Study of *Rasayana* functions of Pomegranate (*Punica granatum* L.) with a focus on iron metabolism and enhancement of healthy lifespan

A Thesis Submitted for the Degree of Doctor of Philosophy

By

S. P. Balasubramani
(Reg No: 100100034)

Institute of Trans-disciplinary Health Sciences and Technology
(An expression of FRLHT),
74/2, Jarakabande kaval, Post Attur, Via Yelahanka,
Bangalore-560 106, Karnataka
India

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CERTIFICATE

The research presented in this thesis entitled **"Study of *Rasayana* functions of Pomegranate (*Punica granatum* L.) with a focus on iron metabolism and enhancement of healthy lifespan"** was conducted at the Institute of Trans-Disciplinary Health Sciences and Technology of FRLHT, Bangalore, India under my supervision and guidance. It is hereby certified that the thesis submitted is a bonafide record of the research conducted by **S. P. Balasubramani** (Reg. No: 100100034) in partial fulfillment of the requirements for a Doctoral degree and has not previously been used for the basis for any other degree, diploma or any other academic qualification in any university.

Place: Bangalore

Date:

Prof. Padmavathy Venkatasubramanian

Research Guide

Institute of Trans-Disciplinary Health Sciences
and Technology (An expression of FRLHT),
74/2, Jarakabande kaval,
Attur post, Via Yelahanka,
Bangalore - 560 106,
Karnataka, India

ABSTRACT

Rasayana, a branch of Ayurveda, deals with methods to optimally nourish tissues and delay ageing. An individual following *Rasayana* regimen is said to attain '*Svasthya*' or a healthy and balanced life. Understanding *Rasayanas* can provide insights into managing nutrition and age related health conditions. A trans-disciplinary approach involving Ayurveda and biology was adopted for this thesis work. This trans-disciplinary research model has potential to add new knowledge or dimension to the existing understanding about health and wellness.

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* (pomegranate) *Rasayana* for iron deficiency anemia (IDA)
iii. Scientific validation of the use of *Dadima Rasayana* for wellness

In this thesis pomegranate as a *Rasayana* has been taken up to scientifically understand the effects and mode of action on healthy lifespan as well as on iron metabolism.

Ayurveda literature indicates that *Rasayanas* function by one or more of the three possible mechanisms, including *Agnivyapara* (regulating *agni* that drives metabolic processes of human body), *Srotosodhana* (clearing the channels and increase tissue perfusion of nutrients) and *Poshana* (nourishing tissues).

Iron deficiency anemia (IDA) is one of the major nutritional deficiency disorders affecting the quality of life of >60% of global population particularly women and children. IDA caused by low iron bioavailability leads to decreased oxygen consumption, delayed mental development, suppressed immune functions and decreased cognitive function in human. *Pandu* is an Ayurvedic correlate of IDA caused primarily due to '*mandagni*', an improperly functioning digestive process. *Jatharagni* (*agni* present in stomach), is responsible for digestion and assimilation.

A list of 40 possible *Rasayana karmas* (effects) was identified from Ayurveda literature. Pomegranate (*Dadima*) was the *Rasayana* selected for this study because it is considered as a '*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 the management of *pandu* (an Ayurvedic correlate for IDA). Ayurvedic texts indicate *dadima* as a *Rasayana* with '*rocana*' (appetizer), '*dipana*' (digestive stimulant) and '*agnidipaka*' (improves digestive fire) properties. Based on the general mode of action of *Rasayanas* and the specific *Rasayana karmas* of pomegranate scientific experiments were designed.

Fresh juice prepared from the arils of pomegranate (PJ) was subjected to qualitative and quantitative phytochemical analysis. Carbohydrates, fixed oils, flavonoids, glycosides, phenolics, tannins, phytosterols, proteins, amino acids and resins were present in PJ. Further, PJ had TDS (140 ± 3.4 g/l), TSS (13.96 – 15.08 ^{o}Brix), total

phenolics (1.48 ± 0.008 g/l), total organic acids (3.0 – 3.4 % w/w), ascorbic acid (AA; 10.12 – 13.8 mg/100 ml) and total iron (0.7 – 0.9 mg/100 g) content.

Since Biomedicine and Ayurveda are based on different epistemologies, interpretation of *Rasayana* concepts using modern scientific tools is a challenge. However, certain functional aspects can be studied using established scientific models. *In vitro* cell free and cell based (Caco-2 and HepG2 cell lines) models are extensively used in modern biomedicine to study bioavailability. A cell free *in vitro* model simulating the gastric and intestinal digestive process and measuring dialyzable iron was used to study the *agni* enhancing potential (*agnivyapara*) of PJ. *In vitro* digestion simulation indicated that PJ (with ~13 mg/100 ml AA) increased dialysability of iron by >3 fold, while the equivalent amount of AA alone was able to enhance the iron dialysability only by 1.6 fold than that in control. Presence of PJ in the dialysates improved iron uptake by about 6 fold and iron assimilation in terms of ferritin, storage iron by 30% when compared to PJ equivalent ascorbic acid in Caco-2 cells. A similar observation was made with HepG2 cells as well. Presence of PJ improved the iron uptake in HepG2 cells by about 3 fold and enhanced iron assimilation by about 50%.

Panduhara (anti-anemic) and *balya* (strength giving) properties of PJ was studied using 'anemic' yeast (*Saccharomyces cerevisiae*) cells. *S. cerevisiae* has been a well-accepted model organism to study iron metabolism. In the current study 'anemic yeast' cells were developed by culturing yeast cells in iron-free medium with bathophenanthroline disulfonate (BPS). The effect of pomegranate juice (PJ) on reversing the 'IDA like' condition in yeast was studied. Culturing iron deficient (ID) cells in the presence of 10% PJ supplemented medium (IDP), improved iron status by

at least 7 fold ($p<0.0001$) and reversed mitochondrial degeneration induced by iron deficiency. Percentage of healthy reticulate mitochondria in IDP cells was >30% higher ($p<0.0001$) than that in the ID cells grown in iron deficient medium (IDD) and at least 14% more than that in ID cells grown in 10% PJ-equivalent iron substituted media. Interestingly, PJ substitution improved the functional ferrous (Fe^{2+}) form as well as the bio-assimilated heme form of iron, but not the ferric (Fe^{3+}) storage form in ID cells. The increase in heme content can be attributed to the *dhatuposhana* activity of pomegranate. Pomegranate's potential role as a nutritional supplement in IDA management and as a hematinic is worthy of further research.

Extended lifespan is associated with increased prevalence of infectious, cardiovascular, metabolic and degenerative diseases in ageing individuals. Lifestyle modifications and strengthening diet and nutrition have been identified as important strategies to improve wellness in ageing individuals. *Svasthya* (wellness) imparting potential of PJ was studied using fruit fly (*Drosophila melanogaster*) model. Lifespan extension (*ayuvardhana*), delay ageing (*vayasthapana*), immunity (*vyadhikshamatva*) and reproductive potential (*vrshya*) were used as the markers of wellness for studying PJ.

Supplementation of standard corn meal with 10% (v/v) pomegranate juice (PJ) extended the lifespan of male and female flies by 18 and 8%, respectively. When male and female flies were mixed and reared together, there was 19% increase in the longevity of PJ fed flies, as assessed by mean survival day (MSD) which was 24.8. MSD for control and resveratrol (RV) groups was at 20.8 and 23.1 days, respectively. A two-fold enhancement in fecundity (*vrshya*), improved resistance to oxidative stress

(H_2O_2 and paraquat induced) and to *Candida albicans* infection (*vyadhikshamatava*) were observed in PJ fed flies. Further, the flies in the PJ fed group were physically active over an extended period of time (*vayasthapana*), as assessed by the climbing assay. PJ thus outperformed both control and RV groups in the lifespan and health-span parameters tested.

Semi-quantitative gene expression analysis of *FOXO*, *TOR*, *JNK*, *AMPK* and Mn*SOD* genes were performed with PJ, RV and control feed fed flies. All the five genes tested had a reduced expression in PJ fed group. Knockdown of *FOXO* and Mn*SOD* indicated that lifespan extension of PJ is independent of *FOXO* but partially dependent on Mn*SOD*. Probably, pomegranate also directly contributes to the fly's anti-oxidant system. Based on these observations, it is likely that PJ might be acting through the inhibition of *TOR* pathway requiring further confirmation.

This trans-disciplinary study has developed models for understanding and testing *Rasayana karmas* linked to IDA and healthy lifespan. The study has added experimental evidence for Ayurveda's claim of using pomegranate in the management of IDA and also for healthy lifespan (using *in vitro* and small organism models). The interpretation of the *Rasayana karmas* in this study is based on current understanding; there is scope for improving them in the future. Using model systems and organisms can certainly facilitate us to dissect the mode of action of *Rasayanas* at molecular level. These models only help in understanding certain aspects of how the *Rasayanas* function and not as a whole. This is a limitation of such Shastra-Science research.

DECLARATION

I hereby declare that the work presented in this thesis entitled **"Study of *Rasayana* functions of Pomegranate (*Punica granatum* L.) with a focus on iron metabolism and enhancement of healthy lifespan"** has been carried out by me under the guidance and supervision of **Prof. Padmavathy Venkatasubramanian**, School of Life Sciences, Institute of Trans-Disciplinary Health Sciences and Technology of FRLHT, Bangalore. The present work has not formed the basis for the award of any other degree, diploma or any other qualification previously. All the research described in this thesis was carried out by me. The particulars given in this thesis are true to the best of my knowledge.

Place: Bangalore

Date:

Balasubramani, S. P.

Institute of Trans-Disciplinary Health Sciences and Technology (An expression of FRLHT),
74/2, Jarakabande kaval, Attur post,
Via Yelahanka, Bangalore - 560 106,
Karnataka, India

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Balasubramani S. P.

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LIST OF ABBREVIATIONS USED

AA	:	Ascorbic acid
AMPK	:	AMP-activated protein kinase
BPS	:	Bathophenanthroline di-sulfonate
DW	:	Dry weight
FOXO	:	Fork head transcription factor
g	:	Grams
h	:	Hour
HPLC	:	High performance liquid chromatography
ID	:	Iron deficient
IDA	:	Iron deficiency anemia
IN	:	Iron normal
JNK	:	C-Jun N-terminal kinases
l	:	Litres
mg	:	Milligram
min	:	Minute
ml	:	Millilitre
MSD	:	Median survival day
ng	:	Nanogram
PJ	:	Pomegranate juice
RV	:	Resveratrol
SOD	:	Superoxide dismutase
TDR	:	Trans-disciplinary research
TOR	:	Target of rapamycin
UN	:	United Nations
WHO	:	World Health Organization
µg	:	Microgram
µl	:	Microlitre
µM	:	Micrmolar

Chapter 1

INTRODUCTION

This chapter sets the background for the thesis work. A brief description about the public health issues like population ageing and iron deficiency anemia (IDA), the role of nutrition in combating these health issues and the need for research in holistic nutrition like Ayurvedic *Rasayana* are summarized. The specific objectives and thesis overview are also described.

1.1 Population ageing, a public health problem

Ageing is defined as a continuous process that includes loss of functional capability and the increase in risk of disease and death (Franco et al., 2009). World Health Organization (WHO) statistics indicate that the human population is progressively ageing (United Nations, 2013). Decrease in the mortality and fertility rate of individuals has been contributing to the addition of older individuals to the world population (Turner, 2009). The global share of older people (aged 60 years or over) was 11.7% in 2013 and is expected to reach 21.1% by 2050 (United Nations, 2015). Even though the incidence of infectious and parasitic diseases has decreased to a significant extent, the burden of non-communicable diseases (NCD) has increased drastically. Ageing, diet and life style changes have been contributing to the development of NCD's like cancer, cardiovascular diseases, diabetes, Alzheimer's disease, Parkinson's disease and dementia (Salive and Guralnik, 1997). This increases the burden of health care costs and decrease productivity. Thus, health care of ageing population poses major social and economic consequences.

1.2 Active ageing

Public health agencies and geriatrics researchers have started focusing on “active ageing” or “healthy ageing” or “successful ageing”. WHO defines active ageing as the process of optimizing opportunities for health, participation and security in order to enhance quality of life as people age (WHO, 2002). A ‘healthy ageing phenotype’ can be defined as ‘the condition of being alive, while having highly preserved functioning metabolic, hormonal and neuro-endocrine control systems at the organ, tissue and molecular levels (Lara et al., 2013).

Active ageing allows people to realize their potential for physical, social, and mental well-being throughout the life course and to participate in society, while providing them with adequate protection, security and care when they need (WHO, 2000). Focus on active ageing is also said to improve ‘longevity dividend’, which is defined as "the sum of health, social and economic benefits that result from slower ageing” (Olshansky et al., 2007). Researchers indicate that the investments and interventions for healthy ageing would have greater effect on quality of life than compared to disease specific treatment approaches (Kaeberlein et al., 2015).

1.3 Nutrition for wellness and healthy ageing

Wellness is defined as the state of being in good health. Focus of health care has shifted from disease centric to wellness. It is widely accepted that healthy living is not just a ‘fate effect’ but a result of interweaving between behavioral, environmental and genetic factors (Boccardi et al., 2016). In this context nutrition and life-style are the behavioral determinants of healthy ageing. Optimal nutrition plays a significant role in determining the wellbeing of young, delay age related degeneration and reduce the risk of contracting disease (World Health Organization, 2002). The current literature

evidence suggests that diet composed of fruit, vegetables, fish, whole grains and starchy low-fat staple foods are likely to play a key role in promoting aspects of wellness and healthy ageing including life expectancy (Jong et al., 2014). This dietary pattern is also shown to lower risk of cardio-metabolic diseases and adverse cognitive outcomes.

Molecular studies have shown that irreversible ageing phenotype arises because of the accumulation of macromolecular damage within the cell (Richardson and Schadt, 2014). Several nutritional components including primary and secondary metabolites of plants have been experimentally shown to reduce such damage, or enhance the organism's capacity to repair the damage, leading to reduced risk of age-related diseases and extended longevity (Mathers, 2015). An optimal intake of micronutrients and metabolites is also said to impart metabolic harmony, which in turn prevents disease and imparts wellness (Ames, 2003).

1.4 Iron deficiency anemia (IDA) – another public health issue

Iron is an essential micronutrient, as it is required for adequate erythropoietic function, oxygen transport, oxidative metabolism, DNA synthesis and cellular immune responses (Munoz et al., 2009). Human body does not have mechanism to synthesize iron, so diet is the only source of iron for all physiological requirements. Systemic iron status in the organism is maintained by regulation of iron absorption and storage, but there is no known regulated mechanism for iron excretion from the body (Siah et al., 2006). Increased iron requirements, limited external supply and increased blood loss may lead to iron deficiency (ID) and iron-deficiency anemia (Gomollon and Gisbert, 2009).

