CHAPTER 8

Agnivardhana action of Dāḍima and Vṛkṣāmla

This chapter explains about the studies undertaken to evaluate and compare the agnivardhana action with reference to enhancement of iron bioavailability by Dāḍima and Vṛkṣāmla using in vitro models
8.1 Introduction

‘Agnivardhana’ has been mentioned as one of the pharmacological actions of Dāḍima (Punica granatum) as per Ayurveda (Sastry, 1997b; Sharma, 2006b). ‘agni’ or the ‘digestive fire’ is responsible for digestion, metabolism and absorption of ahara (food) in the body. Derangement in agni can lead to many diseases, one of them being ‘pāṇḍu’. Pāṇḍu can be roughly correlated to iron deficiency anemia (Murthy, 2001a). One of the important causes of pāṇḍu is the derangement in jāṭaragni, which can be equated to the digestion in the proximal gastrointestinal tract (Dwarakantha, 1996). Dāḍima (P. granatum) is often prescribed to be taken as food supplement in the management of pāṇḍu (Sastry, 2004).

Vṛkṣāmla (G. indica) has been suggested as the APD for Dāḍima, both with agnivardhana properties and use in the Ayurvedic management of digestion and metabolic disorders (Chunekar, 2004).

Iron Deficiency Anemia (IDA) is the result of long-term negative iron balance and is the most common nutritional disorder in the world (WHO, 2000). The amount of iron ingested, its availability for absorption in the body and the iron status of the individual determine the amount of iron absorbed from the diet. Bioavailability is the process whereby an ingested nutrient becomes available for digestion, absorption, transport and utilization (Benito and Miller, 1998). Certain chemicals of plant origin like L-Ascorbic acid (AA) or Vitamin C and citric acid are known iron bioavailability enhancers, while phytates inhibits iron bioavailability (Allen and Ahluwalia, 1997).

Venkatasubramanian et al. (2014) have used a modified protocol of Miller et al., 1981 and Glahn et al., 1998 to demonstrate the agnivardhana effect of Amalaki (Phyllanthus emblica) in terms of iron dialysability using cell-free (Miller et al., 1981) model and iron uptake in Caco2 cell-based in vitro models (Glahn et al., 1998) in the gastrointestinal tract simulated.
conditions (Venkatasubramanian et al. 2014). Not only intestinal cells, but also the hepatocytes play an important role in iron homeostasis, including its transport and storage. In Iron deficiency anemia, iron depletion occurs in the serum and liver. In liver iron is stored as ferritin (Takami and Sakaida, 2011). Therefore the influence of dialysates of Dāḍima and Vṛkṣāmla on the uptake of iron in HepG2 cell model was studied.

The protocol of Venkatasubramanian et al. (2014) was followed to compare the AD-APD pair Dāḍima and Vṛkṣāmla with reference to agnivardhana action. Ingested iron available for the use in metabolic processes or deposition in storage forms like ferritin is a key factor in iron nutrition (Dreosti, 1993), therefore ferritin has been used as an indicator of iron uptake in the cell-based models (Glahn et al., 1998).

Dāḍima (P. grantatum) is rich in organic acids (Rahimi et al., 2012). Vṛkṣāmla (G. indica) is also enriched with organic acids and hydroxycitric acid (Jayaprakasha and Sakariah, 2002). P. granatum and G. indica was found to contain 3.2±0.2 and 12.6±0.8% w/w of organic acids respectively (section 6.6.2.3). Juice of P. granatum showed the presence of ascorbic, citric and gallic acids (section 6.6.2.5 and Fig. 6.10), the three major acids in pomegranate. Ascorbic acid and hydroxylcitric acid, a type of citric acid were detected in the rinds of G. indica (section 6.6.2.6 and Fig. 6.10). Several pharmacological actions of G. indica were attributed to the above mentioned organic acids (Parthsarathy and Nandakishore, 2014). The Ayurvedic properties like amla rasa, maintenance of pitta and therapeutic indications like dipana of both drugs mentioned in Ayurvedic classics and lexicons (Sastry, 1997a; Sharma, 1982) indicate their potential as iron bioavailability enhancers and in the management of IDA.
8.2 Materials and Methods

