Chapter 9

Development of *Nardostachys jatamansi* based nutraceutical product and evaluation of the product for its efficacy
Introduction

A nutraceutical is any substance that is food or part of a food and provides medical or health benefits, including the prevention and treatment of a disease (DeFelice, 1995). According to the World Health Organization, over 80% of world’s population (4.3 billion people) relies on traditional plant based systems of medicines as phytochemicals, nutritional constituents or as functional foods (Allen, 1997; Kasbia, 2005). Nutraceuticals have a nutritional role in the diet and benefits to health may arise following long term usage of foods (Whitman, 2000). Plant derived nutraceuticals are of great importance in the present system of medicine and healthcare (Pandey et al., 2010). Thus, nutraceuticals are becoming popular and widely accepted adjunct to conventional therapies thereby enhancing health.

Plants have been utilised for their protective and healing properties since ages. Ayurveda and traditional folk medicines stand tall to these claims. Consumption of phytochemicals has remarkably increased in the current decade owing to their myriad beneficial and health promoting properties. Plant derived phytochemicals are a rich source of antioxidants and consumption of natural antioxidants is associated with reduced risks of cancer, cardiovascular disease, diabetes, and other diseases associated with ageing (Veerapur et al., 2009; Ashokkumar et al., 2008). In recent years consumption of the natural phytochemicals present in berry crops, teas, herbs, oilseeds, beans, fruits and vegetables has markedly increased (Kitts et al., 2000; Muselik et al., 2007).

Nutraceuticals provide nutritional benefits in a compact and convenient form and could be stored for considerable duration without spoilage and ease to carry (Prasad, 2014).

In the preceding chapters, a comprehensive detail of NJE with respect to its metabolite profile, its anti-anxiety activities as well as its biodistribution pattern has been shown. Hence, in the present study we envisaged at developing a nutraceutical with herbal additives of NJE that apart from attenuating anxiety would also provide general nutrition thereby relieving people of the stigma of consuming drugs.
Materials and Methods

Good quality raw materials i.e. soya flour, powdered sugar, maltodextrin, skimmed milk powder and glucose, cardamom powder, chocolate flavour (INRE4512/13A, Akras Flavours India Private Limited, India) were procured from local market.

Plant Material and preparation of Extract

*Nardostachys jatamansi* was extracted with 70 % ethanol as described in the chapter 2. The lyophilized powder of the 70 % ethanol fractions was used for nutraceutical preparation.

Preparation of NJE enriched drink mix

For the preparation of 1 kg of *Nardostachys jatamansi* based drink mix, roasted soya flour, powdered sugar, maltodextrin, skimmed milk powder, glucose, cardamom powder and chocolate flavour were mixed as mentioned in Table 9.1. Soya flour was roasted in an aluminium pan at low flame, and sieved to get a fine powder.

Table 9.1: Ingredients for preparation of NJE based drink mix

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>25 g</th>
<th>1000 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soya flour</td>
<td>1 g</td>
<td>40 g</td>
</tr>
<tr>
<td>Sugar</td>
<td>19 g</td>
<td>760 g</td>
</tr>
<tr>
<td>Maltodextrin</td>
<td>1 g</td>
<td>40 g</td>
</tr>
<tr>
<td>Skimmed milk powder</td>
<td>3 g</td>
<td>120 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>1 g</td>
<td>40 g</td>
</tr>
<tr>
<td>NJE</td>
<td>250 mg</td>
<td>10 g</td>
</tr>
<tr>
<td>Chocolate flavour</td>
<td>0.08 g</td>
<td>3.2 g</td>
</tr>
<tr>
<td>Cardamom flavour</td>
<td>0.4 g</td>
<td>16 g</td>
</tr>
</tbody>
</table>
Fig. 9.1

All the above ingredients were mixed thoroughly with NJE as per standardisations. The product was packed in polypropylene pouches (Fig. 9.1) and analysed for physicochemical, microbiological, sensory attributes and for its potential anxiolytic effects in mice.

**Proximate composition**

Proximate composition in the NJE based drink mix was analysed according to Official Methods of Analysis of AOAC INTERNATIONAL (2012).