Iron deficiency anemia (IDA) affects more than 2 million people worldwide (Kassebaum et al., 2014). The reported prevalence indicates that globally about 40% of pre-school children, 30% in reproductive age women and 38% of pregnant women suffer from iron deficiency (Camaschella, 2015). IDA affects cognitive development, immunity, physical productivity and increased morbidity (Lopez et al., 2016). Maternal mortality and low birth weight are observed in pregnancy linked IDA (Bond, 2016). For the past several decades, governments and WHO has been trying various intervention strategies to combat IDA. Prescribing iron rich foods, distribution of iron-folic acid tablets, fortification of commonly consumed food materials are some of the strategies adopted (Walter et al., 1993; Sloan et al., 2002; Thuy et al., 2003; Zimmermann et al., 2003). But, these interventions have not made a significant impact in reducing IDA. The major reason behind the failure of iron supplementation programmes could be the lesser bioavailability of iron (Nair and Iyengar, 2009). Researchers have been focusing on iron bioavailability enhancers. Ascorbic acid (Cook and Reddy, 2001) and Na-EDTA (Thuy et al., 2003) are some of the iron bioavailability enhancers. Adding them with iron salts can decrease palatability and aggravate side effects like nausea and vomiting (Galloway and McGuire, 1993; Ekstrom et al., 2002).

1.5 Need for research in holistic nutrition

Modern biomedicine does not emphasize the significance of nutrition as a major cause of a wide range of health disorders. Subsequent to infliction with disease, it relies more on target specific drug preparations to re-orient physiology. But, any deviation from the normal might have caused several molecular changes in the complex network of human body. For example, in the case of IDA, iron deficiency may not

just be due to iron unavailability but may also be due to lack of the potential to absorb iron in the system. In the latter case, supplementation of iron may not make any impact. Optimizing the system and its absorbing capacity should be the prime motive.

Similarly, in the case of ageing, several debilitative conditions converge. Rather than individually treating each of the chronic diseases, it would be better if we can directly target ageing. While targeting such complex phenotypes, a holistic nutrition can play a very vital role. Such nutrition can normalize and optimize the system as a whole, rather than looking at specific targets.

Holistic nutrition is an approach to develop a healthy balanced diet while taking into account the person as whole. The goal of holistic nutrition is to facilitate a complete recovery plan as well as build a strong body for lifelong optimal health. There is a need to look at novel and innovative solutions that can address all aspects of wellbeing and which are sustainable.

1.6 *Rasayana* for wellness

Ayurveda is a traditional medical system of India. Treatment principles of Ayurveda are based on the whole system approach and gives equal importance to the individuals, food and medicine. One of the eight disciplines of Ayurveda, called *Rasayana* is specifically for rejuvenation (Udupa, 2004). The *Rasayana tantra* includes some common good living practices and use of herbal or herbo-mineral preparations for wellbeing of an individual (Payyappallimana and Venkatasubramanian, 2015). They have been in practice for several thousands of years but the exact mode of action of the *Rasayanas* based on Ayurvedic principles has not been studied except for few a general bioactivity studies (Balasubramani et al., 2011).

It was hypothesized that understanding *Rasayana* concept and studying the selected aspects and mode of action will add new dimension to the science of rejuvenation particularly in ageing and nutrition related disorders like IDA. The findings of such study will help in development of low-cost, plant-based easily acceptable interventions for IDA and also for healthy ageing and wellness using pomegranate, which is mentioned as *Rasayana* in Ayurveda.

1.7 Objectives of the study

With the above hypothesis, the following were fixed as the objectives of this study:

(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

1.8 Thesis overview

Chapters in the current thesis have been laid out as below. The methodology, findings and discussions are self-contained in each chapter.

Chapter 2 - Methodology: Overall trans-disciplinary research approach followed in understanding the concept of *Rasayana* by theoretical and experimental studies using *dadima Rasayana* are presented in this chapter.

***Chapter 3 - Review of Literature*:** This chapter introduces the biomedical aspects of wellness, ageing and iron deficiency anemia (IDA), followed by literature review on the models used for ageing and iron metabolism related studies. A detailed review on the current status of research on pomegranate also forms a part of this chapter.

Chapter 4 - Rasayana and its role in the management of 'Pandu' and 'Svasthya': This chapter is a review of classical Ayurveda literature and also recent texts to understand the Ayurvedic concepts of *Rasayana*, *Pandu*, *Svasthya* and also the *Drvyaguna* (Ayurveda pharmacology) of *Dadima*.

Chapter 5 - Chemical Standardization of Pomegranate juice: This chapter describes the preparation and standardization of pomegranate juice (PJ) used for experimental studies. Phytochemical standardization, total dissolved solids (TDS), total soluble solids (TSS), total phenolics, total acids, ascorbic acid content and iron content in PJ are presented.

Chapter 6 - PJ increases iron bioavailability and uptake in in vitro models: The models selected and experiments performed to study the *agni* enhancing property of PJ with reference to iron bioavailability and uptake are presented. Cell free and cell based (Caco-2 and HepG2) *in vitro* models have been used.

Chapter 7 - PJ improves iron status and ameliorates iron deficiency induced cellular changes in Saccharomyces cerevisiae: Experimental studies performed to understand the *panduhara* and *dhatuposhana* properties of PJ with reference to improving iron status, heme content, ATP content and healthy mitochondria in anemic yeast (*Saccharomyces cerevisiae*) cells are summarized.

Chapter 8 - PJ enhances healthy lifespan in Drosophila melanogaster: Experiments conducted in *D. melanogaster* model to study the effect of PJ on lifespan extension (*ayuvardhana*), delay ageing (*vayasthapana*), immunity (*vyadhikshamatva*) and reproductive potential (*vrshya*) are described. The possible molecular mechanisms for longevity have also been explored.

***Chapter 9 - Conclusions*:** Overall summary and future directions for *Rasayana* research are presented in this chapter.

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Chapter 2

GENERAL METHODOLOGY

2.1 Introduction

The focus of this study is to understand the concept of *Rasayana*, identify appropriate models to test pomegranate, a *Rasayana* for its anti-anemic (*Panduhara*) and healthy lifespan (*Svasthya*) enhancing properties. This chapter briefly describes the overall trans-disciplinary research (TDR) strategy followed in this doctoral study. The detailed descriptions of methods used for theoretical understanding of *Rasayana*, experimental studies to analyse the anti-anemic and healthy lifespan enhancement by pomegranate are described under the respective chapters.

2.2 Trans-disciplinary research (TDR) strategy

TDR transgresses boundaries between scientific disciplines and between science and other societal fields and includes deliberation about facts, practices and values (Wiesmann et al., 2008). Thus, TDR is defined as research that includes cooperation within the scientific community and a debate between research and the society at large. TDR model deals with scientific studies derived from 'real world problems' (Jaegar and Scheringer, 1998). In this thesis, TDR includes research into Ayurvedic knowledge system to address real life problems like anemia and healthy ageing.

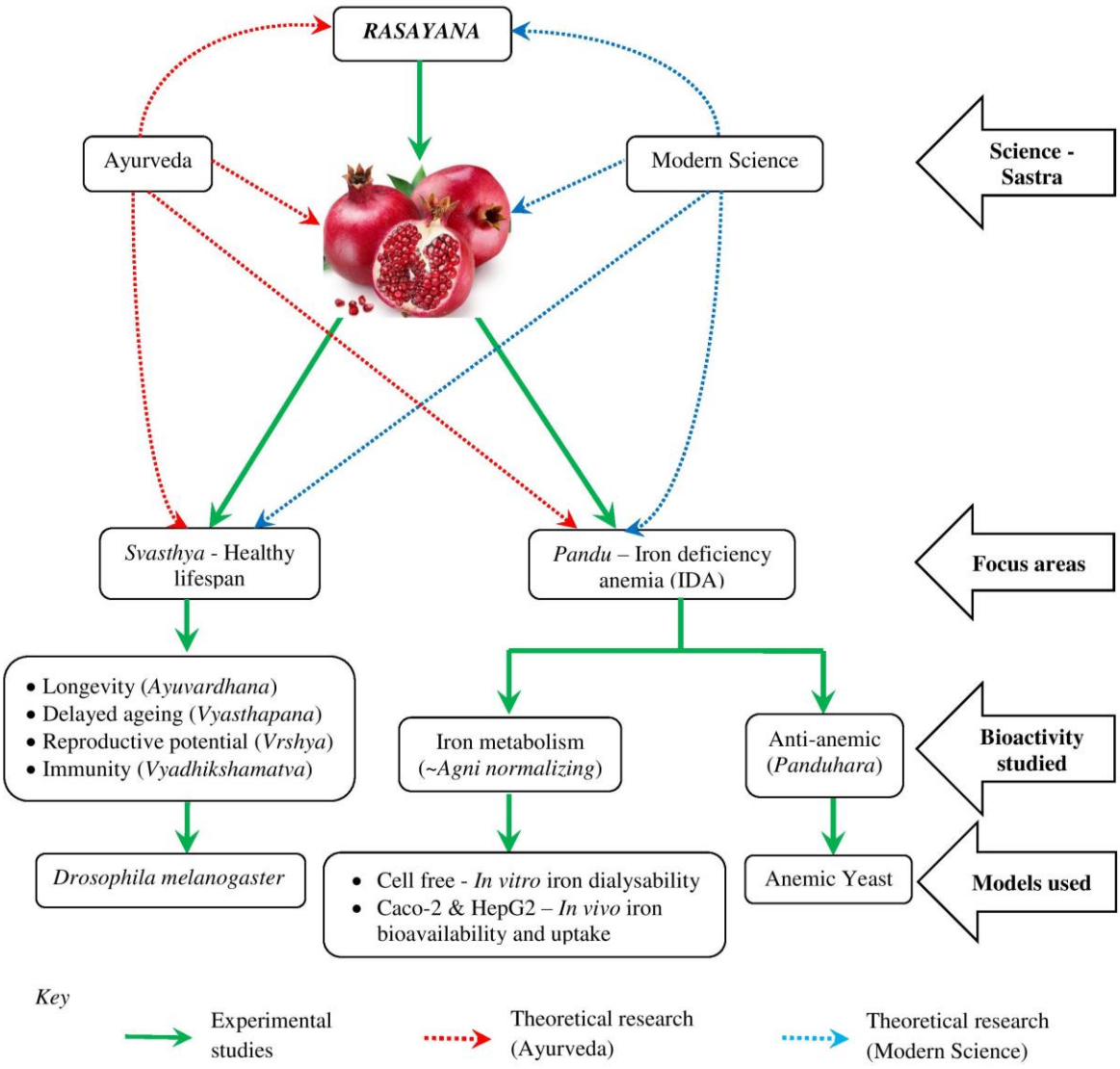

Figure 2.1: Trans-disciplinary research strategy adopted to study of *Rasayana* function of Pomegranate (*Punica granatum* L.) with a focus on iron metabolism and enhancement of healthy lifespan.

To understand the concept of *Rasayana* and to test pomegranate (*dadima*) for its *Rasayana* properties, an appropriate TDR strategy was followed, facilitating dialogue between Ayurveda practitioners and contemporary bio-science. Figure 2.1 gives an overview of the strategy adopted for this study

Concepts involved in *Rasayana* were understood by reading Ayurveda texts and also through discussions with Ayurveda practitioners and scholars. A TDR strategy was framed based on the interactions with Ayurvedic experts and modern scientific understanding in order to identify and test *Rasayana Karmas* of pomegranate for its anti-anemic and healthy lifespan enhancing properties using *in vitro* and *in vivo* relevant models.

2.3 Steps in TDR strategy adopted for this study

Steps involved in the trans-disciplinary understanding of *Rasayana* with a focus on iron metabolism and enhancement of healthy lifespan are mentioned below.

2.3.1 Literature review

Review of modern biomedical literature on iron metabolism, iron deficiency anemia, ageing, wellness, nutrition and pomegranate from published research papers are presented in chapter three. It gives the current understanding on the subject areas and its anticipated utility.

2.3.2 Stating the research sub-problems and solutions:

To understand the concept of *Rasayana* with a focus on iron metabolism and enhancement of healthy lifespan the following sub-problems and solutions were identified.

2.3.2.1 Understanding the concept of *Rasayana* and *Pandu*

Solution: It is important to understand the inter-cultural perspective on subjects chosen per Ayurveda and modern biomedicine before undertaking TDR on *Rasayanas*. A structured literature survey and discussion with Ayurveda experts was performed to understand the concept of *Rasayana*, *Pandu* and *Svasthya* according to Ayurveda and its correlation with modern biomedicine (chapter 4). Prescription of pomegranate based preparations for *Pandu* and its mentioning as *'Nitya Rasayana'* prompted its selection for experimental studies.

2.3.2.2 Standardization of pomegranate juice

Solution: Scientific techniques used in herbal standardization (phytochemical, TDS, TSS, total phenolics, total acids, ascorbic acid content and iron content) were followed to standardize pomegranate juice (chapter 5).

2.3.2.3 *Agni* enhancing potential of pomegranate juice (PJ)

Solution: Literature survey and discussion with Ayurveda experts indicated digestion and metabolism as one of the functions of *agni*. Pomegranate that is used in management of IDA is recommended as '*pathya*' (nutritive supplement), is known to have the ability to enhance '*agni*' through its '*dipana*' and '*pachana*' properties. Cell free in vitro digestion of iron, Caco-2 and HepG2 cell based iron uptake models were used to study the *'agni'* enhancing potential of PJ (chapter 6).

2.3.2.4 *Panduhara* property of PJ

Solution: Iron deficient or anemic Yeast (*Saccharomyces cerevisiae*) cells were generated and were used to study the anti-anemic (*panduhara*) property of PJ in terms of iron and heme contents (chapter 7).

2.3.2.5 Healthy lifespan inducing property of PJ

Solution: Drosophila melanogaster model was used to study the healthy lifespan inducing property of PJ in terms of lifespan extension (*ayuvardhana*), delay ageing (*vayasthapana*), immunity (*vyadhikshamatva*) and reproductive potential (*vrshya*) (chapter 8).

2.3.3 Integration of research findings

Findings of each of the above mentioned studies helped to provide an integrated understanding the concept of *Rasayana* and how it can be used in iron metabolism and healthy lifespan enhancement using *dadima Rasayana* (pomegranate juice) (chapter 9).

2.4 References

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Wiesmann U, Biber-Klemm S, Grossenbacher-Mansuy W, Hadorn GH, Hoffmann-Riem H, Joye D, Pohl C, Zemp E. Enhancing transdisciplinary research: A synthesis in fifteen propositions. In: Hadorn GH, Hoffmann-Riem H, Biber-Klemm S, Grossenbacher-Mansuy W, Joye D, Pohl C, Wiesmann U, Zemp E, eds. Handbook of Transdisciplinary Research. Netherlands: Springer, 2008.

Chapter 3

REVIEW OF LITERATURE

3.1 Introduction

This chapter is a compilation of bio-medical literature related to

(i) Health, wellness, ageing and models for ageing research (section 3.3)

(ii) Iron metabolism and iron deficiency anemia (IDA) - models for IDA research (section 3.4) and

(iii) Modern scientific research on pomegranate (section 3.5)

The objective of this chapter is to analyze and understand the current status of research in the aspects related to the thesis objectives. Review of Ayurveda literature regarding *Rasayana*, *Svasthya* (wellness), *Pandu* and *Dadima* are presented in the following chapter (chapter 4).

3.2 Methodology

A search was performed in PubMed and Google with the terms like ‘wellness’, ‘health’, ‘ageing’, ‘nutrition’, ‘models for ageing research’, ‘iron metabolism’, ‘iron deficiency anemia’, ‘models for anemia research’ and ‘pomegranate’. No restriction was placed on the dates of articles for shortlisting. The results yielded a huge amount of literature, both scholarly as well as lay. Research and review articles which give an overall understanding on the above said aspects were used to compile this review chapter. Websites of World Health Organisation (www.who.int) and United Nations (www.un.org) was visited for definitions, facts and figures.

