. The nanospheres have...
a matrix type structure in which the active ingredient is dispersed throughout (the particles), whereas the nanocapsules have a polymeric membrane and an active ingredient core. Nanonization possesses many advantages, such as increasing compound solubility, reducing medicinal doses, and improving the absorbency of herbal medicines compared with the respective crude drugs preparations [11-20].

By combining nanotechnology to the phospholipid complexation the particle size of the phospholipid complex can be reduced to nano size range and this will give an added advantage of increase rate of dissolution leads to immediate absorption of the phytoconstituents.

This is the first time we are reporting the application of nanotechnology for the development of phospholipid based herbal formulation showing better efficacy. With a view to enhancing bioavailability and efficacy, Delphinium denudatum nanophytosome (DNP) have been prepared which is advanced herbal product produced by binding individual components of Delphinium denudatum aqueous fraction to phosphatidylcholine, followed by nanosizing by hi pressure homogenization resulting in a product that is better absorbed, and thus, produces better results than the conventional herbal extract. Hence, the present research work is aimed at evaluating the anticonvulsant and other neuropharmacological activity of nano sized Delphinium denudatum phospholipid complex in rodents.

Materials and method

Animals: Swiss Albino mice strain (either sex) weighing 18-30 gms were procured from central animal house facility of Hamdard University with prior approval from CPCSEA Approval No. JH/ CPCSEA/31-01-2000/Project No. 393/2007-2010. Animals were housed in groups of 6 per cage and maintained at 20–30ºC and 50–55% humidity in a natural light and dark cycle, with free access to food and water. The experiments were performed during the light cycle in awake, freely moving animals that were adjusted to laboratory conditions before proceeding with the experiments.

Drugs: Pentylentetrazole were procured from Sigma (St. Louis, MO, USA), Sodium Valproate generous as a gift sample from Wockhardt research centre (Aurangabad, India), Phospholipon 90H were provided by Lipoid GmbH (Germany) as a gift sample. All other chemicals were procured from Merck (Mumbai, India).

Collection and identification of plant material

Dried roots of Delphinium denudatum collected from M/S Green Earth product Ltd. Greater Kailash New Delhi and a voucher specimen no. NISCAIR/RHMD/CONSULT/- 2008-09/1099/130/Delphinium denudatum/Dated 07NOV 2008 was deposited in the herbarium of NISCAIR, New Delhi after the identification and taxonomical authentication by Dr. H.B. Sing, Head, raw material herbarium & museum, NISCAIR, New Delhi.

Delphinium denudatum Extract Preparation

Delphinium denudatum roots aqueous fraction was prepared as per the method reported by M. Raza et al. 2001 (Figure 1) [21].

Formulation development

The phospholipid complex of the obtained aqueous fraction (AF) was prepared with Phospholipon 90H (Hydrogenated Soy Phosphotidylcholine) at AF: Phospholipid ratio of 1:2. Weighed amount of AF (10gms) and SPC (20gms) were taken in a 1000 ml round bottom flask and 1000 ml of ethanol was added. The mixture was refluxed at a temperature not exceeding 50ºC for 2 h. The resultant solution was dried under vacuum to remove traces of solvents in rotary evaporator apparatus. The collected mass was suspended in to the 200 ml of Dist. Water and homogenised by Hi pressure homogenizer at 5000-25000rpm for 5 cycles to achieve the minimum particles sized. The homogenised nanosuspension was freeze dried to get the Delphinium denudatum nanophytosomes (DNP).

Characterization of Nanoparticles

The size of nanophytosomes was determined by dynamic light scattering (Nano ZS90, Malvern, UK), taking the average of five measurements, whereas zeta-potential was estimated on the basis of electrophoretic mobility under an electric field, taking an average of three measurements.

Transmission electron microscopy (TEM)

Contrast for TEM was achieved by adding 1-dodecane (20%, w) in major oil (ethyl butyrate) as a dopant to the nanophytosome. A 1000-fold dilution of stock nanophytosome suspension was dripped onto a carbon-coated gold grid, and left overnight to allow moisture evaporation. The grids were then placed in a sealed container where OsO4 vapor was diffused through the polysiloxane/silicate to stain the 1-dodecane in the nanophytosome for not less than 4 h. TEM images were obtained at 75 kV. Data were compared with images of three independently made formulations.