**Total calories**

1g of sample was weighed into a crucible and 6 cm of nichrome wire and 6 cm of cotton thread is tied to the electrodes which was then placed in the Bomb calorimeter and closed tightly. Oxygen gas was filled (20 psi pressure) for combustion by using semi auto gas filling device. The bomb calorimeter filled with oxygen is now placed in water jacket and electrodes are connected. The initial weight was noted and
the sample was burnt automatically using a crucible, to get the calorific value. The experiment was carried out in triplicates to get concordant values (Buskirk and Mendez, 1980).

**Colour measurement**

The difference in colour of NJE drink mix and a control drink mix (without NJE) were measured by Hunter colorimeter D-65 illuminant (Hunter Associates Laboratory, Inc, Reston, VA). The colorimeter was calibrated using standard white and black tiles. Samples were taken and pressed one by one against instrument (sample) port, making sure that it completely covers the area to be measured. Four readings for each composition of sample were taken. Both crust and crumb colour was taken. L*, a*, and b* values were recorded at the 2 cut surfaces of each slice. Measurement was made at the four points on the bread loaf. The results were expressed as a mean value of all the samples (Francis and Clydesdale, 1975).

$L^*$ measures lightness and varies from 100 for perfect white to zero for black, approximately as the eye would evaluate it.

The chromaticity dimensions (a* and b*) give understandable designations of colour as follows:

- **a*** measures redness when positive, gray when zero, and greenness when negative.
- **b*** measures yellowness when positive, gray when zero, and blueness when negative.

**Microbial analysis**

The NJE drink mix along with a control drink mix (without NJE) before and during storage at 37 °C, were subjected to microbial analysis in terms of total plate count, coliforms, yeast and moulds, using the methods recommended by the American Public Health Association, 1976. TPC was determined using dextrose tryptone agar (DTA) after incubation for 48 h at 30 °C. Yeast and moulds were estimated with the help of acidified potato dextrose agar (PDA), after incubation at 30 °C for 4 - 5 days. Spore formers were determined after killing the vegetative cells by keeping the samples in boiling water bath for 10 to 20 min and subsequent incubation at 37 °C and 55 °C for 48 h after incubation (Harrigan, 1998).
Sensory evaluation for overall acceptability

Sensory analysis was performed in terms of colour, aroma, taste, texture and overall acceptability in our laboratory, with 20 semi-trained panelists (aged 24 to 50 years old) comprising of both the sexes (10 men and 10 women). The NJE based drink mix was evaluated for colour, aroma, taste, texture and overall acceptability.

The drink was analysed by panelists on a 9 point hedonic scale to rate the individual attributes numerically. Scores were assigned from ‘like extremely’ point 9 to ‘dislike extremely’ point 1 (Lawless and Heymann, 2010).

Thermal analysis

Differential Scanning Calorimetry (DSC)

Thermal analysis of NJE based drink mix was performed on a DSC-2010 (TA Instruments Inc., New Castle, DE, USA) as described in chapter 4.

Thermo gravimetric Analysis (TGA)

The non-isothermal thermo gravimetric analysis (TGA Q50, TA Instruments, DE, USA) was used to measure the amount and rate of change in weight of the sample as a function of increasing temperature under a controlled atmosphere with conditions explained in chapter 4.

Experimental design for anxiolytic experiments

Mice were administered NJE based drink, control drink or diazepam (standard anxiolyte; 1 mg/kg) 14 days followed by anxiolytic experiments viz. OFT, EPM on 13th day, LDB and VCT on 14th day. After VCT mice were sacrificed by cervical dislocation and the brain tissue was immediately dissected out, washed with ice cold isotonic sodium chloride and drained thoroughly and stored at -80°C until further use (Fig. 9.2).
Elevated plus maze test

EPM test is the commonly used behavioural paradigm to assess the anxiolytic behaviour in rodents. The test was carried out as described in chapter 2.

Open Field Test

Open field test is a measure of spontaneous locomotor activity by measuring the total number of line crossings/total ambulatory distance and the behavioural analysis was carried out as described in chapter 2.

Light dark box test

Light dark box test was conducted as described in chapter 4.

Vogel’s conflict test (VCT)

An elaborate description of the apparatus and behaviours recorded is given in detail in chapter 5.