3.3 Health and Wellness

WHO defines health as "State of complete physical, mental, and social wellbeing, and not merely the absence of disease or infirmity". Wellness is the integration of different components like mental, social, emotional, spiritual, and physical that determines individuals' potential to live a quality and productive life (Edlin and Golanty, 2004). Wellness enables the individual to perform all life activities without limitation and can function independently irrespective of community support (Juechter and Utne, 1982). Focusing on strengths and learning to accommodate weaknesses are essential keys to maintain individuals' health and wellness. Bouchard and Shephard (1994) defines wellness as positive health pertaining to the capacity to enjoy life and withstand challenges. Witmer and Sweeney (1992) defined wellness in terms of life tasks that include self-regulation, work, friendship, spirituality and love. Myers et al., (2005) define wellness as being a way of life oriented towards optimal health and well-being in which the body, mind, and spirit are integrated by the individual to live more fully within the human and natural community.

Physical, emotional (mental), intellectual, social, occupational, environmental and spiritual are the different dimensions of wellness (Hatfield and Hatfield, 1992). Wellness and better quality of life is possible regardless of the health status. It is possible to possess wellness while being ill or possessing a debilitating condition (Payne and Hahn, 1998). Healthy life-style is the prerequisite for wellness. Regular physical activity, good nutrition and stress management form components of healthy life-style that improve quality of life (figure 3.1) (Corbin and Pangrazi, 1998).

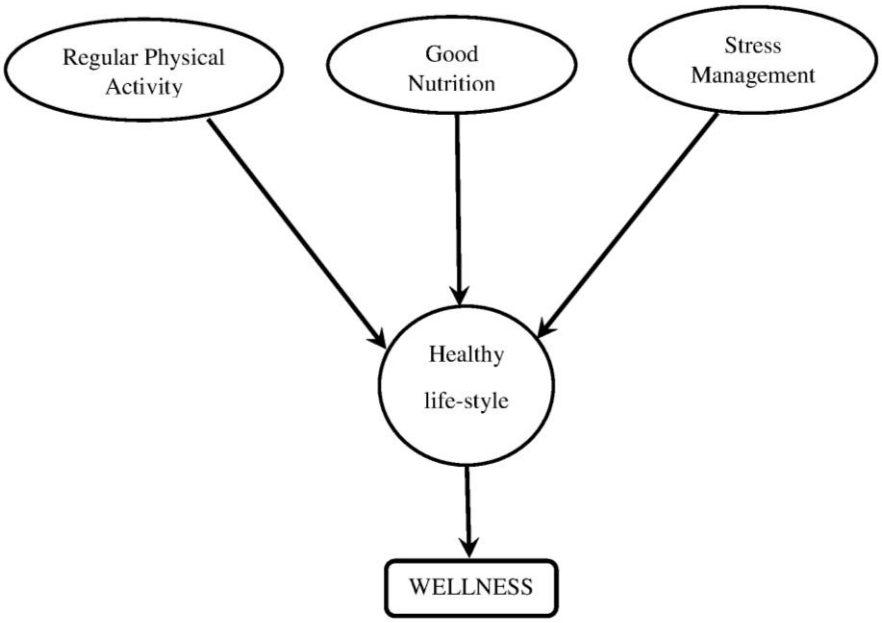


Figure 3.1: A model for optimal health and wellness

3.3.1 Determinants of wellness

Wellness itself is considered as a determinant of the quality of life (Sarvimaki and Stenbock-Hult, 2000). Wellbeing enables the individual to work effectively, enjoy leisure time, be healthy, resist diseases and meet emergency situations (Grewal et al., 2006). Body composition, flexibility, endurance, strength, balance, coordination, speed, power and reaction time are some of the parameters used in describing wellness (Bowling and Iliffe, 2006). Integration of physical, psychological, social, intellectual, spiritual, occupational and environmental dimensions (figure 3.2) contribute to the wellness of the individuals (Adams et al., 1997). Importance of maintaining health related quality of life is of much relevance in older age when health status changes as a result of ageing processes and chronic diseases (Lin et al., 2016).

Figure 3.2: Dimensions of wellness

3.3.2 Ageing and diseases

Aging is defined as a progressive, irreversible, endogenous and deleterious process that occurs post-maturation (Strehler, 1962). This turns young healthy adults into older, more frail adults, increasingly susceptible to environmental challenges (such as extreme temperature or disease-inducing infectious agents) and increased risk of death (Buffenstein et al., 2008). Loss of physical abilities, loss of strength, reduction in the rate of aerobic capacity, reduction in flexibility of joint movement, balance disorders, loss of fat free mass and increase in fat mass are observed with old age (Baeza et al., 2009; Milanović et al., 2013). With several biomedical advancements and public health interventions, the current century has witnessed a steep increase in the average lifespan of humans (Oeppen and Vaupel, 2002). It is estimated that 901 million

individuals aged 60 years or above were living in 2015 and this would increase to 1.4 billion by 2030 and to 2.1 billion by 2050 (United Nations, 2015). This increase in the lifespan is associated with increased incidence of various non-communicable diseases like cardiovascular conditions, diabetes, obesity, osteoarthritis and neurodegeneration (De Grey, 2007; Dillin et al., 2014). Increased lifespan also increases the physical and financial dependency of aged individuals on care takers. This is directing the biomedical scientists, not only to treat the various diseases that are associated with ageing, but also to promote healthy ageing (Partridge et al., 2011). Maintenance of health and fitness is necessary for graceful or healthy ageing (Baeza et al., 2009).

3.3.3 Theories of Ageing

The hallmarks of ageing are genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and altered intercellular communication (López-Otín et al., 2013). Several theories have been proposed for ageing process. They are:

i. Evolutionary theory considers aging as a result of a decline in the force of natural selection (Haldane, 1941),
ii. Free radical theory considers accumulation of endogenous oxygen radicals generated in cells as a reason for the aging and death of all living beings (Harman, 2003).
iii. Mitochondrial theory is an extension of free radical theory. Mitochondrial DNA mutations accumulate progressively during triggering an exponentially increasing oxidative damage and dysfunction, which ultimately culminate in death (Miquel et al., 1980).

iv. Gene regulation theory states that senescence is the resulting of changes occurring in the gene expression (Kanungo, 1975).

v. Telomere theory - Telomere sequences stabilize chromosomal ends. The attrition of chromosomal termini, caused by loss of telomerase, can lead to breaks and damage chromosome leading to cellular senescence (Harley et al., 1990).

vi. Inflammation hypothesis - Inflammation is an innate defense mechanism against stress. Uncontrolled stress can over-activate the inflammatory process leading to physiological damage and ageing (Franceschi et al., 2000a).

vii. Immune theory proposes that ageing is indirectly controlled by a network of cellular and molecular defense mechanisms (Franceschi, 1989).

viii. Neuro-endocrine theory – Loss of bidirectional communication between nervous and immune system results in loss of homeostasis leading to death (Fabris, 1991)

ix. Neuro-endocrine - immune theory – Plasticity of neuro-endocrine and immune system is lost on ageing resulting in auto-immune pathology that eventually leads to death of the individual (Franceschi et al., 2000b).

x. Caloric restriction is a mechanism by which organisms can extend the life-span by reducing the calorie intake without compromising the essential nutrients intake (Weindruch et al., 1986).

Changes at the molecular level are responsible for the development of pathological conditions associated with age related diseases (Dillin et al., 2014).

3.3.4 Healthy ageing

The main characteristic of ageing is the gradual loss of functions (Vaupel, 2010). As there are no strong theories to link the loss of physical function and death to genetic programmes, it is possible to delay or reduce this functional decline, improve health and wellbeing both physically and mentally (Longo et al., 2015). Any internal or external factor can alter the physiology and influence the ageing process of an individual (MacNee et al., 2014). Some factors might have negative influence and thus accelerate ageing, while there are also positive factors which can delay or facilitate healthy ageing (Baeza et al., 2009). Progressive ageing can be postponed by avoiding risk behaviors like smoking, excessive alcohol consumption, and obesity, which speeds up the onset of diseases linked to age (Shammas, 2011). Further, health promotional activities like healthy diet and exercise patterns, can contribute to reduce ill effects of ageing, an increase in life expectancy and better health (Shammas, 2011; Fontana and Partridge, 2015). Such benefits are most effective when healthy lifestyles are adopted early in life, however, positive effects can occur at any age (Kumar and Preetha, 2012).

3.3.5 Nutrition, a prerequisite for health and wellness

The provision of nutrients in the womb and the food that we eat from birth onwards influences the size, shape and endurance of the human body throughout the life course (Uauy and Solomons, 2005). Nutrition have direct influence on the rate at which an individual grows and matures from conception to adult life and also on physical and mental development (WHO, 2003). Holistic nutrition refers to healthy food for optimum health and wellbeing (Haas and Levin, 2006). Hallmarks of holistic nutrition include unrefined, unprocessed, organic and locally grown whole foods (Seedorf et

al., 2007). Research indicates that many chronic illnesses like diabetes, obesity, arthritis, heart disease and high blood pressure can be prevented through diet (WHO, 2002). The potential impact of dietary manipulation on the maintenance of physical and cognitive function between middle and old age has profound consequences for optimization of health, independence and well-being for the latter years (Charlton, 2002).

3.3.6 Healthy-lifespan enhancers

Rapamycin, a metabolite produced by soil bacteria is the first to be identified as a lifespan enhancer (Bjedov et al., 2010). It is a small molecule inhibitor of the protein kinase mTOR (mechanistic target of rapamycin) (Kennedy and Lamming, 2016). This compound has extended healthy lifespan from yeast to mammals and is presently in the human clinical trials (Johnson and Kaeberlein, 2016). Resveratrol is a polyphenolic compound (Takaoka, 1939) which has been tested for its healthy lifespan enhancing properties across various model organisms (Bhullar and Hubbard, 2015) and is presently in the human trials (Park and Pezzuto, 2015).

Several foods or food components have been identified for imparting healthy lifespan (Lee et al., 2015). Plant-based diets like vegetables and fruits, whole grains, pulses, nuts and seeds are said to have health benefits (Leonov et al., 2015). Several phytochemicals present in plant based foods like epicatechin, quercetin (Pallauf and Rimbach, 2013) and curcumin (Ferrari, 2004) have already been identified as lifespan enhancers and anti-ageing molecules in model organisms. Healthy nutrition has been shown to reverse age related epigenetic changes and prevent disease onset (Park et al., 2012).

3.3.7 Healthy ageing research

Several researchers are involved in identifying life-span increasing interventions in terms of drugs, dietetics and practices. In spite of rigorous research, still there are several unresolved mysteries in ageing mechanisms. As ageing is a complex phenomenon, several physiological pathways need to be analysed simultaneously. Even though using human for anti-ageing intervention studies is most appropriate, it is impractical due to ethical issues, long natural lifespan, life-style, diet, environmental influences and genetic heterogeneity. Thus research into aging and longevity can begin using short-lived animal models. Exploration and experimentation take place using these species because life span studies can be carried out in a short period of time, subsequent to which promising life span enhancers are further tested in higher animal models. Only after finding success in the above screening, potential interventions make it to human clinical trials.

Biogerontologists have identified a few model organisms which occupy different positions in animal evolution but exhibit conserved regulatory processes in ageing (Murthy and Ram, 2015; Mitchell et al., 2015). However, the observations are restricted on only those areas in which their use is most appropriate.

The three primary criteria employed for selection of animal models are:

i. Feasibility of the model
ii. Whether it can address the specific question under investigation, and
iii. Whether the findings can be generalized across various species

The fundamental biology of cells, regulation of metabolism and mechanisms of aging are similar across widely separated species (Carmona and Michan, 2016). Thus

research in lower animals can still be relevant to human cellular biochemistry, and provide insight into human aging.

Table 3.1: Model organisms used in ageing studies

Common name	Scientific name	Aspects studied	Reference[1]
Yeast	*Saccharomyces cerevisiae*	Genetic pathways and interventions	Gershon and Gershon, 2000
Star ascidian or golden star tunicate	*Botryllus schlosseri*	Immunology, stem cell biology, evolutionary biology and regeneration	Rinkevich et al., 2013; Murthy and Ram 2015
Ciona	*Ciona intestinalis*	Ageing and regeneration	Jeffery, 2012
Hydra	*Hydra vulgaris*, *H. oligactis*	Longevity and immortality	Bellantuono et al., 2015; Tomczyk et al., 2015
Monogonont rotifers	*Brachionus manjavacas*	Life-span and health span	Snell et al., 2014
Sea urchins	*Strongylocentrotus franciscanus*, *S. purpuratus*, *Lytechinus variegatus*	Longevity	Bodnar, 2009
Daphnia	*Daphnia pulicaria*, *D. pulex*	Stress and longevity, dietary restriction	Kim et al., 2014; Schumpert et al., 2014
C. elegans	*Caenorhabditis elegans*	Ageing mechanisms and interventions	Tissenbaum, 2015
Fruitfly	*Drosophila melanogaster*	Ageing mechanisms and interventions	Brandt and Vilcinskas, 2013; Balasubramani et al., 2014[2]
Zebrafish	*Danio rerio*	Tissue regeneration, longevity and interventions	Gilbert et al., 2014
Turquoise killifish	*Notho branchius furzeri*	Ageing and disease	Kim et al., 2016
Mice	*Mus musculus*	Ageing mechanisms, age related diseases and interventions	Vanhooren and Libert, 2013
Naked mole-Rat	*Heterocephalus glaber*	Mechanisms of longevity, potential therapies	Gallagher et al., 2011
Monkey	*Rhesus macaques*	Age related pathology and interventions	Roth et al., 2004

[1]References shown are only indicative
[2]Publication from this thesis

3.3.8 Model organisms for ageing research

From yeast to monkey, several organisms are used in ageing research. Studies have shown that dietary restriction, without malnutrition, can extend lifespan, and delay the onset of age-related pathologies in almost all the model organisms including yeast (Lin et al., 2004), *C. elegans* (Houthoofd and Vanfleteren, 2006), drosophila (Partridge et al., 2005), and mammalian models (Robertson and Mitchell, 2013). Studies on both the effects and causes of ageing in model organisms can yield valuable insights into the molecular and cellular processes that underlie ageing in humans. The list of various *in vivo* models used in healthy ageing research and the aspects studied are presented in table 3.1.

3.3.9 Ageing pathways are conserved during evolution

Genes of *daf-2* which code for the insulin / insulin like growth factor - 1 (*IGF-1*) pathway was found to be involved in extending the life-span of long-lived *C. elegans* mutants (Dorman et al., 1995). *C. elegans* which had mutations downregulating *IGF-1* expression not only lived longer but also were looking young (Herndon et al., 2002). It was later identified that the *IGF-1* pathway is conserved during evolution and is involved in the lifespan enhancement of similar mutants in *Drosophila* and mice (Piper et al., 2008). *IGF-1* is a nutrient sensitive pathway which controls growth, metabolism and reproduction. It was identified that the life extending mutations in mice alter growth hormone (GH) and insulin growth factor-1 (*IGF-1*) signalling (Bartke, 2011). Some of the preliminary experiments indicate that one of the key effectors of *IGF-1*, forkhead transcription factor (*FOXO*) has influence on the lifespan of organisms including humans. In *Drosophila*, inhibiting insulin/*IGF-1* signalling systemically or increasing the activity of *FOXO* specifically in adipose

tissues increases lifespan (Kenyon, 2010). In *C. elegans*, overexpression of *daf-16*, a *FOXO* transcription factor increases lifespan (Gami and Wolkow, 2006).