HPTLC fingerprinting

Phospholipid complex of Delphinium denudatum aqueous fraction and pure extract were dissolved in methanol and the methanolic solutions were spotted on the Silica Gel 60F254 pre-coated TLC plates and chromatogram was developed in chromatographic chambers using dichloromethane: methanol 99:1 as solvent system at a room temperature of 30ºC, at an angle of 70º. After development of chromatogram, the plates were scanned with the help of Camag TLC scanner IV at wavelength of 366 and the Rf values of the spots were recorded (Figure 2).

**Figure 1:** Picture of the crude herb Delphinium denudatum

**Figure 2:** Comparative HPTLC Fingerprinting of DNP, Aqueous Fraction and Lipid
Neuropharmacological studies

Anticonvulsant activity: Pentylenetetrazole (PTZ, 70mg/kg, i.p.) and increased current electroshock (ICES) models in Swiss Albino mice (4 week, 25-35 g) were used to evaluate anticonvulsant activity. PTZ-induced seizures were induced as described in the previous report [22]. Briefly, mice were given PTZ at a dose of 70 mg/kg i.p., this being the dose that produced seizures in all the animals treated with the drug. The animals were observed immediately after PTZ injection for a period of 30 min. The observations included: latency to and incidence (%) for (i) myoclonic jerks and (ii) clonic generalized seizures with fall. The ICES test, as proposed by Kitano et al. [23] and modified by Marwah et al. [24], was used to evaluate the anticonvulsant effect of the drugs. Starting with a current of 2 mA, electroshock was delivered to each mouse via ear electrodes as a single train of pulses (square wave, 20Hz for 0.2 second) with a linearly increasing intensity of 2 mA/2s. The current at which tonic hindlimb extension (HLE) occurred was recorded as the seizure threshold current (STC). If tonic HLE was not observed with a current of 30 mA, electroshock was terminated and this cutoff current was used in the analysis. The prepared DNP was evaluated against the uncomplexed aqueous fraction for anticonvulsant, anxiolytic and antidepressant activity at dose level of 400mg/kg and 800 mg/kg (p.o.).

Test for motor coordination: The rotarod test according to Lima et al. was used to determine the effect of LSO on motor coordination [25]. Effects on motor function were assessed in a rotarod test using a rod with a diameter of 3 cm rotating at a constant speed of 10 rpm. Mice were trained to walk continuously on the rod for a period of 120s. The trained animals were then evaluated for motor coordination at 30, 60, 90, and 120 min after oral administration of 500mg/kg, 1000mg/kg and 2000mg/kg of the DNP. The time each animal could walk continuously on the rod was recorded. Control groups were used with mice only receiving distilled water (10mL/kg, i.p.).

Elevated plus maze test: Mice of either sex were divided into six groups of six animals each. Two groups received the DNP at doses of 400mg/kg and 800 mg/kg, p.o., two groups will receive aqueous fraction in the dose of 400mg/kg and 800 mg/kg, p.o., one group will receive placebo of DNP while the control group received normal saline (10 mL/kg, p.o.). The animals were trained for the elevated plus maze activity for that each animal were placed individually at the end of either of the open arms and the time spent by the animal into open arm and number of entries into open arm was noted.

Forced swimming test: The FST was based on the method of Porsolt et al. [26-28]. Briefly, mice were trained to swim for 15 minutes in glass beakers (height: 15 cm, diameter: 11 cm) containing fresh water (temperature: 22±2°C) to a height of 6 cm. This constituted the “pretest session.” Twenty-four hours later, each animal was re-exposed to the swimming condition in a similar environment in a 6-minute “test session.” The animal’s vigorous attempts to leave the swimming environment were interspersed with bouts of immobility, signifying “behavioral despair.” In this procedure, the duration of total immobility is measured for each animal.