Neurotransmitters estimation by HPLC

GABA

Reverse phase HPLC analysis were performed on a Waters 2465 RP-HPLC (Milford MA, USA) with a waters 515 HPLC pump and coupled to a waters 464 pulsed electrochemical detector. The protocol for analysis was as described in chapter 5.
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**Monoamine neurotransmitters**

Monoamine neurotransmitters viz 5-hydroxytryptamine or serotonin, norepinephrine and dopamine levels in mouse brain were estimated by RP-HPLC, coupled to an electrochemical detector and the detailed methodology followed was as described in chapter 5.

**Statistical analysis**

All the results obtained were expressed as mean ± SD (n = 8). Data was evaluated by one-way ANOVA followed by Tukey’s Honestly Significant Difference Post hoc test using SPSS 16.0 software. *p* value less than 0.05 was considered significant.

**Results**

**Proximate composition**

The proximate analysis of NJE based drink mix is presented in Table 9.2.

**Table 9.2: Proximate composition of NJE based drink mix**

<table>
<thead>
<tr>
<th>Content</th>
<th>NJE based drink mix (100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy</td>
<td>388 Kcal</td>
</tr>
<tr>
<td>Protein</td>
<td>9.2 g</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>74.88 g</td>
</tr>
<tr>
<td>Fat</td>
<td>5.64 g</td>
</tr>
<tr>
<td>Moisture</td>
<td>4.88 g</td>
</tr>
<tr>
<td>Total ash</td>
<td>5.4 g</td>
</tr>
<tr>
<td>Crude fibre</td>
<td>0.93 g</td>
</tr>
</tbody>
</table>

The moisture content of the drink was found to be 4.88 %. The protein was 9.2 % mainly from soya and skimmed milk powder, the fat content was 5.64 % again mainly from soya flour, and the carbohydrates were 74.88 %. The total ash content was 5.4 % which reflects the total inorganic matter present in the food sample (Akpanyung, 2005). A 100 g serving would give 388 Kcal of energy mainly from the carbohydrates present.
Colours measurement

The colour measurement of NJE based drink mix or control drink was carried out on a HunterLab colorimeter with L* a* b* values shown in Table 9.3. The L* value of the sample was lesser than control and indicated that addition of NJE made the product darker. The a* value indicated that NJE based drink mix was much greener than control and the b* value indicated that there was a significant decrease in yellowness of the product on addition of NJE. However, on subsequent storage there was an increase in the darkness of the product.

Table 9.3: L* a* b* values of NJE based health drink and control drink

<table>
<thead>
<tr>
<th></th>
<th>Control drink</th>
<th>NJE based drink mix</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>3 months</td>
</tr>
<tr>
<td>L*</td>
<td>90.25±2.6</td>
<td>88.43±2.1</td>
</tr>
<tr>
<td>a*</td>
<td>-0.74±0.05</td>
<td>-0.78±0.03</td>
</tr>
<tr>
<td>b*</td>
<td>21.11±0.8</td>
<td>22.86±0.7</td>
</tr>
</tbody>
</table>

Microbiological analysis

The microbial load of NJE based health mix initially and during storage was analysed in terms of total plate count (TPC), coliforms, yeast and moulds. The microbial load was within the acceptable limit and the product was safe from microbial contamination and the microbiological report is given in Table 9.4.

Table 9.4: Microbiological report of NJE based drink mix

<table>
<thead>
<tr>
<th></th>
<th>TPC (CFU/g)</th>
<th>Coliforms (CFU/g)</th>
<th>Yeast and Moulds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control drink mix (Initial)</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>NJE based drink mix (Initial)</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>Control drink mix (3 months)</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>NJE based drink mix (3 months)</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>Control drink mix (6 months)</td>
<td>2.0 x 10^2</td>
<td>3.0 x 10^1</td>
<td>Nil</td>
</tr>
<tr>
<td>NJE based drink mix (6 months)</td>
<td>1.0 x 10^2</td>
<td>2.0 x 10^1</td>
<td>Nil</td>
</tr>
</tbody>
</table>
Sensory evaluation for overall acceptability

The sensory evaluation of control mix (without NJE) and NJE based drink was evaluated initially and during storage (3 and 6 months) in terms of colour, aroma, taste, texture and OAA on a 9-point hedonic scale. The OAA scores are shown in Table 9.5. The NJE based drink had a good OAA score initially (7.8 ± 0.21) which was not significantly different after 6 months of storage of the product at RT (7.6 ± 0.23). In comparison to control mix, NJE based mix had a lower sensory score due to addition of NJE which tastes bitter.