Target of Rapamycin (*TOR*) pathway is another signaling mechanism identified to play a role in lifespan extension. Inhibition of *TOR* pathway was found to increase lifespan across yeast and several multicellular organisms (Kapahi et al., 2004; Kaeberlein et al., 2005). *TOR* is an amino acid sensing pathway. Rapamycin, an inhibitor of *TOR* extended the lifespan in mice (Harrison et al., 2009). McCormick et al., (2011) identified that mutations in *TOR* signalling extend lifespan in yeast, *C. elegans*, *Drosophila* and mice.

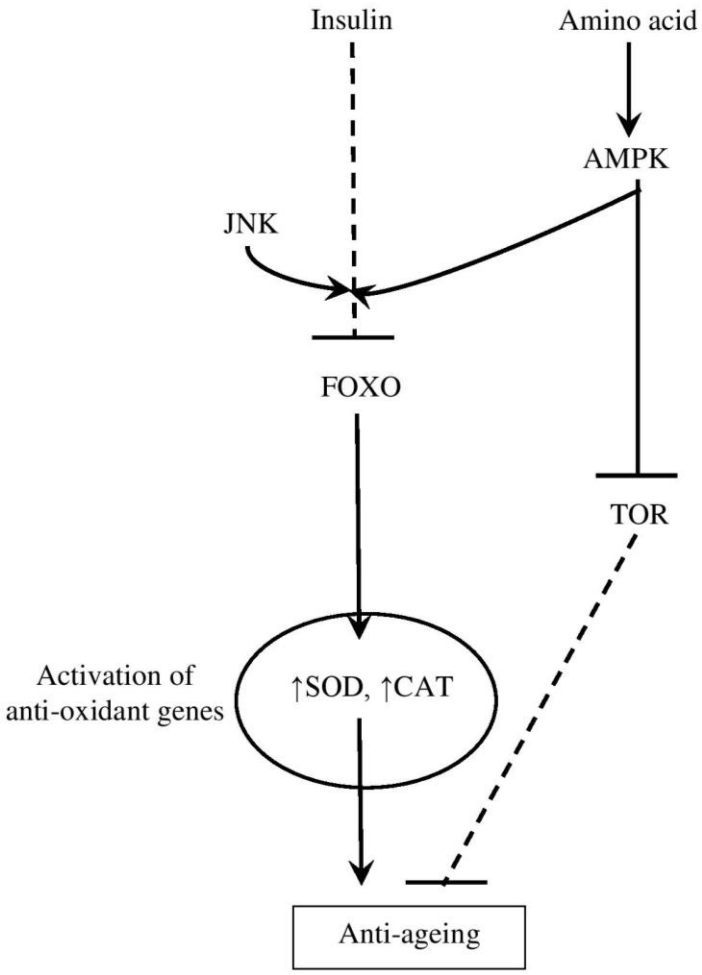


Figure 3.3: Genetic pathways involved in lifespan extension and anti-ageing. Activation of *FOXO* and downregulation of *TOR* is indicated to have anti-ageing effect.

Exploring the downstream effectors of *TOR* indicated the involvement of *AMPK* activity and translation (McCormick et al., 2011). *AMPK* (5' adenosine monophosphate-activated protein kinase) is a nutrient and energy sensor enzyme that plays a role in cellular energy homeostasis. Overexpressing *AMPK* extends lifespan in *C. elegans* (Apfeld et al., 2004), and the anti-diabetic drug metformin is an *AMPK* activator and shown to extend lifespan in mice (Anisimov et al., 2008). Overexpression of sirtuins (*sir2*) have also been reported to extend lifespan in yeast, worms and flies (Kenyon, 2005). Sirutins are NAD+ dependent protein deacetylases and are said to function by gene silencing at telomeres during ageing (Dang et al., 2009). Figure 3.3 presents an overall view of the genetic pathways involved in lifespan extension and anti-ageing.

3.3.10 *Drosophila* model

In 1900's, *Drosophila melanogaster* (commonly known as fruit fly) was introduced as a model organism to research genetics, developmental biology, signal transduction and cell biology (Ashburner et al., 2005). Even though, genome of *D. melanogaster* is only 5 % of the size of a typical mammalian genome, gene families and pathways are shared with mammals (Miwa and Cohen, 2006), as well as many tissues and organ systems (De Velasco et al., 2004; Matthews et al., 2005). Availability of genetic manipulation methods like mutagenesis screens, RNA interference (RNAi), and transgenesis have facilitated aging research in *D. melanogaster* (Venken and Bellen, 2005). There are abundant publicly available resources, including thousands of *D. melanogaster* strains provided by the Bloomington Stock Center, Indiana University, USA as well as many cell lines, clone libraries, antibodies, and microarrays making this organism ideal for ageing research. There is also an exhaustive database

containing information relevant to *D. melanogaster* genetics, development and molecular biology (Drysdale, 2008).

Drosophila has physiological, genetic and anatomical similarities with human (Iliadi et al., 2012). *D. melanogaster* has >60% of homologous genes (of 13,601 genes) with humans, these have been analyzed to identify sequences related to those causing human diseases (Jafari et al., 2006). It has large numbers of induced and spontaneous mutations with only four chromosomes in a small genome. These facts make *Drosophila melanogaster* model suitable to study the genetics of disease, degeneration, and ageing processes, as results can yield insights into molecular pathways of ageing in humans (Miwa and Cohen, 2006). There are several advantages for using *Drosophila* in aging research (Beckingham et al., 2005; Miwa and Cohen, 2006). They include

i. Tiny body size with short lifespan (<3 months) (figure 3.4)
ii. Easy maintenance of flies including male and female differentiation (figure 3.4)
iii. Multiple clones with identical genetic make-up can be generated to avoid batch to batch variation in observations
iv. Possibility of manipulating environmental and genetic factors to alter life span
v. Simple assays to observe physiological and behavioral changes
vi. Huge literature is available on ageing studies using flies
vii. Availability of genetically altered stocks to study the effect of specific genes
viii. Several molecular genetic techniques have been developed for fly genetics including GAL4/UAS for targeted gene expression (Duffy, 2002)
ix. Complete genome sequence of *Drosophila* genome is available

x. Flies have proven to be successful in dissecting complex developmental biological phenomenon

xi. The demarcation between development and adulthood is much clearer in insects than other model organisms (adulthood being defined as eclosion from the pupal case)

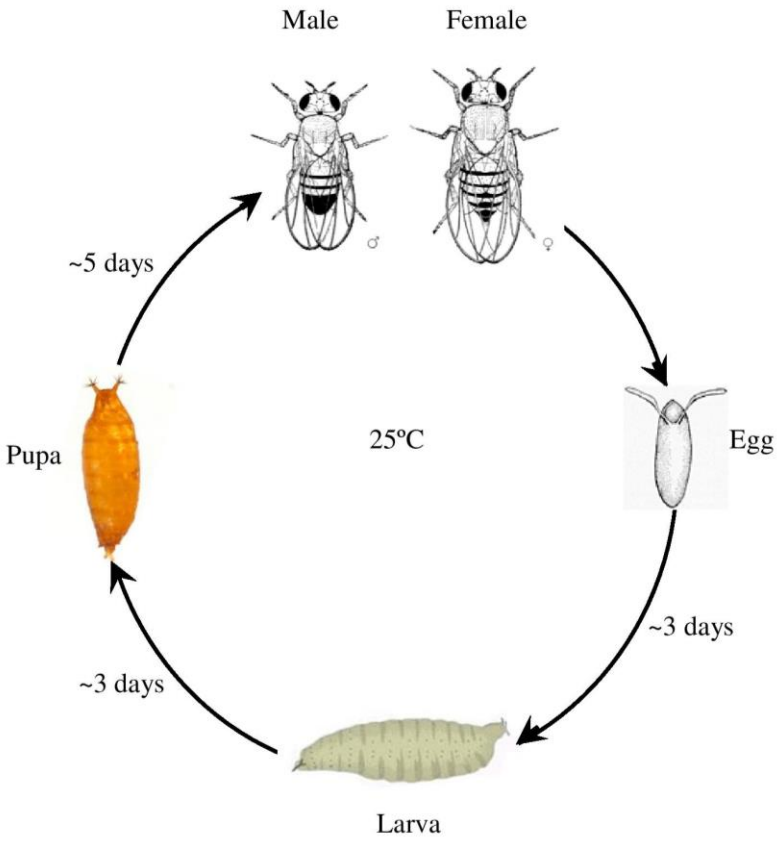


Figure 3.4: Life cycle of *Drosophila melanogaster*

3.3.10.1 *Drosophila* GAL4/UAS system

GAL4 system is a targeted gene expression system consists of two components: (i) GAL4, a transcriptional activator from yeast, which is expressed in a tissue-specific manner and (ii) UASG, a transgene under the control of the upstream activation sequence that is bound by GAL4 (Duffy, 2002). Expression can be controlled by

growing the flies in low temperature. GAL4 UAS and the gene of interest are brought together in a simple genetic cross (Elliott and Brand, 2008). The progeny of the cross is tested for the expression of the gene of interest in specific tissues or whole organism.

3.3.10.2 *Drosophila* as a human ageing model

Pletcher et al., (2002) profiled the transcripts of *Drosophila* on a genome-wide scale over a range of adult ages, finding that at least 6 % of the genes show changes in transcription with age spanning about 400 loci that are involved.

The anti-ageing property of resveratrol, a plant-derived compound, well established by its presence in some types of wine was initially described based on experiments with *Drosophila* (Bauer et al., 2004). Changes in diet composition, dietary and caloric restriction, were also found to extend fruit fly lifespan (Tatar, 2011). Increase in median lifespan, extended survival of flies (Peng et al., 2011), fertility (Chandrashekara et al., 2011), stress resistance (Peng et al., 2012), physical performance (Wang et al., 2013) and quantity of food intake (Bahadorani and Hilliker, 2008) are the commonly assessed parameters in anti-ageing studies. Several molecular mechanisms have been proposed for dietary or drug-mediated longevity enhancement. In particular, FOXO (Partridge et al., 2011), TOR (target of rapamycin) and AMPK signaling (Bjedov and Partridge, 2011) pathways are mainly involved in lifespan-prolonging effects of many treatments, as determined by experiments conducted on fruit fly models. Observations from the reported studies indicate that "many" pathways are involved in ageing in fruit flies. Several herbal preparations have been identified as life and health span enhancers using *Drosophila* model (table 3.2). In 2010, Priyadarshini et al., developed an insect specific Rasayana and tested

for its potential to increase longevity in *Drosophila* model. They found 50% increase in lifespan by feeding the insect specific Rasayana to flies.

The current understanding of ageing biology and the experimental studies conducted indicate that novel strategies need to be followed to develop 'anti-agathic' (anti-ageing) interventions. With conserved ageing pathways and other biological tools, *Drosophila* has a strong potential to be used in aging research.

Table 3.2: Herbal based dietary supplements with lifespan enhancing properties tested in *Drosophila*

Diet source	Reference
Prunetin (isoflavone)	Piegholdt et al., 2016
Naringenin (bioflavonoid)	Chattopadhyay et al., 2016
Herbal extract-SC100 (*Astragalus membranaceus* root, *Pterocarpus marsupium* bark, pine bark oligo-proanthocyanidins, and L-theanine)	Villeponteau et al., 2015
Proanthocyanidins from *Kunlun Chrysanthemum* flowers	Jing et al., 2015
Cinnamon	Schriner et al., 2014
Nordihydroguaiaretic Acid (a lignin from *Larrea tridentate*)	Spindler et al., 2015
Curcumin	Chandrashekara et al., 2014; Siddique et al., 2014; Wang et al., 2014; Shen et al., 2013 Soh et al., 2013; Lee et al., 2010; Suckow et al., 2006
Green tea polyphenols	Lopez et al., 2014
Artemisinin and curcumin	Das et al., 2014
Curcuma longa and *Emblica officinalis*	Rawal et al., 2014
Ludwigiaoctovalvis	Lin et al., 2014
Viscum album coloratum (Korean mistletoe)	Lee et al., 2014
Cranberry	Sun et al., 2014 Wang et al., 2014
Pectin	Shaposhnikov et al., 2014
Lutein (carotenoid)	Zhang et al., 2014
Rhodiola rosea	Schriner et al., 2013
Organically raised bananas, potatoes, raisins and soy beans	Chhabra et al., 2013

Incarvillea younghusbandii root extract	Pan et al., 2012 Pan et al., 2008
Sesamin	Zuo et al., 2013
Black rice	Zuo et al., 2012
Cynomorium songaricum	Liu et al., 2012
Amalaki Rasayana	Dwivedi et al., 2012
Emblica officinalis	Pathak et al., 2011
Blueberry extract	Peng et al., 2012
Resveratrol	Bass et al., 2007; Wang et al., 2013
Rosa damascena	Schriner et al., 2012
Aloe vera and resveratrol	Chandrashekara et al., 2011
Orange and lemon juice	Fernández-Bedmar et al., 2011
Epicatechin	Si et al., 2011
Glucose and polyphenols	Ortega-Arellano et al., 2011
Nectarine	Boyd et al., 2011
Apple polyphenols	Peng et al., 2011
Genistein	Altun et al., 2011
Water and ethanol extracts of *Stachys lavandulifolia*	Altun et al., 2010
Rasayana diet	Priyadarshini et al., 2010
Black tea theaflavins	Peng et al., 2009
Cocoa	Bahadorani and Hilliker, 2008
Caffeine	Nikitin et al., 2008
Green tea catechins and broccoli	Li et al., 2008
Rhodiola	Jafari et al., 2007

3.4 Iron Metabolism, Iron Deficiency and iron deficiency anemia (IDA)

This section of the review summarises the current understanding on iron metabolism, iron deficiency and IDA.

3.4.1 Iron, an essential micronutrient

Iron plays a vital role in several physiological processes including oxygen transport and storage, oxidative metabolism and cellular proliferation (Cairo et al., 2006). Its most important property is the reversible one-electron oxidation-reduction reaction between the two common oxidation states, reduced ferrous (Fe^{2+}) and oxidized ferric (Fe^{3+}), allowing it to coordinate electron donors and to participate in redox processes (Hentze et al., 2004). This reductive property of iron also accounts for its potential toxic effects. Reactions with oxygen can lead to the formation of unstable intermediates with unpaired electrons. These free radicals, particularly the hydroxyl radical OH*, react with DNA, proteins and lipids causing their destruction (Puntarulo, 2005).

Iron is also an essential nutrient for all known pathogens, freely available iron in the system may increase virulence of pathogenic microorganisms (Jurado, 1997). The human body has developed complex metabolic processes to absorb, transport and store iron ensuring a ready supply for cellular growth and function, but limiting its participation in reactions that produce free radicals and its availability to invading pathogens (Camaschella and Strati, 2010). Iron is an essential component of haemoglobin. Diet is the only source of iron (Scheers, 2013).

3.4.2 Diet as the iron source

Two forms of iron are present in diet:

i. Inorganic or non-haem iron, which occurs as ferric hydroxide complexes loosely bound with proteins, amino acids or organic acids of herbs.

   During acid-pepsin digestion in the stomach, hydrochloric acid in the stomach as well as organic acids in food split the inorganic form of iron from its combination with organic molecules and reduce to ferrous state. Presence of reducing substances like vitamin C facilitate this process. Presence of phytates can result in the formation of insoluble salts and prevent absorption (Nair and Iyengar, 2009).

ii. Haem iron present in animals is bound to porphyrin in haemoglobin. Haem iron is absorbed intact into the intestinal epithelial cells and the iron is split off from the haem moiety within the epithelial cell (West and Oates, 2008).

