Chapter 9

CONCLUSION

Traditional health systems like Ayurveda can provide cost-effective solutions for wellness and to prevent diseases. However, since they are based on different epistemologies from biomedicine, bridging the two types of knowledge systems through scientific research poses several challenges. While Ayurveda is a holistic system that looks at human beings as a reflection of the cosmos, biomedicine look at structure-function relations in the human body. This thesis has attempted to understand a concept of Ayurveda called Rasayana and apply it for wellness and management of iron deficiency anemia.

The objectives of this doctoral thesis were:

i. To understand the logic behind the functioning of Ayurvedic Rasayana products

ii. To develop appropriate in vitro and in vivo models to study dadima Rasayana for iron deficiency anemia (IDA)

iii. Scientific validation of the use of dadima Rasayana for wellness

9.1 Trans-disciplinary understanding of Rasayanas and Rasayana Karma

A trans-disciplinary research strategy involving Ayurveda and biology was adopted for this thesis work. This approach has an advantage of adding new dimensions to the existing knowledge. Literature survey of Ayurveda texts and discussions with Ayurveda scholars helped in understanding the functional logic of Rasayanas. Forty possible Rasayana karmas were identified from Ayurveda literature. These Rasayana karmas (effects) contribute to the ‘svasthya’ or wellbeing of an individual.
Ayurveda literature survey indicated that *Rasayanas* act by three possible mechanisms to produce health benefits to the individual (Sharma, 2001). They are:

i. *Agnivyapara*: by regulating *agni* (~fire) that drives transformations in the body. Metabolic functions are one of the major sites of *agni*.

ii. *Srotosodhana*: by clearing the macro and micro channels thereby increasing tissue perfusion of nutrition and other impulses, and

iii. *Poshana*: by improving the ‘*Rasa*’ (the nutrient essence of food) quality and give optimal nourishment to tissues.

Apart from tissue nourishment or perfusion, it is also to create a homeostasis in human body by facilitating optimal functioning of anabolic and catabolic activities. Ayurvedic texts indicate that the streamlining of *agni*, *srotos* and *poshana* functions will lead to optimal functioning of tissues or organs, delay ageing and enhance quality of life.

**9.1.1 Pomegranate, a Rasayana**

Pomegranate (*dadima*) was the *Rasayana* selected for this study because it is considered as one of the ‘*Nitya Rasayana*’, which can be consumed on a daily basis throughout life for wellness. Further, pomegranate has been indicated to have *panduhara* (anti-anemic), *balya* (promotes strength) and *dhatuvrddhikara* (promotes optimal growth of tissues) effects. Ayurveda texts also prescribe intake of fresh pomegranate juice as a supplement in management of *pandu*. *Dadima* as a *Rasayana* with ‘*rocana*’ (appetizer), ‘*dipana*’ (digestive stimulant) and ‘*agnidipaka*’ (improves digestive fire) properties have been indicated in Ayurveda texts.
9.1.2 Experimental models to interpret Rasayana Karmas

Based on the theoretical understanding of the Rasayana karmas (functions) cell free in vitro, human cell lines (Caco-2 and HepG2), yeast (Saccharomyces cerevisiae) and fruit fly (Drosophila melanogaster) models were developed for this study, as summarized in table 9.1.

Table 9.1. Summary of models used to study selected functional aspects of Rasayana karmas of pomegranate