Statistical Analysis: Statistical analysis of prepared formulation DNP as compared to pure extract and control was evaluated by the Tukey test using GraphPad software and the results were expressed as the mean±S.E.M. A P value of less than 0.05 was considered significant. The results of all the experiments were presented as percent change.

Result and Discussion

Formulation and development: The prepared DNP was found dark brown in colour. The odour and flavour of DNP is slightly lipidic. The prepared DNP was analysed by HPTLC for the presence of the same components as present in the extract. The Rf values of Pure extract were found 0.10, 0.15, 0.23, 0.35 and 0.40 However for DNP the Rf values shift due to change in the polarity due to complex formation showing Rf values at 0.19,0.28,0.37,0.46,0.53 and 0.94. The additional spot showing Rf value of 0.94 can be considered for the lipid fraction remained in the formulation.

Particle Size: The particle size of the prepared DNP was around 500 nm. Most of the DNP were less than 800 nm in size. (representative TEM shown in Figure 3). Although the transmission electron microscopy was only used to test a small part of the overall sample, the results obtained were in good agreement with the generality.

Anticonvulsant activity

Pentylenetetrazole Test: We found an improved anticonvulsant action with DNP as compared to aqueous fraction in PTZ model of seizures as per the results given in Table 1. The DNP elicited significant increase in seizure threshold and significantly prolonged the latency to myoclonic jerks and clonic generalized seizures as compared to
control, aqueous fraction and placebo treated groups. The improved efficacy of DNP as compared to AF ascertained by ANOVA and Tukey’s test. For myoclonic jerks the statistical significance level between the DNP and AF was found $F(6, 41)=119.8$, ***$p<0.001$ and for Generalized clonic seizures the statistical significance level between the DNP and AF was found $F(6, 41)=166.7$, ***$P<0.001$.

ICES test: The DNP significantly increased the threshold current to produce hind limb tonic extension and decreases the mortality percentage due to electroshock at all the doses tested in mice as compared to control, Uncomplexed Extract and placebo treated groups. However, there was a significant dose dependent reduction in the recovery period following the convulsions, the effect being highest at the dose of 800 mg/kg and least at 400 mg/kg, p.o. which can be ascertained by statistical significant level of $F(6, 41)=22.10$, ***$P<0.001$.

Elevated Plus Maze: In the elevated plus maze test, the DNP treatment markedly increases the number of open arm entries at all doses administered as compared to control, aqueous fraction and placebo treated groups. The increased in the number of entries and time spent in the open arm of the maze indicates that the DNP enhances the anxiolytic effect of the aqueous fraction (Figure 4 A&B).

Table 2: Effect of DCP on ICES in mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>ICES (mA)</th>
<th>Recovery Period (Sec.)</th>
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<tr>
<td>I</td>
<td>Dist. Water</td>
<td>10ml/kg</td>
<td>10.03±0.87</td>
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<tr>
<td>II</td>
<td>Sodium Valproate</td>
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<td>III</td>
<td>DD aqueous Fraction</td>
<td>400mg/kg</td>
<td>14.72±0.84</td>
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<td>IV</td>
<td>DD aqueous Fraction</td>
<td>800mg/kg</td>
<td>16.24±0.98</td>
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<td>V</td>
<td>DNP</td>
<td>400mg/kg</td>
<td>18.41±0.65</td>
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<tr>
<td>VI</td>
<td>DNP</td>
<td>800mg/kg</td>
<td>21.11±0.78</td>
<td>15.28±4.56</td>
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<td>VII</td>
<td>Placebo</td>
<td>800mg/kg</td>
<td>10.71±0.49</td>
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Neurotoxicity assessment: DNP did not induce a statistically significant disturbance in motor coordination or fall-off time up to a

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Figure 4A: Elevated Plus Maze Test (Percentage of time spent in Open arm)

Elevated Plus Maze Test (Number of Open arm entry)

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Figure 4B: Elevated Plus Maze Test (Number of Open arm entry)