Most of the common diets have both facilitators of iron absorption as well as inhibitors. Phytates, phosphates and tannins from vegetables foods are iron absorption inhibitors while ascorbic acid and animal protein including meat, help the absorption of iron (Nair and Iyengar, 2009).

3.4.3 Iron metabolism in humans

Major site of iron absorption is the proximal part of duodenum (figure 3.5) (Munoz et al., 2009). Absorption can also happen from distal parts of the duodenum, jejunum and proximal ileum. Soluble ferrous iron can also be obtained from colon. In stomach and small intestine, all ferric iron in the food is converted to the soluble ferrous form before it can be absorbed (Frazer and Anderson, 2005).

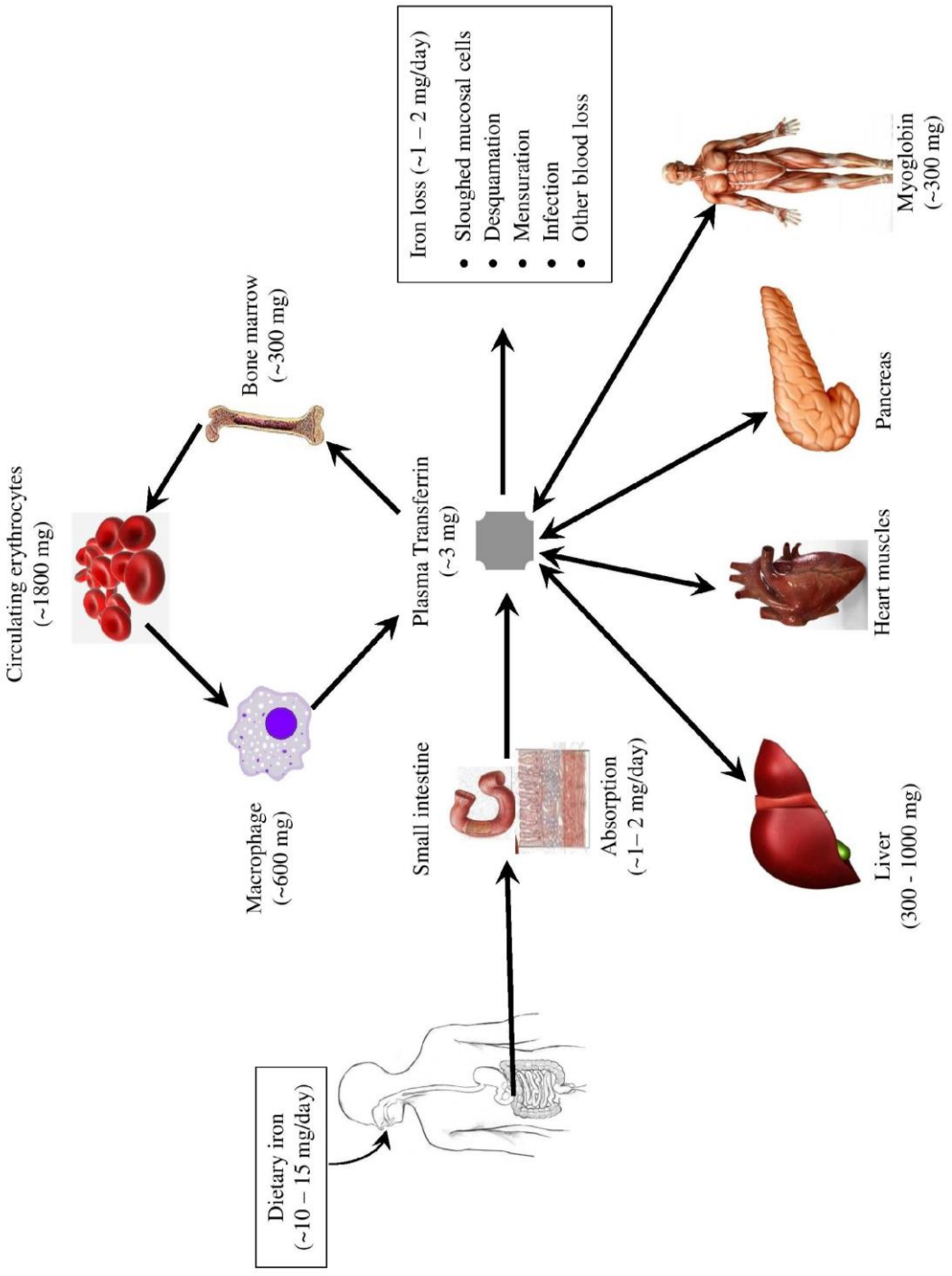


Figure 3.5. Iron metabolism in humans

3.4.4 Iron absorption and storage

Iron taken up by the brush border of the enterocyte rapidly passes into the cell. The quantity of iron transferred from the lumen into the enterocytes depends upon the availability of receptors on the brush border (Mackenzie and Garrick, 2005). Unlike inorganic iron, haem can enter the enterocyte directly and it is broken down to release iron (West and Oates, 2008).

Specific carriers (ferroportin) in the enterocytes transfer iron to the serosal side and deliver to plasma transferrin (Wessling-Resnick, 2006). Excess iron in the cell which is not transferred from the enterocyte is stored as ferritin, which acts as a slowly exchangeable pool of iron with a half-life of about 4 days (Theil, 1990). Ferritin iron is also lost by the desquamation of cells. The gut mucosa plays a regulatory role in iron absorption which depends upon the saturation of the mucosal cells with iron through hepcidin, which regulates entry of iron into circulation (Wessling-Resnick, 2006). The state of repletion of body stores, degree of erythropoiesis and hypoxia influence iron absorption (Tussing-Humphreys et al., 2012).

3.4.5 Internal iron exchange

Iron absorbed into the blood stream is carried by transferrin and is transported to the sites of use and storage (Munoz et al., 2009). In the adult male, 200 mg of iron is liberated daily from catabolized erythrocytes and is recycled by the transport system to the bone marrow for incorporation into new red blood cells (figure 3.5) (Knutson and Wessling-Resnick, 2003). The daily turnover of plasma iron is about 35 mg; only a small portion (1 – 2 mg) of it is derived from the diet even when absorption has been at a maximum (Munoz et al., 2009). The total amount of functioning tissue iron in the adult is 300 mg and a significant amount is replaced daily to replete the losses

(Hentze et al., 2010). About 1 mg of iron a day is lost from the body in urine, faeces, sweat, and cells shed from the skin and the gastrointestinal tract (Institute of Medicine Panel on Micronutrients, 2001). Menstrual losses amount to about 20 mg a month. Increased requirements of pregnancy (500 – 1000 mg) contribute to the higher incidence of iron deficiency in women of reproductive age (Koenig et al., 2014).

Human beings normally have 40–50 mg Fe/kg body weight (Bothwell et al., 1979). Iron is stored in the body in the form of ferritin and haemosiderin (figure 3.5). Both forms are available to replace lost iron but ferritin is more readily available than haemosiderin (Saito et al., 2012). This latter form of stored iron is nearly fixed and takes many years to disappear. Body stores of iron are distributed as approximately a third in the liver, a third in the marrow and another third between spleen, muscle and other tissues (Zimmermann and Hurrell, 2007).

During pregnancy the placenta is a site of significant iron transfer which is carried out through placental receptors, from where it is then transferred to the fetus (Koenig et al., 2014). Because of the requirements of pregnancy and losses in menstrual blood, the iron requirements of a woman in the reproductive period of life are at least twice those of a man or of a post-menopausal woman (Lopez et al., 2016).

3.4.6 Iron Deficiency

Iron deficiency tends to be most common nutritional disorder in the world (Lopez et al., 2016). It may be a resultant of any one or more of the following reasons (Munoz et al., 2009).

i. Inadequate iron intake
ii. Body's excess iron demand to meet the requirements of growth, e.g. in pregnancy, during infancy and at adolescence
iii. Infections that may interfere with the activity of the bone marrow or increase erythropoiesis like *Helicobacter pylori* infection
iv. Blood loss or haemolysis
v. Surgical procedures that alter the anatomy of the stomach and duodenum
vi. Malabsorption syndromes

3.4.7 Iron deficiency to anemia (IDA)

The end result of a long period of negative iron balance or reduced iron intake or increased iron loss or increased physiologic requirements for iron leads to IDA (Lopez et al., 2016). Generally, hemoglobin measure is used in diagnosis of IDA (table 3.3) (WHO, 2008). IDA development may have prelatent, latent and disease stage (figure 3.6) (Zimmermann and Hurrell, 2007).

Table 3.3: Normally used cut-off for hemoglobin to define anemia (WHO, 2008)

Group	Cut-off value
Children aged 0.5-5 years	<11.0 g/dl
Children aged 5-11 years	<11.5 g/dl
Children aged 12-13 years	<12.0 g/dl
Men	<13.0 g/dl
Non-pregnant women	<12.0 g/dl
Pregnant women	<11.0 g/dl

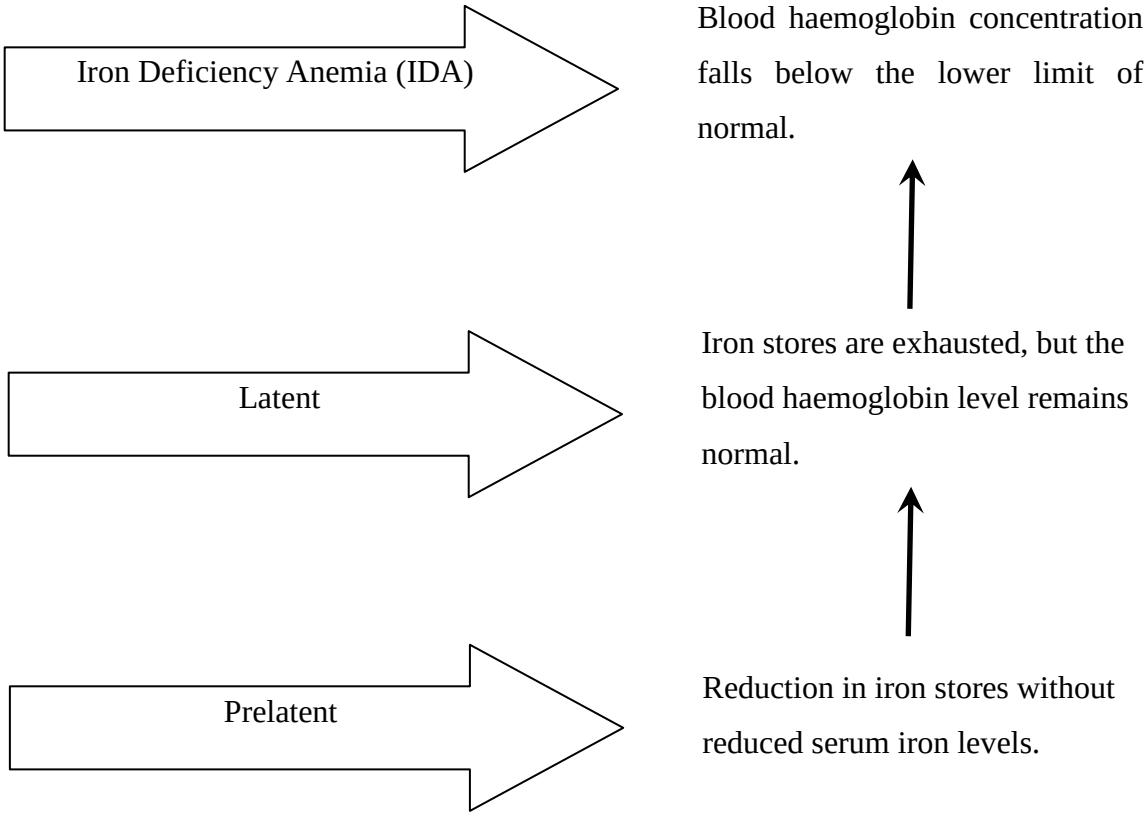


Figure 3.6: Stages in the development of iron deficiency

3.4.8 Signs and symptoms

Fatigue and diminished capability to perform hard labor, poor scholastic performance, cold intolerance, reduced resistance to infection, attention deficit disorder and symptoms of comorbid cardiac or pulmonary disease are observed with IDA individuals (Lopez et al., 2016).

3.4.9 Diagnosis of IDA

IDA is diagnosed by testing hemoglobin content, complete blood count, serum iron binding capacity and serum ferritin (Zimmermann and Hurrell, 2007).

3.4.10 Management of IDA

Treatment of IDA consists of correcting the underlying etiology and replenishing iron stores by any of the following methods (Balarajan et al., 2011):

i. Oral ferrous iron salts
ii. Parenteral iron for patients who are either unable to absorb oral iron or who have increasing anemia despite adequate doses of oral iron
iii. Transfusion of packed RBCs for patients who are experiencing significant acute bleeding or are in danger of hypoxia and/or coronary insufficiency

Inspite of having efficient diagnosis methods and iron supplementation programmes, still a majority population suffers from IDA. Researchers are still in the lookout for strategy to combat IDA.

3.4.11 Models used in iron metabolism studies

Several *in vitro*, *in vivo* and *ex vivo* models are used for laboratory based iron metabolism studies. They are summarised in table 3.4.

Studying iron metabolism in humans would be the most appropriate way to develop management strategies for IDA, but due to practical and ethical reasons models are used to studying iron metabolism. It must be remembered that models cannot fully replace human studies but they can certainly give leads of what can happen in human. Every model have its own advantages and limitations.

Table 3.4: Models used in iron metabolism studies

Assay	Experiment details	Parameter / Marker	References
In vitro	Cell free digestion & dialysis	Dialysable iron	Miller et al., 1981
	Caco-2 cells	Iron absorption, uptake, iron stores (ferritin)	Glahn and Van Campen 1997
	HepG2 cells		Scheiber-Mojdehkar et al., 2003
	BeWo cells		Heaton et al., 2008
	Caco-2/HepG2 combined		Scheers et al., 2014
Ex vivo	Fragments of gut	Iron absorption	Goddard et al., 1997
	Brush border membrane and vesicles		Simpson et al., 1986
	Perfused duodenal segment		Garcia and Diaz-Castro, 2013
	Everted gut sacs		Moshtaghie and Taher, 1993
In vivo	Yeast (*Saccharomyces cerevisiae*)	Iron metabolism	De Freitas et al., 2003; Jo et al., 2009; Balasubramani et al., 2015*
	Zebrafish (*Danio rerio*)	Iron metabolism	Ferri-Lagneau et a., 2012; Zhao et al., 2014
	Mouse	Iron metabolism	Fiorito et al., 2012
	Rat	Iron metabolism	Fleming et al., 1998
	Pig	Iron absorption and metabolism	Miller and Ullrey, 1987
	Broiler Chicken	Iron absorption and metabolism	Tako et al., 2010
	Rhesus Monkey	Gestational iron metabolism	Gloub et al., 2006

*Publication from this thesis

3.4.12 *In vitro* iron bioavailability and uptake

The cell-free in vitro dialysis method described by Miller et al., (1981) is a two step process consisting of gastric digestion with pepsin and HCl at physiological temperature (37ºC) and with pH2. The second step simulates intestinal phase, where digestion at physiological temperature (37ºC) is performed with pancreatin and bile

salts at pH7. The digestate is then passed thru a dialysis membrane and the dialysate is then tested for iron dialysability. To further study the effect of test food on iron uptake, the dialysate is then passed through cell lines to study iron uptake. Several studies have used cultured Caco-2 cells as a surrogate for enterocytes of the small intestine or HepG2 hepatoma cells to study iron uptake (García and Díaz-Castro, 2013).