<table>
<thead>
<tr>
<th>Rasayana Karma of Dadima</th>
<th>One of the Probable functional correlations</th>
<th>Function / Marker selected</th>
<th>Model used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agnivyapara (Jataragni)</td>
<td>Improves digestion &amp; uptake of nutrients</td>
<td>Bioavailability of iron</td>
<td>Cell free &amp; cell based (Caco-2 and HepG2) <em>in vitro</em> assays</td>
</tr>
<tr>
<td>Dhatuposhana</td>
<td>Nourishes tissues</td>
<td>Ferritin</td>
<td>Caco2 and HepG2 cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Yeast</td>
</tr>
<tr>
<td>Panduhara (overall)</td>
<td>Anti-anemic</td>
<td>Iron status</td>
<td>Anemic Yeast (<em>Saccharomyces cerevisiae</em>)</td>
</tr>
<tr>
<td>Ayuvardhana</td>
<td>Life-span extension</td>
<td>Longevity</td>
<td><em>Drosophila melanogaster</em> lifespan</td>
</tr>
<tr>
<td>Vayasthapan</td>
<td>Delay ageing</td>
<td>Delay in age related functional decline</td>
<td><em>D. melanogaster</em> climbing assay</td>
</tr>
<tr>
<td>Vyadhirikshamatva</td>
<td>Immunity</td>
<td>Resistance against infection</td>
<td><em>Candida albicans</em> infection in <em>D. melanogaster</em></td>
</tr>
<tr>
<td></td>
<td>Stress resistance</td>
<td>Resistance against free radical stress</td>
<td>Paraquat and H₂O₂ induced stress in <em>D. melanogaster</em></td>
</tr>
<tr>
<td>Vrshya</td>
<td>Fecundity</td>
<td>Reproductive ability</td>
<td>Off spring production in <em>D. melanogaster</em></td>
</tr>
</tbody>
</table>
Experiments were designed and results were interpreted with due consideration of the 
*Rasayana karma* of *Dadima* (pomegranate) described in Ayuredic texts.

### 9.2 Phytochemical standardization of PJ

Fresh juice prepared from the arils of pomegranate was subjected to qualitative and 
quantitative phytochemical analysis. Qualitative phytochemical analysis indicated the 
presence of carbohydrates, fixed oils, flavonoids, glycosides, phenolics, tannins, 
phytosterols, proteins, amino acids and resins. Quantitative estimation of TDS, TSS, 
total phenolics, total organic acids, ascorbic acid and total iron content was estimated. 
While the iron content of PJ (0.7 – 0.9 mg/100 g) was found to be almost equal to that 
of date palm fruits (1 mg/100 g), PJ has almost three times higher phenolics content 
than that of dates (~0.4 g/l gallic acid equivalent) (Saafi et al., 2009). The high 
content of phenolics in PJ has been linked to several of its biological activities like 
anti-microbial, anti-oxidant, cardio protective etc. Organic acids play a major role in 
improving digestion and absorption, of which ascorbic acid and citric acid are already, 
established iron bioavailability enhancers. However, experiments in this thesis 
indicate that PJ has at least five times lesser vitamin C content than orange or grape 
fruit juices. Phytochemical standardization was performed not just for identification 
and quantification of active metabolites but also for batch to batch control of quality 
of PJ. Quantity of ascorbic acid estimated was used to represent the concentration of 
PJ in the bioactivity experiments.
9.3 IDA and Pandu

Chronic diseases and micronutrient deficiencies lead to weakness and accelerate ageing (Ames et al., 2005). IDA is one such condition. Apart from reduction in hemoglobin content and RBC count, physical weakness, immune compromise and reduced intellectual abilities also occur due to IDA. According to the modern scientific understanding apart from lesser intake of iron rich foods, low bioavailability and absorption of iron from diet are also considered as the major reasons for IDA. Therefore, iron supplement tablets with bioavailability enhancers like vitamin C, citric acid, folic acid etc., are used in IDA management programs. However, these are not showing adequate effectiveness, instead the iron folic acid tablets used in health programs report side effects including constipation and nausea.

Pandu is roughly correlated to IDA in Ayurveda and is said to occur due to improper digestion or low digestive fire (mandagni). Ayurveda suggests supplementation of diet (pathya) with fresh juice prepared from amla, dates, grapes and pomegranate in the management of pandu (Sharma, 2001).

9.3.1 Rasayanas improve digestion and metabolism

Optimizing the functions of agni is one of the Rasayana karmas. Ayurveda recognizes 13 types of ‘agni’ in human body which includes jatharagni, responsible for digestive functions happening in stomach and intestine (Murthy, 2003). Maintenance of agni is important to be healthy. Derangement of agni causes formation of ‘ama’, undigested or improperly digested food material or biological waste. Ama impairs homeostasis and results in development of diseases. Thus,
impairment in any of the *agni* may result in disease manifestations in body. A properly functioning *agni*, avoids formation of *ama* and prevents disease development. Ayurvedic texts indicate that *dadima* (pomegranate) has the ability to regulate digestion and metabolism (*agni deepana / pachana* properties), which is regulated by *agni*. To assess the *agni* enhancing potential of PJ, a cell free and human cell line based *in vitro* models were used. The models selected are already well accepted in contemporary science for studying dialysability and uptake of iron.