8.2.1 Chemicals

All the general chemicals used for the experiments were of ‘Analytical Grade’. Glassware used were from ‘BOROSIL’. Ascorbic acid (AA) standard required for the study was purchased from HiMedia (Mumbai). Chemicals required for in vitro digestion including porcine pepsin (800-2,500 units/mg protein) and pancreatin (activity= 4 x USP specifications) were purchased from Sigma Chemicals (St. Louis, Mo., U.S.A.). Bile salt (sodium tauroglycocholate) was procured from Loba Chemie, Mumbai. Sodium hydrogen carbonate and hydroxylamine hydrochloride were procure from Fisher Scientific (Mumbai). 6-well plates were purchased from Becton Dickinson, (USA). The dialysis membrane (MW cut-off 6,000 – 8,000 Da) were procured from Fisher Scientific (Pittsburgh, PA). Ferrozine (3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p′-disulphonic acid), phosphate buffered saline (PBS) and Bradford reagent, trichloroacetic acid, triiodothyronine (T3), epidermal growth factor (EGF), PIPES, bathophenanthroline disulfonic acid, sodium selenite, insulin, hydrocortisone, trypan blue, sulphorhodomine B (SRB) and chelex-100 were also obtained from Sigma Chemicals (St. Louis, Mo., U.S.A.). GIBCO (Auckland, New Zealand) supplied MEM (Minimum essential medium), Pen-Strep, Trypsin-EDTA (0.25%), and FBS (foetal bovine serum). Iron standard was procured from Fluka Analytical. Double deionized water used to prepare the reagents.

8.2.2 Plant materials

Details of collection, authentification and processing of drug samples are given in section 6.2.2. Fresh juice of Dāḍima was used for the study. Dry rinds of Vṛkṣāmla (50g) were boiled with 400 ml of water, reduced to 100 ml, filtered and stored at -80°C.
8.2.3 In vitro digestion and iron bioavailability

In vitro digestion was performed as per the protocol given by Venkatasubramanian et al., (2014) with modifications from Miller et al., (1981) to suit a 6-well plate.

8.2.3.1 Test and Control Mixtures

HCl (0.01 N) was the blank for the study. Control (FeSO4) was a mixture of 5 ml ferrous sulphate (FeSO4.7H2O) stock solution (2 mg Fe equivalent). Test mixtures were prepared with FeSO4 (0.5 mg/ml i.e., equivalent to 100 μg/ml of iron) + 0.175 mg/ml of AA (the optimum concentration of AA with respect to iron dialysability); 0.5 mg/ml of FeSO4 + different concentrations of either Dāḍima or Vṛkṣāmla (dry weight equivalent). AA or test material was added to the mixture just prior to the start of digestion.

The control and test mixture’s were adjusted to pH to 2.0 by adding 6M HCl or 1M NaHCO3. Then they were incubated at 37 °C for 10 min in a shaking water bath (Remi Laboratory instruments, Mumbai). Then, 0.64 mL (1.60 g of pepsin brought to 10 mL with 0.1N HCl) was added to each of the samples and incubated for 2h at 37 °C, shaking at 200 rpm.

The above pepsin digest mixture was added with pancreatin-bile salt mixture (0.5 mL) (40 mg of pancreatin and 250 mg bile suspended in 10 mL of 0.1M NaHCO3). The mixture was brought to alkaline pH of 7.5 by titrating against 1M NaHCO3 and the titratable volume of 1M NaHCO3 was recorded.

In vitro digestion and dialysis were carried out in 6-well plates. The dialysis membrane were opened (MW cut-off 6,000 – 8,000 Da) and fixed to the base of a glass ring insert. Previously recorded titratable volume of 1 M NaHCO3 was added to the wells. They were made up to 2.5 mL by adding distilled water. Two ml of the pepsin digest (obtained from the
previous step of digestion) was placed on the upper chamber of the insert. The glass insert with membrane was then just immersed in the 1M NaHCO₃. Then the plates were incubated until the pH of the digest increased to 5 or for 30 min at 37 °C on a shaking water bath. After the pH of digest reached 5, 0.5 mL of pancreatin-bile salt mixture was then added to the samples. After addition of pancreatin-bile mixture, they were again incubated for 2 h in the same condition. After completion of the incubation, the glass insert was taken out from the wells. Then the dialysate remaining in wells was collected and the iron content was estimated. The same dialysate was further used for cell based iron bioavailability studies.

8.2.3.2 Iron estimation

The dialyzable iron in the dialysate was estimated by spectrophotometer, which was considered as a measure of iron bioavailability. Iron in the dialysate was estimated by ferrozine method (Kapsokefalou and Miller, 1991). 100 μL of reducing solution (5% hydroxyl amine in 10% HCl) was added to 250μL of dialysate. It was incubated for 1 h at room temperature. Finally to this mixture, 100 μL of 5 mg/ ml of ferrozene was added and incubated for 15 min. at room temperature. Immediately after the incubation, colour developed was read at 517 nm by using spectrophotometer (Biorad, California, USA).