3.4.13 Caco-2 cell iron assimilation

The Caco-2 cell line is a continuous cells of heterogeneous human epithelial colorectal adenocarcinoma cells (Hidalgo et al., 1989). Although they are derived from colon (large intestine) carcinoma, these cells when cultured under specific conditions become differentiated, polarized and resembles the enterocytes lining the small intestine in their phenotype, morphology and functionality (Pinto et al., 1983). Caco-2 cells express tight junctions, microvilli and a number of enzymes and transporters that are characteristic of enterocytes (Sambuy et al., 2005).

Caco-2 cells are most commonly used as a confluent monolayer on a cell culture insert filter (e.g., transwell) to simulate epithelial cell monolayer that provides a physical and biochemical barrier to the passage of ions and small molecules (Sambuy et al., 2005). These cells are used to develop in vitro model of the human small intestinal mucosa to predict the absorption of orally administered drugs (Artursson, 1990). The correlation between the in vitro apparent permeability across Caco-2 monolayers and the in vivo fraction absorbed is well established (Artursson and Karlsson, 1991).

3.4.14 HepG2 cell iron assimilation

HepG2 is a human liver carcinoma cell line with morphology resembling epithelial cells. The cells secrete a variety of major plasma proteins including albumin, transferrin and fibrinogen. HepG2 cells are a suitable *in vitro* model system for the study of polarized human hepatocytes (Scheers et al., 2014). Because of their high degree of morphological and functional differentiation *in vitro*, HepG2 cells are a suitable model to study the intracellular trafficking, liver metabolism, toxicity and for drug targeting studies (Decaens et al., 2008). Hepatocytes play important role in iron transport, storage and regulation of iron homeostasis. In IDA, iron depletion occurs not only in the serum but also in the liver where it is stored as ferritin (Takami and Sakaida, 2011).

Any enhancement in the level of dialysed iron is likely to proportionately enhance iron levels in the hepatocytes, provided the dialysed iron is taken up. The unique feature of both Caco-2 and HepG2 cell lines is the formation of ferritin, iron storage protein (Arosio et al., 2009). Increase in ferritin is an evidence for iron uptake by cells because cells produce ferritin in response to intracellular iron. Recently, *Amla* (Indian gooseberry), a vitamin C and polyphenol rich fruit was shown to enhance iron dialysability and uptake using cell free, Caco-2 and HepG2 cell based *in vitro* models (Venkatasubramanian et al., 2014).

3.4.15 Yeast, *Saccharomyces cerevisiae* as a model organism

As reviewed by Karathia et al., (2011), model organisms help us:

i. To overcomes ethical and experimental constraints that hold for the target life form
ii. They provide a framework on which to develop and optimize analytical methods that facilitate and standardize analysis, and
iii. They are representative of a larger class of living beings for the biological process the researchers are interested in.

Humans have cultivated yeast since the dawn of agriculture to make beer, bread, and wine. As a domesticated microorganism and sexual eukaryote, the budding yeast *Saccharomyces cerevisiae* is one of the most widely used single cellular, tractable model organisms (Mell and Burgess, 2002). Several studies have been reported with *S. cerevisiae* model for understand ageing (Murakami and Kaeberlein, 2009), regulation of gene expression (Biddick and Young, 2009), signal transduction (Hohmann et al., 2007), cell cycle (Nasheuer et al., 2002), metabolism (Brocard-Masson and Dumas, 2006; Lopez-Mirabal and Winther, 2008), apoptosis (Owsianowski et al., 2008), neurodegenerative disorders (Miller-Fleming et al., 2008) and also for drug discovery (Ross-Macdonald, 2003). Yeasts belong to the kingdom of fungi and share a common cellular architecture and rudimentary life cycle with multicellullar eukaryotes (Mell and Burgess, 2002). As non-pathogenic, non-motile microorganisms, yeasts are grown in batch liquid culture, isolated as colonies derived from single cells on solid media and also manipulated in the laboratory (Ross-Macdonald, 2003). The generation time is about 90 min, so large populations of individuals can rapidly be grown and analysed (De Freitas et al., 2003). Like all

eukaryotes, yeast cells have numerous membrane bound organelles, including a nucleus, endosymbiotic mitochondria, the peroxisome, and the organelles of the secretory pathway. The budding yeast carries its genome of nearly 6000 genes in 12 mb of DNA on 16 chromosomes in the nucleus (De Freitas et al., 2003). About 30% of genes implicated in human disease may have orthologs in the yeast proteome (Foury, 1997). Proteins involved in iron import, distribution and export are conserved from *S. cerevisiae* to humans (De Freitas et al., 2003; Askwith and Kaplan, 1998), hence it has been used as a model to study iron metabolism.

3.4.15.1 Iron metabolism in *S. cerevisiae*

The steps involved in iron metabolism in *S. cerevisiae* are:

i. Iron is acquired at the plasma membrane by low affinity (Fe^{3+}), high affinity (Fe^{2+}) or by siderophore mediated mechanism (figure 3.7).
ii. Obtained iron accumulates in the cytosol as Fe^{3+} and also as Fe-S (iron sulphur cluster)
iii. The reduced iron (Fe^{2+}) enters mitochondria for several physiological mechanisms including heme production
iv. Excess iron is stored in vacuole as Fe^{3+} (figure 3.7)
v. In the absence of cytosolic iron, *Aft1* activates the transcription of the iron regulon which results in increased cellular iron uptake and increased cytosolic iron pools
vi. Iron is also obtained by recycling of heme
vii. In case of reduced iron in the growth media, stored iron in the vacuole will be transported to cytosol and utilized

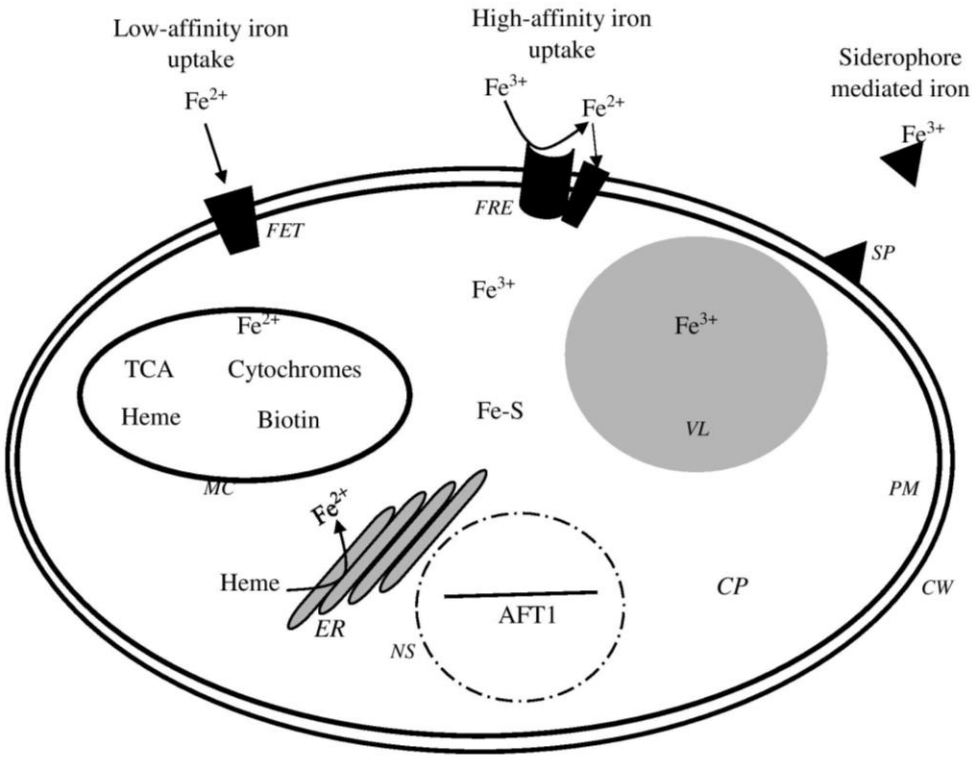


Figure 3.7: Iron metabolism in *S. cerevisiae.* CW – cell wall, PM – plasma membrane, CP – cytoplasm, MC – mitochondria, VL – vacuole, NS – nucleus, FET and FRE – iron transporters, SP – siderophore, AFT1 – transcription factor and Fe-S – iron sulphur cluster.

3.4.15.2 *S. cerevisiae* as a model to study iron metabolism

Based on the similarities in the iron transport mechanisms, Askwith and Kaplan (1998) have indicated that the pathways of iron metabolism are conserved from yeast to humans. Several homologous proteins have been identified in human and yeast.

NFU1 and *ISU1* are genes whose protein products have been identified to play a role in iron homeostasis by helping in assembly, insertion and / or in repair of mitochondrial Fe-S clusters (Schilke et al., 1999). This protein domain is conserved in many organisms and establishes the fact that yeast and human share similar iron metabolism pathways.

Table 3.5: Homologous genes that affect iron metabolism in both S. cerevisiae and Human*

Phenotype of yeast disruption	Protein	Human homolog	Phenotype of mammalian disruption
Deficient growth on low Fe	Fet3p	Ceruloplasmin	Deficient iron mobilization
Deficient growth on low Fe/Cu	Ctr1p	hCtr1p	Not known
	Atx1p	Hah1p	Not known
	Ccc2p	Menkes (Atp7ap)	Severe copper deficiency
		Wilson (Atp7bp)	Copper overload
Deficient growth on low Mn	Smf1p/Smf2p	Nramp2p	Microcytic anemia due to deficient iron transport
Deficient growth in non fermentable carbon source due to mitochondrial iron overload	Yfh1p	Frataxin	Decreased amount of protein cause Friedreich's ataxia

*Adopted from Askwith and Kaplan (1998).

Because of the similar iron metabolism pathways, some of the human iron metabolism disorders have been simulated in *S. cerevisiae* model for better understanding (table 3.5). One of such example is Friedreich's ataxia (FA), a genetic disorder. It is caused by mitochondrial dysfunction and free radical toxicity, with consequent mitochondrial damage, axonal degeneration and cell death. Mutation in the FRDA gene causes deficiency of fratixin, a highly conserved nuclear encoded protein localized in mitochondria and cause the disease (Duclos et al., 1994). A FRDA gene yeast homologue (YFH1) was identified using the complementation tests (Babcock et al., 1997). Upon deletion of YFH1 gene, yeast cells accumulated iron in the mitochondria, similar to the pathological conditions observed in human (Foury and Cazzalini, 1997). Further, the YFH1 knockout yeast cells were found to be sensitive to free radicals (Rotig et al., 1997). When the YFH1 gene was reintroduced to the yeast cells, they exported the excess iron from mitochondria to the cytosol

(Becker and Richardson, 2001). This broadened the understanding of the disease and currently yeast based FA model is used to screen iron chelators for potential application in treatment of FA (Wong et al., 1999).

3.4.15.3 Effect of natural products on iron metabolism in *S. cerevisiae*

Effect of natural products like desferroxamine (Yun et al., 2000), curcumin (Minear et al., 2011), and sampangine (Huang et al., 2011) on cellular iron metabolism has been elucidated using *S. cerevisiae* as model.

3.4.15.4 Iron deficiency in *S. cerevisiae*

Parsons and Hickmans (1933), cultured yeast cells repeatedly in iron free medium and observed that the cells were white in colour instead of brown and also assumed mycelial form (figure 3.8). They named these cells as 'anemic yeast'. The cells reverted to their normal growth characters on returning them to iron containing normal media. Iron chelators, BPS (Bathophenanthroline disulfonic acid disodium salt hydrate) and ferrozine have been used in several studies to induce iron deficiency leading to the formation of 'anemic' yeast cells (Cowart et al., 1993; Jo et al., 2009). Under iron deficient conditions yeast undergoes an overall metabolic reorganization that includes recycling of heme iron, release of stored iron from the vacuole and down-regulation of iron-dependent processes (Kaplan et al., 2006). In *S. cerevisiae* iron deficiency was found to limit growth (Philpott and Protchenko, 2008), alter metabolic pathways (Shakoury-Elizeh et al., 2010) and reduce energy production in mitochondria (Jo et al., 2009). Holmes-Hampton et al., (2013) studied the response of *S. cerevisiae* cells to low and excess iron, which is summarized in table 3.6.

(a) (b)

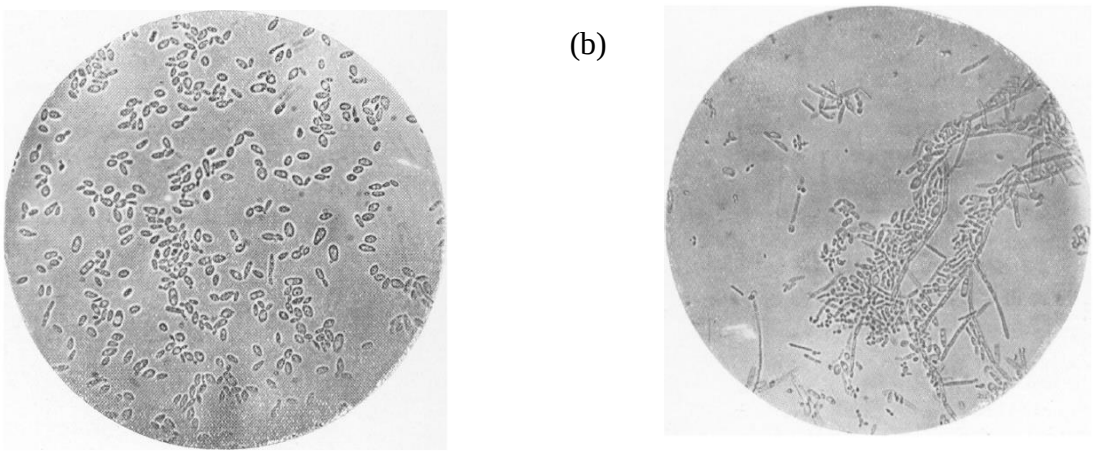


Figure 3.8: Microphotograph of normal (a) and anemic yeast (b) cells

(Source: Parsons and Hickmans, 1933)

Table 3.6: Iron content of S. cerevisiae cells grown in iron normal and iron deficient conditions (Holmes-Hampton et al., 2013)

Form of Iron	Fe normal medium	Fe deficient medium
Fe^{2+}	~100 µM	Present
Vacuolar Fe^{3+}	~300 µM	Absent
Mitochondria	Fe^{2+} ions and substantial amounts of Fe^{3+} nanoparticles	Fe-S clusters and Fe^{2+}
Fe content	400 – 450 µM (even at 250-fold excess Fe in media)	~150 µM

3.5 Pomegranate

This section summarises the available literature on phytochemistry and bioactivity of pomegranate with specific focus on pomegranate juice.

Figure 3.9: Pomegranate fruits and arils

3.5.1 Taxonomy

Kingdom	:	Plantae
Phyllum	:	Angiosperms
Class	:	Eudicots
Sub-class	:	Rosids
Order	:	Myrtales
Family	:	Punicaceae
Genus	:	*Punica*
Species	:	*granatum*
Botanical Name	:	*Punica granatum* L.