Table 9.5: Sensory scores for OAA of NJE based drink

<table>
<thead>
<tr>
<th>OAA</th>
<th>Initial</th>
<th>3 months</th>
<th>6 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control NJE based</td>
<td>8.1±0.32</td>
<td>7.8±0.21</td>
<td>7.9±0.27</td>
</tr>
<tr>
<td>drink</td>
<td>Control</td>
<td>7.7±0.22</td>
<td>7.7±0.24</td>
</tr>
<tr>
<td></td>
<td>NJE based</td>
<td>7.6±0.23</td>
<td></td>
</tr>
<tr>
<td></td>
<td>drink</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Thermal analysis

Differential Scanning Calorimetry (DSC)

The DSC curve of NJE based health drink is shown in Fig. 9.3. The thermal process occurred between 30 ºC to 200 ºC. The product showed a melting temperature of 161.29 ºC with an endothermic peak at that temperature in the DSC curve (ΔH = 70.98 J/g).

Fig. 9.3: DSC curve of NJE based drink mix.
Thermo gravimetric Analysis (TGA)

The TGA analysis was carried out at temperatures between 30 °C to 300 °C. Loss in mass of the sample was observed at two temperatures as depicted in Fig. 9.4 with a residue of 60 %.

Fig. 9.4

![TGA curve of NJE based drink mix.](image)

Fig. 9.4: TGA curve of NJE based drink mix.

Anxiolysis experiments

Elevated plus maze test

One way ANOVA revealed a significant difference between the groups (p < 0.05) with respect to the time spent on open arms. Mice orally fed with NJE based drink spent significantly higher time on open arms of EPM in comparison to the control drink. Diazepam (1 mg/kg) as reported showed the maximum effect (Fig. 9.5).

Fig. 9.5

![Effects of administration of NJE based drink, control drink and diazepam (1 mg/kg) on the behaviour of mice in EPM measured as the time spent by mice on open arms.](image)

Fig. 9.5: Effects of administration of NJE based drink, control drink and diazepam (1 mg/kg) on the behaviour of mice in EPM measured as the time spent by mice on open arms. * p < 0.05 versus control, # p < 0.05 versus control drink.
Open Field Test

An increase in the number of line crossings is a measure of anti-anxiety nature of a drug. As observed the number of line crossings made were significantly higher ($p < 0.05$) in the NJE based drink fed mice with significantly higher time spent on the central zone of the apparatus in comparison to control drink (Fig. 9.6).

**Fig. 9.6**

![Graph showing number of line crossings](image-url)

**Fig. 9.6: Effects of administration of NJE based drink, control drink and diazepam (1 mg/kg) on the behaviour of mice in OFT measured as the number of line crossings.** * $p < 0.05$ versus control, *# $p < 0.05$ versus control drink.

Light dark box test

One way ANOVA revealed a significant difference between the groups ($p < 0.05$) with respect to time spent in the light compartment of LDB. A significantly higher time was spent by mice orally fed with NJE based drink in the light compartment of LDB. Diazepam (1 mg/kg) similarly caused an increase in the time spent in lit box of LDB (Fig. 9.7).

**Fig. 9.7**

![Graph showing time spent in light compartment](image-url)

**Fig. 9.7: Effects of administration of NJE based drink, control drink and diazepam (1 mg/kg) on the behaviour of mice in LDB measured as the time spent in the light compartment.** * $p < 0.05$ versus control, *# $p < 0.05$ versus control drink.
Vogel’s conflict test

The number of licks made and subsequent shocks accepted in VCT were significantly higher ($p < 0.05$) for the NJE based drink fed mice in comparison to control drink fed mice. These results clearly suggest that consumption of NJE based drink could help alleviate anxiety (Fig. 9.8 A and B).