### 9.3.2 PJ improves *in vitro* dialysability of iron

A cell free in vitro model which simulates the stomach, intestinal digestion and uptake using a dialysis membrane was used to study the *Agni* enhancing potential of PJ. In vitro experiments performed indicated that PJ (with ~13 mg/100 ml AA) increases bioavailability of iron by >3 fold, while the equivalent amount of AA alone was able to enhance the iron dialysability only by 1.6 fold. This shows that PJ has other phytoconstituents that enhance bioavailability, which is one of the characteristic functions of *Rasayanas*.

### 9.3.3 PJ improves iron assimilation by Caco-2 and HepG2 cells

Cultured Caco-2 cells form a monolayer, express tight junctions, form microvilli and resemble the enterocytes in the small intestine of human. Absorption and uptake of nutrients or drugs are commonly studied using this cell line. PJ improved iron uptake by about 6 fold and iron assimilation in terms of ferritin was enhanced by 30% when compared to PJ equivalent ascorbic acid. A similar observation was made with HepG2 cells as well. PJ improved the iron uptake in HepG2 cells by about 3 fold and enhanced iron assimilation by about 50%. HepG2 is liver carcinoma cell line and they
are routinely used in iron metabolism studies. Modern biomedicine considers liver as a vital organ playing a role in iron transport, storage and regulation of iron homeostasis (Takami and Sakaida, 2011). Ayurveda indicates that, ‘rasa’, the nutrient essence of food gets transformed into ‘rakta dhatu’ (blood). The red colour of the blood is said to be imparted by ‘ranjaka pitta’ which is predominantly present in liver and spleen (Sharma, 2001). Thus, Ayurveda also considers liver as a major site involved in formation of blood.

The observations from the cell free and cell based iron bioavailability experiments indicate that, PJ is an agni enhancing Rasayana. Probably PJ has the ability to optimize jataragni, the digestive and absorptive fire by which iron uptake and assimilation is improved. Ayurveda considers proper metabolism as a key for health (Dwarakantha, 1986). Ayurveda’s suggestion for intake of PJ during pandu may be to improve the digestion and metabolism of iron. Perhaps this holistic approach of Ayurveda may be more effective than the allopathic approach of adding bioavailability enhancers and as hypothesized PJ was found to be more effective than the equivalent ascorbic acid. This also reinforces that PJ has multiple biologically active molecules such as punicalin, punicalagin, catechin, rutin, quercetin, ellagitannins, ellagic acid, gallic acid, citric acid and not just ascorbic acid.

9.3.4 PJ has anti-anemic (Panduhara) property

One of the karmas of PJ is panduhara, which means anti-anemic. Yeast (Saccharomyces cerevisiae) is a single cellular organism which has similar iron metabolism pathways like that of human. Iron deficiency (ID) alters cellular metabolic pathways, particularly glucose metabolism, amino acid biosynthesis and
lipid biosynthesis are altered in ID cells (Shakoury-Elizeh et al., 2010). Apart from metabolic changes, morphological and genetic changes also have been reported.