Common Name	:	Pomegranate (English) Anar (Hindi) Dalimbe (Kannada) Matalam (Malayalam) Madulai (Tamil) Danimma (Telugu)
Commonly used edible part	:	Arils / seeds

3.5.2 Pomegranate, an ancient medicinal fruit

Pomegranate fruit is known as the "jewel of winter". It is a native of the Himalayas in northern India, but it has been cultivated and naturalized since ancient times over the entire Mediterranean region. Use of pomegranate has been recorded over hundreds of years. It is considered to be a symbol of life, health, longevity, femininity, fecundity, knowledge, morality, immortality and spirituality (Mahdihassan, 1984).

The major bioactive phytochemical component classes identified in pomegranate fruit are anthocyanins and hydrolyzable tannins, specifically ellagitannins (Tzulker et al. 2007). The major anthocyanins in pomegranate juice across several Iranian cultivars were delphinidin 3,5-diglucoside, cyanidin 3,5-diglucoside, pelargonidin 3,5-diglucoside, delphinidin 3-glucoside, cyanidin 3-glucoside, and pelargonidin 3-glucoside (Alighourchi et al. 2008, Mousavinejad et al. 2009).

In traditional medicines, pomegranate is considered "a pharmacy unto itself," and is used as an antiparasitic agent (Naqvi et al., 1991), a "blood tonic," and to help in aphthae, diarrhea and ulcers (Caceres et al., 1987). The potential therapeutic properties of pomegranate are wide-ranging and include treatment and prevention of cancer (Johanningsmeier and Harris, 2011), cardiovascular disease (Aviram and Rosenblat, 2012), diabetes (Bagri et al., 2009), dental conditions (Sastravaha et al.,

2005), erectile dysfunction (Forest et al., 2007), protection from ultraviolet (UV) radiation (Afaq et al., 2005), in infant brain ischemia (West et al., 2007), Alzheimer 's disease (Hartman et al., 2006), male infertility (Turk et al., 2008), arthritis (Rasheed et al., 2010) and obesity (Lei et al., 2007). The properties and actions of Pomegranate according to Ayurveda are reviewed in chapter 4.

3.5.3 Bioactive phytochemicals present in pomegranate juice (PJ)

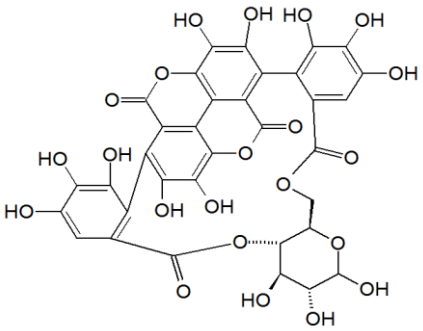
Pomegranate juice is rich in phytochemicals like flavonoids, anthocyanins, organic acids, vitamins, amino acids and minerals which are known to constitute to its biological actions. Published literature on the bioactive phytochemicals are presented in the following section. The review presented here is only on pomegranate juice (PJ) and not on other pomegranate based preparations.

3.5.3.1 Phenolics

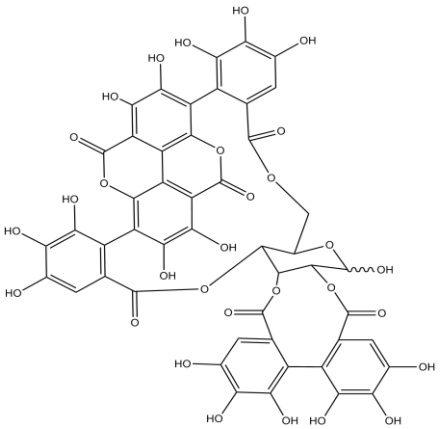
Phenolics are aromatic benzene ring compounds with one or more hydroxyl groups. They impart stress resistance to the plant. They also play an important role in plant development and pigment biosynthesis (Bhattacharya et al., 2010). In PJ, phenolics form the major class of bioactive metabolites. Literature indicates that the total phenolic content of PJ is in the range of 14.4 – 1008.6 mg Gallic Acid Equivalent/100ml (Tezcan et al., 2009). This is at least 10 to 20 fold higher than the phenolics content of apple fruit varieties (Boyer and Liu, 2004). The different classes of phenolics present in PJ are:

Tannins: Tannins are large polyphenolic compounds containing hydroxyls and other chemical groups like carboxyls. They form strong complexes with various macromolecules (Chung et al., 1998). Punicalin, punicalagin, ellagic acid, gallic acid, galloyl glucose (hydrolyzable tannin) are the major tannins present in PJ (Wang et al., 2004)

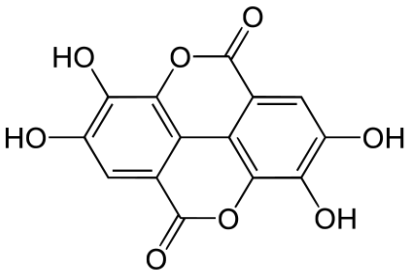
Punicalin



Punicalagin



Ellagic acid



Gallic acid

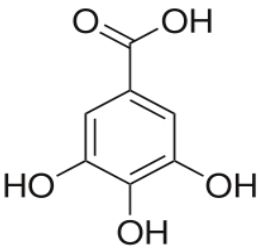
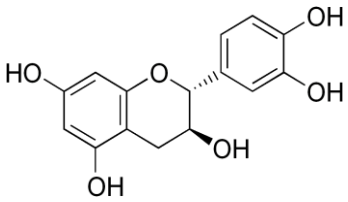


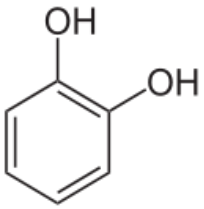
Figure 3.10: Tannins present in PJ

Flavonoids: Flavonoids are polyphenolic molecules containing 15 carbon atoms forming two phenyl rings and one heterocyclic ring. They are predominantly anti-oxidants and are soluble in water (Kumar and Pandey, 2013). PJ has been reported to contain flavonoids like catechin, catechol, epicatechin, epigallocatechin 3-gallate, flavan-3-ol, isoquercetin, procyanidin, quercetin and rutin (Wang et al., 2004)

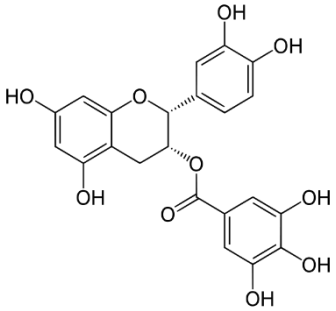
Catechin



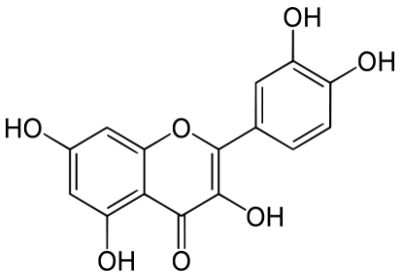
Catechol



Epicatechin gallate



Quercetin



Rutin

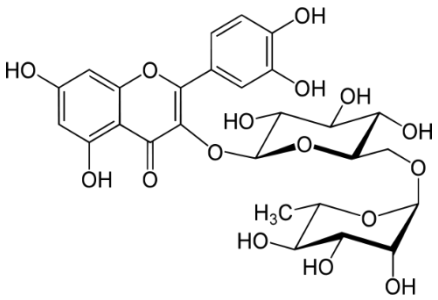
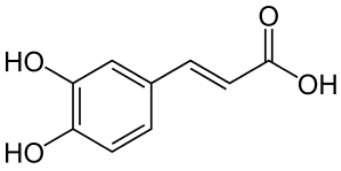


Figure 3.11: Flavonoids present in PJ

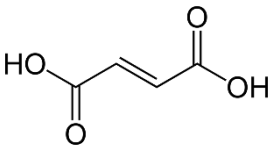
Anthocyanins: Anthocyanins are water-soluble vacuolar pigments. Cyanidin 3- O-glucoside, cyanidin 3,5-di- O-glucoside, delphinidin 3-O-glucoside, delphinidin 3,5-di-O-glucoside, pelargonidin 3-O-glucoside, pelargonidin 3,5-di-O-glucoside are some of the anthocyanins present in PJ. They are reported to have anti-oxidant (Tsuda et al., 1996), enzyme inhibition (Tusda et al., 2003), cardiac protection (Kong et al., 2003) and cytokine modulatory functions (Rossi et al., 2003).

Phenolic acids: Phenolic acids or phenolcarboxylic acids are types of aromatic acids. They contain a phenolic ring and an organic carboxylic acid functional group. Caffeic acid, fumaric acid, p-coumaric acid and chlorogenic acid are some of the commonly found phenolic acids in PJ. Phenolic acid metabolites are said to have anti-oxidant, anti-microbial and anti-tumor activities (Heleno et al., 2015).

Caffeic acid



Fumaric acid



Chlorogenic acid

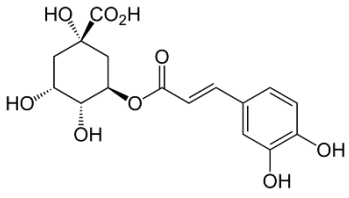
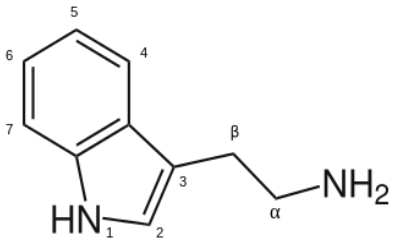


Figure 3.12: Phenolic acids present in PJ

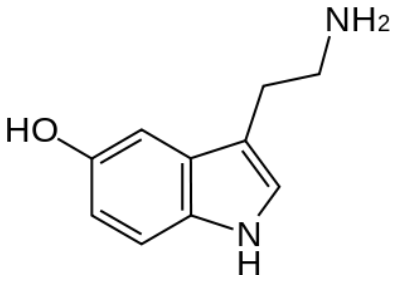
3.5.3.2 Alkaloids

Alkaloids are a class of naturally occurring chemical compounds that contain nitrogen atoms. Tryptamine, serotonin and melatonin are the major alkaloids present in PJ (Wang et al., 2004). They have been linked to anti-oxidant, anti-inflammatory and hepatoprotective property of PJ (Johanningsmeier and Harris, 2011)

Tryptamine



Serotonin



Melatonin

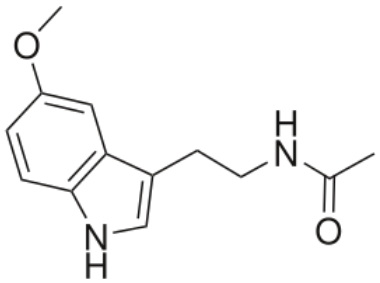
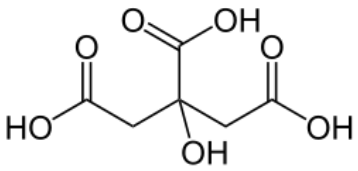


Figure 3.13: Alkaloids present in PJ

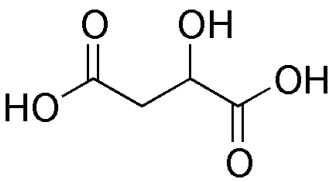
3.5.3.3 Organic acids

An organic acid is an organic compound with acidic properties. PJ contains citric acid in the range of 0.46 – 3.6 mg/100ml (Tezcan et al., 2009). Other organic acids like ascorbic acid, malic acid, tartaric acid, isocitric acid, oxalic acid and succinic acid have also been reported from PJ (Prakash and Prakash, 2011). Organic acids have been reported to influence bioavailability of micronutrients especially iron (Salovaara et al., 2002)

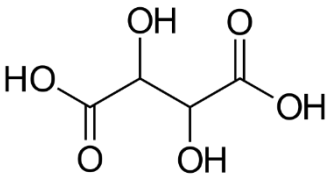
Citric acid



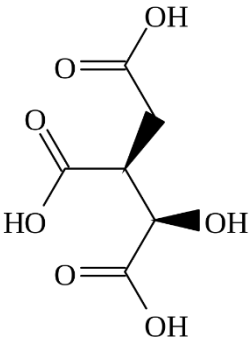
Malic acid



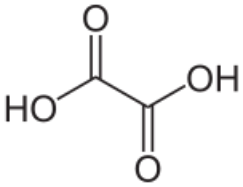
Tartaric acid



Isocitric acid



Oxalic acid



Succinic acid

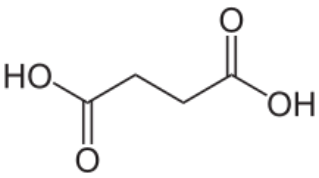


Figure 3.14: Organic acids present in PJ

3.5.3.4 Sugars

Sugar content of juices are expressed in ºB (degree Brix). One degree Brix is 1 gram of sucrose in 100 grams of solution and represents the strength of the solution as percentage by mass. The sugar content of PJ is in the range of 13.68 – 15.18 ºBrix (Akbarpour et al., 2009). Glucose and Fructose forms the major portion of sugars found in PJ (Ozgen et al., 2008). Literature also indicates the presence of minor quantities of sucrose and sorbitol in pomegranate juices (Vegara et al., 2014). Sugars have been shown to improve iron bioavailability in *in vitro* models (Christides and Sharp, 2013).

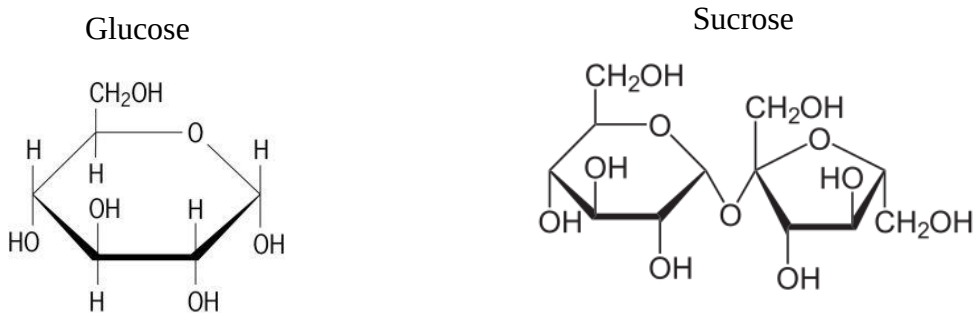


Figure 3.15: Sugars present in PJ

3.5.3.5 Vitamins

Presence of vitamin C (ascorbic acid) in the range of 9.68 – 19.8 mg/100 ml has been reported in PJ (Morton, 2013). Vitamin C is one of the established iron bioavailability enhancers (Hallberg et al., 1989). Vitamin E also has been reported to be present in PJ (Elfalleh et al., 2011). These vitamins have anti-oxidant effect (Johanningsmeier and Harris, 2011).

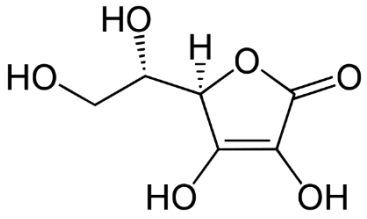


Figure 3.16: Structure of L-Ascorbic acid

3.5.3.6 Amino acids

PJ has been reported to contain amino acids like proline (37.1 – 73.8 mg/100ml), serine (64.1 – 84.6 mg/ 100ml), alanine (25.2 – 54.5 mg/100ml), arginine, tryptophan, leucine, asparagine, glutamine and aspartic acid (Elfalleh et al., 2011). Presence of amino acids increase the nutritive value of PJ.