8.2.4 In vitro iron bioavailability studies in Caco2 and HepG2 cells

The control and test dialysates obtained from the simulated digestion procedure (Section 8.2.3.1) were used to study the iron uptake in human colorectal adenocarcinoma cell line (Caco2) and Hepatocellular carcinoma (HepG2) cells. The protocol followed was as explained by Venkatasubramanian et al., 2014, which was a modified method described by Glahn et al. (1998).
8.2.4.1 Culture and maintenance of Caco2 and HepG2 cells

The Caco2 (passages 35–45) and HepG2 (passages 20–30) cells were obtained from NCCS, Pune, India. Both Caco2 and HepG2 cells were maintained in growth medium (GM) at 37 °C in 5% CO₂ in a CO₂ incubator (Thermo Scientific Forma Steri-Cycle, Waltham, USA). GM was made up of minimum essential medium (MEM) with 20% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin solution. For in-vitro iron bioavailability assay, cells were seeded in 6-well plates (1.0 x 10⁵ cells/well), and cultured for 5 days with changing the medium every two days. After 5 days, the cells were transferred to GM containing growth factors (GF) (10mmol/L PIPES, 4mg/L hydrocortisone, 5mg/L insulin, 5μg/L sodium selenite, 34μg/L T3, 20μg/L EGF) and incubated for a further 12 days till it reached 95% confluency.

8.2.4.2 Iron bioavailability studies

On the day of iron bioavailability assay, the cells were serum starved in MEM containing only GF for 2 hrs (media without FBS- Fetal Bovine Serum) and exposed to 1 mL of test mixture (1:1 ratio of filter sterilized dialysate and MEM with GF). This was incubated for 18 h at 37°C in a CO₂ incubator with 5% CO₂ (Thermo Scientific Forma Steri-Cycle, Waltham, USA).

8.2.4.2 Harvesting Caco2 and HepG2 cell monolayers

After the incubation, the medium was removed. Then the cells were washed with 1 mL of ice cold saline (0.9% NaCl). Then they were washed thrice with 1 mL of stop solution. Stop solution is a mixture of 140 mmol/L NaCl and 10 mmol PIPES with pH6.7, maintained at 4°C. Finally, the cells were washed three times with1 mL of removal solution to ensure the removal of any residual iron. Removal solution is a mixture of 140 mmol/L NaCl, 10mmol/L PIPES, 5 mmol/L bathophenanthroline disulfonic acid - pH 6.7.
8.2.4.3 Cell lysate preparation

Harvested Caco2 and HepG2 cells were solubilized in 50 uL of 0.5 N NaOH for and stored at -80°C until use. About 10 uL of cell lysate was used for ferritin estimation.

8.2.4.4 Estimation of ferritin in cell lysate

Ferritin in cell lysate was estimated by ELISA method, using commercially available Spectroferritin kit (Ramco Laboratories, INC., Stafford) following kit protocol. Briefly, 10 uL of cell lysate taken in anti-ferritin antibody coated micro wells and was mixed with 200 uL of conjugated antihuman ferritin and incubated on a normal rocker, set at 200 rpm, for 2 hours at room temperature. After incubation the cells were washed with deionized water by filling each micro well with water and shaking to decant. After washing 200 uL of the substrate solution (phenylphosphate disodium, 4-amino-antipyrine in 10% diethanolamine) was added to each well and incubated for 30 minutes at room temperature. Color developed by adding 100ul of 0.24% Potassium Ferricyanide to each micro well was measured using a spectrophotometer (Bio-Rad, California, USA) at 500 nm.
8.3 Results

8.3.1 Iron bioavailability in cell-free digestion model

Both *P. granatum* and *G. indica* enhanced iron bioavailability in cell-free digestion model. Figure 8.1 gives the percentage of dialyzable iron in the presence of different concentrations of ADs and APDs. Effect of both *P. granatum* and *G. indica* on iron bioavailability at the doses of 250 mg, 500 mg and 1 g were statistically significant compared to the ascorbic acid (AA) (P<0.05). At 250 mg (raw drug weight equivalent) *G. indica* showed the highest iron bio availability, 43.25±0.002% in terms of dialyzable iron. At that dose, *P. granatum* samples showed comparatively lesser bio- availability, 8.70±0.01%. *P. granatum* showed maximum bio availability of 24.07±0.01% at 500 mg.