As a part of this thesis work, ‘anemic yeast’ or ID cells were generated by culturing yeast cells in iron-free medium with bathophenanthroline di-sulfonate (BPS). These cells had 89% reduction in iron and 39% reduction in heme content. With the use of mitochondrial GFP cells, it was also observed that ID cells had a higher proportion of cells with clumped mitochondria, which is an indication of the cells entering into apoptosis. The ID cells thus generated were then cultured in medium containing normal iron (20 µg/100 ml), 10% PJ (with about 90 µg/100 ml) or medium with iron content equal to 10% PJ (90 µg/100 ml). While, iron supplementation itself was reversing the cells functional (Fe\(^{2+}\)) and storage (Fe\(^{3+}\)) form iron to normal quantity, PJ group showed increase in the functional iron in the form of heme. PJ was also found to significantly enhance the ATP content and the proportion of cells with the healthy reticulate mitochondria in anemic yeast. Dhatuphoshana is one of the karmas of pomegranate. It means pomegranate has ability to improve the quality and quantity of cells / tissues. Maintaining mitochondrial health is mandatory for maintaining the integrity of cells and tissues (Ames et al., 2005). The enhancement in the proportion of cells with healthy reticulate mitochondria can be considered as one of the markers for the PJ’s Dhatuphoshana and Vayasthapana properties. The ATP generation and heme production which are mitochondrial functions, were found to be higher in the ID cells treated with PJ. Earlier literature has indicated that increase in heme content has a role in improving the iron uptake by cells (Huang et al., 2011). A recent publication by Riaz and Khan (2016) also claims that pomegranate juice has anti-anemic activity
and has ability to increase hemoglobin content in rabbits. The ‘hematinic’ activity of PJ observed in this study needs further research to understand the mode of action. The current study has generated experimental evidence for Ayurveda’s use of pomegranate as a panduhara Rasayana and developed models for various Rasayana karmas of pomegranate. In spite of several iron containing drugs available in the market for IDA, majority of the population suffers from anemia. Consuming pomegranate might be a culturally acceptable and easily available solution for the management of anemia. Further PJ might remove the ID induced physiological debilities like weakness and low energy and may improve iron assimilation.

9.4 Wellness and healthy lifespan (Svasthya)

Wellness is the optimal state of health of an individual wherein he or she achieves a conscious and self-directed fullest potential. It is a dynamic process of change and growth and does not merely mean free from illness. Therefore, it is important for everyone to achieve optimal wellness in order to manage stress, reduce the risk of illness and ensure positive interactions. Traditionally, ageing was considered as a natural and universal process. But recent research considers ageing as a ‘disease complex’ with symptoms of several debilitative conditions (Bulterijs et al., 2015). Longevity, productivity, strength, disease resistance and endurance are some of the parameters used to measure wellness of individuals (Thompson et al., 2011). Wellness in the young age ascertains active and healthy ageing of individuals by avoiding disease and disability.
Ayurveda considers wellness as a state arising from the equilibrium state of the three humors (vata, pitta and kapha), tissues (dhatu) and metabolic waste products (mala). The above with an optimal functioning digestive power followed by healthy soul, calm mind and active sense organs is said to impart ‘svasthya’ (Sharma, 2001). In Ayurveda, svasthya means healthy, contented and balanced life in one’s own natural state.

9.4.1 Rasayanas for wellness

Increase in the life expectancy in the current century has simultaneously evidenced decrease in quality of life. Several age-related degenerative diseases such as diabetes, Alzheimer’s, Parkinson’s, arthritis, atherosclerosis, and so on have increased prevalence. This condition has led to search of mechanisms for wellness and healthy living. Rasayana herbs hold potential for rejuvenation and imparting wellness. Ayurveda’s claim of imparting wellness has not been scientifically tested. Recently, researchers have started using models like Drosophila to study longevity extension and healthy ageing by Ayurvedic Rasayanas.

9.4.2 PJ enhances healthy lifespan in Drosophila melanogaster

For this thesis work, fruitfly (Drosophila melanogaster) was identified as a model to study the wellness imparting potential of PJ. Drosophila has physiological and genetic similarities (>60%) with human and it is easy to maintain in large numbers for experimentation. Flies were cultured in the medium supplemented with PJ and were assessed for various healthy living parameters including longevity, fecundity, resistance against stress, survival against infection and age related functional decline.
The current study indicated that, PJ enhanced the life-span of the *Drosophila* by 19%, higher (p<0.0001) than even the already identified life-span enhancing molecule resveratrol. This enhancement did not cause ‘trade-off’ with the reproductive potential of the flies. In fact, PJ fed flies produced more off-springs when compared to control and resveratrol fed groups. It was also observed that, the enhancement in lifespan was not due to calorie restriction. *Vrshya*, or improving reproduction is one of the properties of *Rasayanas*. Traditional texts indicate that *Rasayanas* can improve the quality of life without compromising the day-to-day activities of the individual. PJ fed flies also showed a better survival against free radical induced stress and infection (*Vyadhikshamatava*). Climbing or negative geotaxis assay indicated that PJ feeding reduced the age related functional decline and sustained the stamina of young flies when they grow old.