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Figure 5: Forced Swimming Test
We have selected the aqueous fraction of the delphinium denudatum extract as per the earlier reports shows dose-related increase in anticonvulsant activity of aqueous fractions suggests the presence of anticonvulsant compounds in aqueous fraction. As reported in the literature that most of the bioactive constituents of herbal drugs are water soluble molecules. However, water soluble phytoconstituents like many flavonoids are poorly absorbed either due to their multiple-ring large size molecules which cannot be absorbed by simple diffusion, or due to their poor miscibility with oils and other lipids, severely limiting their ability to pass across the lipid-rich outer membranes of the enterocytes of the small intestine. When these water-soluble phytoconstituent molecules (mainly polyphenoles) converted into lipid-compatible molecular complexes, becomes more bioavailable as compared to simple herbal extracts or fraction owing to their enhanced capacity to cross the lipid rich biomembranes and finally reaching the blood. The lipid-phase substances employed to make phytoconstituent, lipid-compatible are phospholipids from soy, mainly phosphatidylcholine (PC). PC, the principal molecular building block of cell membranes, is miscible both in water and in oil/lipid environments, and is well absorbed orally. The phytosome process has been applied to many popular herbal extract including Ginkgo biloba, grape seed, Hawthorn, milk thistle (Silybum marianum), green tea (Thea sinensis) and ginseng (Panax ginseng). The flavonoid and terpenoid components of these herbal extracts lend themselves quite well for the direct binding to phosphatidylcholine. The flavonoid and terpenoid components of these herbal extracts lend themselves quite well for the direct binding to phosphatidylcholine. Phospholipid aqueous fraction were evaluated by using rotarod test. We found strong bioactivities for the prepared DNP as compared to pure aqueous fraction. These results suggest greater absorption of active components in of delphinium denudatum aqueous fraction prepared using the phospholipids complexation combining with nanozising technique. Prepared formulation didn’t show any neurotoxicity at higher dose level also.

Prepared DNP were standardised for the presence of same phytoconstituents as of aqueous fraction by HPTLC fingerprinting and showing the same fingerprinting profile between DNP and pure delphinium denudatum aqueous fraction. The results are showing the significance enhancement in the bioactivities at lower doses too. The phospholipid complexation of the of herbal extract not only enhances the bioavailability, it also assures delivery to the tissues without compromising safety and this can be clinical benefited

Pretreatment with DNP (400 and 800 mg/kg) significantly alter the seizure latency time to myoclonic jerks and clonic seizures as compared to DDAF. There is a statistical significance

Differences at the same dose level of DDAF and DNP at p<0.001 against PTZ induced seizures. In ICES DNP at lower as well as higher doses not only increases HLTE threshold current but also reduced the recovery time for each animal with the decline in the mortality when compared to DDAF at statistical significance level. DNP also enhances the efficacy against the elevated plus maze as antianxiety model. As showed in the table DNP treated animals move freely in terms of number of open arm entry and spent more time in the open arm of elevated maze as compared to all other groups at p<0.001 DNP shown better antidepressant activity as compared to DDAF with respect to increase in swimming time and decrease in swimming immobility time. From the study, it can be seen that there is a bioactivity of the DNP 400mg/kg is equal or better than DDAF 800mg/kg. This shows that by complexing the herbal extract with phospholipid can leads to dose reduction of the pure extract.

The nanotechnology of drug formulation not only enhances the absorption of poor water soluble drugs but it also improves drug therapeutic effectiveness in pharmaceutical research. Nanoparticle formulation is one of the novel drug delivery systems which possesses various advantages, including increasing drug solubility, enhancing dissolution rate, ameliorating the bioavailability, and decreasing the dosage required for the same effects, compared with the crude or micronized medicine [29].

Conclusion

From these observations, it can be hypothesized that phospholipid complexed delphinium denudatum aqueous fraction which is nanosized may enter the brain to a greater extent due to its enhanced bioavailability than free aqueous fraction. This is in line with the earlier reports that suggested enhanced neuropharmacological action of herbal extract like ginkgo biloba when administered as phospholipid complexed formulation. The augmented action of phospholipid complexed delphinium denudatum aqueous fraction may be attributed to greater ability of the phospholipids carrier to make the herbal extract more bioavailable and tend to cross the blood brain barrier for better efficacy.

Acknowledgement

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References