![Figure 8.1: Increase in dialyzability of iron by *P. granatum* and *G. indica* in cell free model](image)

**Figure 8.1:** Increase in dialyzability of iron by *P. granatum* and *G. indica* in cell free model
8.3.2 Iron bioavailability in Caco2 and HepG2 cells

Both \textit{P. granatum} and \textit{G. indica} enhanced iron bioavailability in Caco2 and HepG2 cells, as observed from the enhancement of iron storage protein, ferritin, upon treatment with dialysates of the respective drugs. The results obtained indicate that the dialysates of \textit{P. granatum} and \textit{G. indica} behave in a similar pattern with respect to iron bioavailability in Caco2 and HepG2 cells, though differ in total ferritin concentration (Fig. 8.2). Amount of ferritin in the lysates of the Caco2 cells treated with \textit{P. granatum} and \textit{G. indica} was 900±10.16 ng/ml and in 715.5±8.38 ng/ml respectively, which is statistically significant compared to the FeSo4 alone control (41.5±2.42 ng/ml) (P<0.001). In the in the lysates of the HepG2 treated with dialysates of \textit{P. granatum} and \textit{G. indica} was 601.02±5.5 ng/mL and 305.24±3.98 ng/mL respectively, which are again significantly high compared to the control sample of FeSo4 (187±3.64 ng/mL). \textit{P. granatum} enhanced the iron storage protein, ferritin by 1.25 and 1.97 times that of \textit{G. indica} in Caco2 and HepG2 cells respectively.

![Estimation of ferritin in Caco2 and HepG2 cells](image)

**Figure 8.2:** Estimation of ferritin in lysates of the Caco2 and HepG2 cells treated with \textit{P. granatum} and \textit{G. indica} dialysates
8.4 Discussion and conclusion

As per Ayurveda, many diseases manifest because of agnimandya (Dwarakanatha, 1996). Dāḍima is one of the highly acclaimed nourishing Rasayanas. It has been told to be the best among fruits. The APD Vṛkṣāmla is also widely used in managing various digestive diseases (Sastry, 1997). As an attempt to establish models to study aspects of rasayanakarma (rejuvenative action) scientists have used both cell-free and cell-based models analyse agnivardhana activity of selected plant drugs (Venkatasubramanian et al., 2014). It was a modified protocol described by Miller et al. (1991) and Glahn et al. (1998), which were well-accepted for studying iron dialysability, uptake and bioavailability. They simulate the stomach and intestinal conditions. They have reported the iron bioavailability enhancing activity of Amlaki (Phyllanthus emblica) with reference to agni management (Venkatasubramanian et al., 2014).

Organic acids like ascorbic acid and citric acid are proved to be enhancers of iron bioavailability which was proved using Caco2 cell models as well (Zhang et al., 2007; Lie et al., 2008). The studied drugs, Dāḍima and Vṛkṣāmla are rich sources of organic acids (Rahimi et al., 2012; Parthsarathy and Nandakishore, 2014), which may be the reason for enhanced iron bioavailability and uptake observed in both cell free and cell based models. The organic acids like ascorbic and citric acids maintain iron in the Fe$^{2+}$ form. This state of iron is favoured for absorption (FAO, 2001). Iron available in the food is a mixture of ferrous/ferric ions and low molecular weight complexes. Dietary iron is initially oxidized in to the Fe$^{3+}$ form during peptic digestion. Further it gets reduced to Fe$^{2+}$ form by the enzyme ferric reductase and gets absorbed into the cells (Morgan et al., 2002). Therefore organic acids maintaining Fe2+ form, probably enhance iron bioavailability (Dada et al., 1998). The fruits and vegetable rich with L-Ascorbic acid (AA) or Vitamin C and citric acid were found to enhance bioavailability of iron (Allen and Ahluwalia, 1997).
The study showed that both *P. granatum* and *G. indica* enhance iron dialysability in the cell-free digestion model, where *G. indica* has showed nearly two folds increase as compared to *P. granatum*. Both of them were enriched with 3.2±0.2 and 12.6±0.8% w/w of organic acids respectively (section 6.6.2.3). Juice of *P. granatum* showed the presence of ascorbic, citric and gallic acids (section 6.6.2.5 and Fig. 6.10). Whereas ascorbic acid and hydroxylcitric acid were detected in the rinds of *G. indica*. In addition, the presence of *P. granatum* and *G. indica*, increased iron uptake in both Caco2 and HepG2 cell lines. This supports the Ayurveda suggestion that, both *Dāḍima* and *Vṛkšāmla* are useful in management of anemia because of their *agnivardhana* function. Since both drugs have increased iron bioavailability and iron uptake in cell free and two cell based study models, *Vṛkšāmla* could be considered as an APD for *Dāḍima* with respect to *dipana* and *agnivardhana* functions. However, this is a preliminary study and requires further *in vivo* studies to confirm the activity.