### 9.4.3 Potential molecular targets of PJ for longevity

Molecular targets for PJ were assessed based on the reported genes in survival related pathways. Over expression of *FOXO*, its upstream (*AMPK* and *JNK*) and downstream (*MnSOD*) targets or down regulation of *TOR* have been indicated for longevity in majority of organisms (Fontana and Partridge, 2015). In the current study, a reduction in the gene expression of both *FOXO* and *TOR* was observed with PJ feeding in *Drosophila* flies. To ascertain these results, *FOXO* and *MnSOD* gene knockdown flies were generated and their survival in PJ substituted media was assessed. It indicated that the biological effects of PJ were independent of *FOXO* and partially dependent on *MnSOD*. Probably, pomegranate also contributes directly to the fly’s anti-oxidant system. These observations indicate that, PJ may be acting through the down
regulation of *TOR* pathway and/or also by compensating *FOXO* function. However, this requires further confirmation. There could be other mechanisms too by which pomegranate imparts health benefits. A recent report by Ryu et al., (2016) indicates that uroliithin A, a metabolic end product of pomegranate ellagitannins induces mitophagy in human myoblasts, prolongs lifespan in *C. elegans* and prevented age-related decline in muscle functions in mouse and rats.

This study has tested PJ’s role in wellness using the *Drosophila* model. Diet based intervention is a most preferred way to promote health and longevity in humans than medicine based interventions (Kieft-de Jong et al., 2014). The results of this study can be correlated to humans, as flies share similar physiological processes and genetic pathways with human. The model developed can be used to test several other *Rasayanas* for their wellness imparting property. Olshansky et al. (2007), reviews that the deceleration in the rate of human ageing, compressing the duration of mortality and morbidity, maintenance of physical and cognitive functions can yield ‘longevity dividend’, social, economical and health bonuses to the society. The benefits are said to be for the current generation and also for all the generations that follow.

A summary of interpretations of the *Rasayana karmas* of PJ with overall observations from cell free, cell based, yeast and *Drosophila* models have been depicted pictorially in figure 9.1.
Figure 9.1: Effect of Pomegranate juice on IDA and wellness
Models have been used in this study for understanding certain aspects of *Rasayana karmas* like *agnivyapara* (digestion and metabolism), *dhatuposhana* (improve quality of cells and tissues by providing nutrition), *panduhara* (anti-anemic), *vrshya* (reproductive potential), *ayuwardhana* (life-span enhancement), *vayasthapana* (delay ageing), *vyadhikshmatva* (immunity) and *svasthya* (wellness and healthy living). These trans-disciplinary research models can be employed to understand functional logic of several *Rasayanas*. Understanding the traditional knowledge in depth and fertilizing with biomedical information can give rise to new knowledge and contemporary applications.

### 9.5 Limitations of this study

Interpretation of Sanskrit terms to models used in this study is based only on the current understanding. There is certainly scope for further improving the understanding and upgrading these models. Practices like *shodhana* (purification) of the individual before *Rasayana* treatment could not be performed in the model used. These are said to add value during *Rasayana* treatment in humans. It is realised that the models used in this thesis can only be used to study parts of holistic ways of functioning *Rasayanas*. The observation from the in vitro and small organism based experiments can be correlated to human only to a certain extent. Trials in human can only be most appropriate, but they will be resource and time consuming. Several experiments to understand the molecular mechanisms can be undertaken in model systems that are not feasible to be done in humans.
9.6 Future directions

1. As nutraceuticals are claimed for improving wellness, further research and human trials can consider testing the nutraceutical or functional food property of pomegranate.

2. Considering Ayurveda recommendations and also from the observations from this study, pomegranate can be considered as a potential herb for developing iron bioavailability enhancers. This would require a collaborative effort of R&D institutions, industry, clinics and government. This study has added value to the Ayurvedic recommendation by establishing a possible mode of action.

3. Ayurveda practitioners and researchers can consider use of the models developed for the study of Rasayanas. There could be many ways to further improve the models.

4. Biomedical scientists can consider the Rasayana karmas identified and tested in this study as parameters for future research on IDA and wellness products.
9.7 References