Fig. 9.8

**Fig. 9.8: Effects of administration of NJE based drink, control drink and diazepam (1 mg/kg) on the behaviour of mice in VCT.** (A) Number of licks (B) Number of shocks. * $p < 0.05$ versus control, # $p < 0.05$ versus control drink.

Brain GABA and monoamine neurotransmitter levels

The levels of GABA (Fig. 9.9) and monoamine neurotransmitters (serotonin, dopamine and norepinephrine; Table 9.6) were significantly elevated following administration of NJE based drink mix in comparison to control drink.
Fig. 9.9: Brain GABA levels following administration of NJE based drink, control drink and diazepam (1 mg/kg). *p < 0.05 versus control, # p < 0.05 versus control drink.

Table 9.6: Brain monoamine neurotransmitter levels following administration of NJE based drink, control drink and diazepam (1 mg/kg). *p < 0.05 versus control, # p < 0.05 versus control drink

<table>
<thead>
<tr>
<th>Monoamine neurotransmitters (ng/g wet tissue weight)</th>
<th>Serotonin</th>
<th>Norepinephrine</th>
<th>Dopamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>691±22</td>
<td>512±21</td>
<td>756±29</td>
</tr>
<tr>
<td>Control drink</td>
<td>709±21</td>
<td>527±14</td>
<td>789±21</td>
</tr>
<tr>
<td>NJE based drink</td>
<td>752±28*#</td>
<td>568±19*#</td>
<td>822±24*#</td>
</tr>
<tr>
<td>Dzp</td>
<td>793±42*#</td>
<td>597±23*#</td>
<td>879±33*#</td>
</tr>
</tbody>
</table>

Discussion

In the current scenario there is an upsurge in stress related diseases owing to life style, working environments, work related stress etc. Aristotle rightly said ‘let food be thy medicine’ hence a careful selection of the right food with the right amount of nutrients is the need of the hour. In the present study, we have attempted to prepare a nutraceutical with herbal additives of *Nardostachys jatamansi* (an anxiolyte) and studied for its physicochemical, sensory, microbiological and thermal properties followed by assessment of its potential anxiolytic effects in mouse models of anxiety in comparison to a control drink (without NJE).

The proximate composition quantitates the different macronutrients in feed/food and is an index to the nutritive value of foods and food ingredients (Madisch and Hofmayer, 2015). The proximate composition showed an enriched
content of proteins, carbohydrates and fat, all of which could serve as ready sources of energy.

Colour, is an important quality attribute in food as it influences consumer’s choices and preferences. Colour measurement of food products has been used as an indirect measure of other quality attributes such as flavour and contents of pigments because it is simple, faster and correlates well with other physicochemical properties (Pathare et al., 2013). The product showed a lesser L* value in comparison to control owing to addition of the functional ingredient i.e. NJE. The a* value measured a much greener and b* value measured a decrease in yellowness again due to addition of NJE. The results are similar to previous reports (Sun-Waterhouse et al., 2010).

Shelf stability of a product is a critical parameter as it is influenced by the ingredients. Any spoilage during storage will mar the quality as well as acceptability of the product. The microbial load of our product was well within the acceptable limits even after storage at RT for 6 months. The OAA score of the product did not differ significantly on storage. This is in accordance with previous reports (Muir and Banks, 2000).

The drink when administered to mice aided in diminution of anxiety as assessed using behavioural parameters. The time spent by mice on the open arms of EPM, the number of line crossings in OFT, the time spent in lit box of LDB and the number of licks made and shocks accepted were all increased on consumption of the drink. All these behavioural parameters are critical indices of the anxiolytic nature of drugs. Addition of NJE as a functional ingredient aided in alleviation of anxiety in mice. Several herbs have been attributed with similar mood elevating, calming, stress relieving properties (Weeks, 2009; Mehta et al., 1991; Woelk et al., 2007; Caso et al., 1995).

NJE based drink was also able to enhance the brain GABA and monoamine neurotransmitter levels further supporting the herb’s anxiolytic actions.

Thus, the developed nutraceutical with herbal additives of Nardostachys jatamansi promises to attenuate anxiety and also provide general nutrition apart from relieving people of the stigma of consuming drugs.