3.5.3.7 Minerals

Presence of mineral nutrients like iron (0.3 – 1.2 mg/100g), phosphorus (8 – 37 mg/100g), copper, sodium, magnesium, potassium, calcium, zinc and manganese is also reported from PJ (Elfalleh et al., 2011). These minerals provide daily requirement of micronutrients.

3.5.4 Biological activity of PJ

From 2000 to June 2016, about 400 research publications have reported bioactivity of PJ. It becomes voluminous to refer all publications and cite it in this thesis. So, a summary of biological activities tested using PJ with most recent reference is presented as a table 3.7 below.

Table 3.7: Reported biological activities of PJ

Model	Activity	Reference
Human	Improve drug metabolism	Abdlekawy et al., 2016
	Anti-diabetic	Shishehbor et al., 2016
	Anti-cancer	Sahebkar et al., 2015
	Anti-oxidant	Sahebkar et al., 2016
	Anti-osteoarthritis	Ghoccchani et al., 2016
	Anti-hypertensive	Asgary et al., 2014
	Improve memory	Bookheimer et al., 2013
	Cardiovascular protection	Aviram and Rosenblat, 2012
	Inhibit cancer metastasis	Wang et al., 2012
	Anti-obesity	Al-Muammar and Khan, 2012
In vivo	Anti-inflammatory in rats	Shah et al., 2016
	Anti-oxidant in rat	Bouasla et al., 2016
	Anti-coagulant, anti-platelet and anti-anemic in rabbit	Riaz and Khan, 2016
	Inhibitory effect on gastrointestinal transit in rat	Souli et al., 2015
	Effective against parkinson's disease in rats	Tapias et al., 2014
	Anti-osteoporosis in mice	Spilmont et al., 2014
	Improve sperm health in rats	Turk et al., 2016
	Enhance bone formation in mice	Monsefi et al., 2012
	Hepatoprotective in rats	Shanban et al., 2014
	Effective against Alzheimer's dosease in mice	Hartman et al., 2006
In vitro	Against cholesterol accumulation in macrophages	Rom and Aviram, 2016
	Anti-atherogenicity in macrophages	Rom et al., 2016
	Pancreatic lipase inhibition	Fabroni et al., 2016
	Anti-cancer in human UBUC T24 and J82 cells	Wu et al., 2016
	Inhibition of cyclooxygenases, xanthine oxidase and acetylcholine esterase in cell lines	Les et al., 2015
	Anti-bacertial against *Staphylococcus epidermis*	Bentanzos-Cabrera et al., 2015
	Neuroprotective in human primary neurons	Braidy et al., 2013
	Protect DNA damage in PC12 cells	Forouzanfar et al., 2013
	Anti-HIV	Neurath et al., 2005

The literature analysis indicates that PJ has been mainly studied for anti-oxidant, anti-inflammatory, anti-cancer and cardioprotective activities. A recent report by Riaz and Khan (2016) claims that pomegranate juice has anti-anemic activity in rabbits. They found a significant increase in the haemoglobin content of rabbits on feeding with PJ (Riaz and Khan, 2016). Even though pomegranate has been extensively studied for individual biological activities, researchers have not tried for testing the PJ's potential in health and wellness promotion.

3.6 Conclusion

To obtain maximum healthy lifespan, maintanence of metabolic harmony is necessary (Ames, 2003). One of the hallmarks of ageing is progressive reduction in the functional reserve of multiple organs and systems. A metabolic tune-up through an optimal intake of nutrients will have health benefits particularly in chronic conditions and during ageing. IDA is one of the major nutrition deficient disorder, caused due to multiple factors. Iron supplementation programmes have not been completely successful in management of IDA. Multifactorial etiology of the disease is one of the major reason for unsuccessful interventions. Optimising digestion and absorption of iron can be an easy way to treat IDA. A new discipline like nutrigerontology has been developed to research on the impact of nutrients, foods, macronutrient ratios and diets on lifespan, ageing process and age related diseases (Aiello et al., 2016). The main aim of this research discipline is to reduce the risk of ageing related diseases and increase the helahty lifespan through diet (Verburgh et al., 2015).

Looking at traditional medicine (TM) for strategies to manage chronic disease conditions and ageing might have a great value. They suggest simple locally available resources for the maintenance of health and wellness. But, the problem with traditional medicine is the lack of scientific understanding on the mode of action. The challenge is to find appropriate models to study TM. Several model systems have been reported to study biological activities of herbal drugs. Each have their own merits and demerits. Even though in vitro models used for iron metabolism studies, they cannot simulate the systemic effects except the rodent and monkey models, yeast could be a simple and good model to study systemic iron metabolism. Based on the convenience of handling and extrapolation of findings to humans, drosophila could be a good model to study healthy ageing aspects.

Literature survey indicates that pomegranate has several bioactive molecules like phenolics, organic acids and anthocyanins. Scientific studies have indicated that pomegranate has anti-oxidant, cardioprotective and anti-inflammatory properties. Based on its phytochemical constituents, it may be a good candidate for wellness and in management of IDA.

3.7 References

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Chapter 4

RASAYANA AND ITS ROLE IN THE MANAGEMENT OF '*PANDU*' AND '*SVASTHYA*'

4.1 Introduction

A trans-disciplinary research strategy integrating Ayurveda (*Sastra*) and biomedical science was followed to conceptualize the experimental research models. This approach promotes theoretical as well as methodological bridging with respect to the core concepts of both the knowledge systems and has the potential to generate new knowledge. An understanding of Ayurvedic concepts for health and wellness is mandatory for designing appropriate experimental models.

This chapter reflects the understanding of the author of this thesis whose education is in conventional Life Science in English medium. It is well understood that there may be gaps in selecting/ phrasing words or sentences that truly reflect the Ayurveda concepts.

This chapter has four different sections:

i. The first section of the chapter deals with *Rasayana* concept, types of *Rasayanas* and mode of action per Ayurveda. *Rasayana karmas* and current status of research on *Rasayanas* are some of the aspects covered.

ii. The second section deals with '*Svasthya*', which can be correlated to 'wellness'. Definition of *Svasthya*, methods to achieve it and indicators of *Svasthya* per Ayurveda are dealt.

iii. Trans-disciplinary understanding of *Pandu*, an Ayurvedic correlate of anemia is dealt with in the third section. This section deals with the aetiology, pathogenesis, symptoms, treatment and methods for the management of *Pandu*.

iv. The last section of this chapter describes the Ayurvedic qualities (*Dravyaguna*) of *Dadima* (pomegranate), the *Rasayana* selected for this study.

4.2 Methodology

The methodology followed for trans-disciplinary understanding of *Rasayana*, *Svasthya*, *Pandu* and *Dadima Rasayana* is detailed below. This chapter is primarily a literature review and an analysis of Ayurvedic entities to get better understanding.

4.2.1 Ayurvedic texts referred

The below were the criteria applied to select Ayurvedic texts for reference.

i. The complete text should be available in published domain- Though a lot of Ayurveda information is available in books, many texts are not complete and have not been published. For this particular study, only published, complete books were selected for reference.

ii. Ayurvedic theoreticians and practitioners should recommend - The books should have been edited by reputed authors and recommended by scholars and practitioners of Ayurveda as standard and authentic.

iii. It should be available in translations in English languages for practical reasons of understanding them.

Following the above criteria, a list of Ayurveda texts (table 4.1) were referred.

Table 4.1: List of Ayurveda texts referred

Book	Chronology	Focus	Editor, year
Charaka Samhitha	15th Century BCE-4th Century CE	*Rasayana*, *Svasthya*, *Pandu*, *Dadima*	Sastry, 1997
Susrutha Samhitha	15th Century BCE-5th Century CE	*Rasayana*, *Svasthya*, *Pandu*, *Dadima*	Acharya, 1992
Ashtanga Sangraha	5th Century CE	*Rasayana*, *Svasthya*, *Pandu*, *Dadima*	Murthy, 2002
Ashtanga Hrdaya	6th Century CE	*Rasayana*, *Svasthya*, *Pandu*, *Dadima*	Murthy, 2001
Chakradatta	11th Century CE	*Rasayana*, *Pandu*	Tripathi and Mishra, 1983
Madanapala Nighantu	13th Century CE	*Dadima*	Dash, 1994
Kaiyadeva Nighantu	14th Century CE	*Dadima*	Sharma, 1979
Raja Nighantu	14th Century CE	*Dadima*	Tripathi, 2006
Bhavaprakasha Samhita	16th Century CE	*Pandu*, *Dadima*	Chunekar, 2004
Materia Medica of Ayurveda based on Ayurveda Saukhyam of Todarananda	17-18th Century CE	*Dadima*	Dash, 1997
Yogaratnakara	18th Century CE	*Rasayana*, *Pandu*, *Dadima*	Sastri, 2002
Bhaishajya Ratnavali	18th Century CE	*Rasayana*, *Pandu*, *Dadima*	Mishra, 2007
Sahasrayogam	19th Century CE	*Pandu*, *Dadima*	Sharma, 2002
Nighantu Adarsha	20th Century CE	*Dadima*	Vaidya, 1968

4.2.2 Understanding *Rasayana*

i. Theoretical understanding of *Rasayanas*, their types, their role in maintenance of physiology and health, and their effect on the living system (*Rasayana karma*) are obtained by referring to the classical Ayurveda texts (table 4.1).

ii. Modern literature related to *Rasayana* research was obtained from PubMed, using the keywords '*Rasayana*' and '*Rasayana herbs*'. Review and research articles reporting bioactivity of *Rasayanas* from 1971 to may 2016 were included for the analysis. Articles pertaining to formulation development and pharmacognosy were not included.

iii. Information related to *Rasayanas* was also obtained by discussion with Vaidyas (Ayurveda practitioners) with more than 10 years of clinical practice and or research.

iv. The list of potential *Rasayana karmas* was searched from FRLHT's Clinically Important Plants of Ayurveda (CIPA 1.0) and Plants of Ayurveda - Materia Medica – *Dravyaguna* (1.05) databases using the keyword '*Rasayana*' and *Rasayana karmas* like '*vayasthapana*', '*balya*', '*thvachya*' etc. The biological effects of different types of *Rasayana* preparations (*Rasayana karmas*) were identified and tabulated.

4.2.3 Trans-disciplinary understanding of *Svasthya*

i. Brihatrayis (works of Charaka, Susrutha and Vagbhata) speak about living healthy being more important than getting inflicted and treated. They have provided features of *Svasthya* and *Svastha* individual in the initial chapters

(*Sutrastana*). Therefore, to have a comprehensive understanding about *Svasthya* or living healthy or being well, the pertinent chapters in the texts were referred (table 4.1).

ii. Information related to the indicators of *Svasthya* and ways to maintain them were obtained from some of the recent textual literature which are appropriately citied and included in the references section.

iii. Information related to *Svasthya* was also obtained by discussion with practicing Vaidyas with more than 10 years of experience.

4.2.4 Trans-disciplinary understanding of *Pandu*

i. The causative factors or aetiology (*Nidana*), pathophysiology (*Samprapti*), signs and symptoms (*Lakshana*) and treatment (*Chikitsa*) of the disease entity *Pandu*, are mentioned in the *chikitsasthanas* of Ayurveda texts (table 4.1)

ii. *Chikitsagranthas* like Chakradatta, Bhaishajya Ratnavali, Yogarathnakara etc, (table 4.1) have dedicated chapters on *Pandu*.

iii. Information related to *Pandu* was also obtained from some of the recent Ayurvedic textual literature which are appropriately cited and included in the references section.

iv. Oral information obtained by discussion with practicing Vaidyas with more than 10 years of experience was used to compile information on *Pandu* and its correlation to IDA.

With reference to IDA, the focus of this thesis was to understand the aetiology, pathology, symptoms, treatment principles of *pandu* and correlation to iron deficiency anemia.

4.2.5 Dravyaguna of *Dadima*

Ayurveda considers '*Rasapanchaka*', five qualities of a substance as the determinant of its biological effect. They include *rasa* (taste), *guna* (qualities), *veerya* (potency of the herb), *vipaka* (taste after digestion) and *prabhava* (biological effect of the herb). *Dadima* (pomegranate) has been indicated as a single drug or a part of the formulation in several Ayurveda prescriptions. *Dadima* is included as one of the entities in the *phala varga* (class of fruits) in classical Ayurveda literature (table 4.1).

i. Information on the *rasapanchaka* of *Dadima* was obtained from the *ahara varga* of classical Ayurveda literature and Nighantus (Lexicons) (table 4.1).

ii. Various pharmacological actions of pomegranate, including *Rasayana karma* and therapeutic action in *pandu* were derived from the verses in *chikitsasthana* of books mentioned in table 4.1.

iii. Some of the recent textual literature were also referred for obtaining information related to *dadima*, which are appropriately cited and included in the references section.

4.3 *Rasayana*

The word *Rasayana* is a combination of two separate words '*Rasa*' and '*ayana*'. '*Rasa*' refers to the 'chyme' (or essence or nutrient) portion obtained through digestion of food, which nourishes all the tissues in the body and '*Ayana*' refers to the channels of exchange or circulation (Sastry, 1997).

तत्र रसायनतन्त्रं नाम वयस्थापनं आयुर्मेधाबलकरं रोगापहरण समर्थं च

Tathra Rasayanatantram nama vayahsthapanamayurmedhabalakaram rogapaharanasamartham ca.

Susruta Samhita, Sutrasthana, 1/7

Rasayana tantra deals with the methods to maintain youthfulness, to increase longevity, intellectual capacity, and physical strength as well as to enable the person to conquer diseases (Acharaya, 1992).

रसायनं च तज्ज्ञेयं यज्जराव्याधिनाशनम् ।

Rasayanam ca tajjnyeyam yajjaravyadhinashanam.

Susruta Samhita, Sutrasthana, 4/13

The methods that help delay ageing and diseases is called *Rasayana* (Acharya, 1992). *Rasayana tantra* represents the basic approach of Ayurveda which comprises of preventive, promotive and curative aspects of health and carries practical methods for management of health and disease (Sharma, 1992).

धीर्घमायुः स्मृतिं मेधामारोग्यं तरुणं वयः ।
प्रभावर्णस्वरौदार्यं देहेन्द्रियबलं परम् ॥
वाक्सिद्धिं प्रणतिं कान्तिं लभते ना रसायनात् ।
लाभोपायो हि शस्तानां रसादीनां रसायनम् ॥

Dheerghamayuh smrtim medhamarogyam tarunam vayah |

Prabhavarnaswaraudaaryam dehendriyabalam param ||

Vaksiddhim pranatim kantim labhate na rasayanat |

Labhopayo hi shastanam rasadinam rasayanam ||

Charaka Samhita, Chikitsa sthana, 1- 1/7-8

The benefits of *Rasayana* are long life, excellent memory, brilliance, health and young age, radiating lustre, pleasing colour, commanding voice, great strength of body and sense organs, influencing speech, reverential attitude and adorable. The benefits are focused on attaining strength (*balya*), life giving (*jivaniya*), bulk promoting (*brmhaniya*) and delaying the ageing process (*vayasthapana*) (Sastry, 1997).

As all *dhatus* (tissues) are formed by subsequent transformation of *rasa dhatu* formed from *ahara* (food), if *rasa* is produced in optimum quality and quantity, then naturally all the tissues in the body will be nourished properly. The process by which *rasa* is carried to all the body tissues for anabolism is called as *rasakriya* (Dwarakanath, 2003).

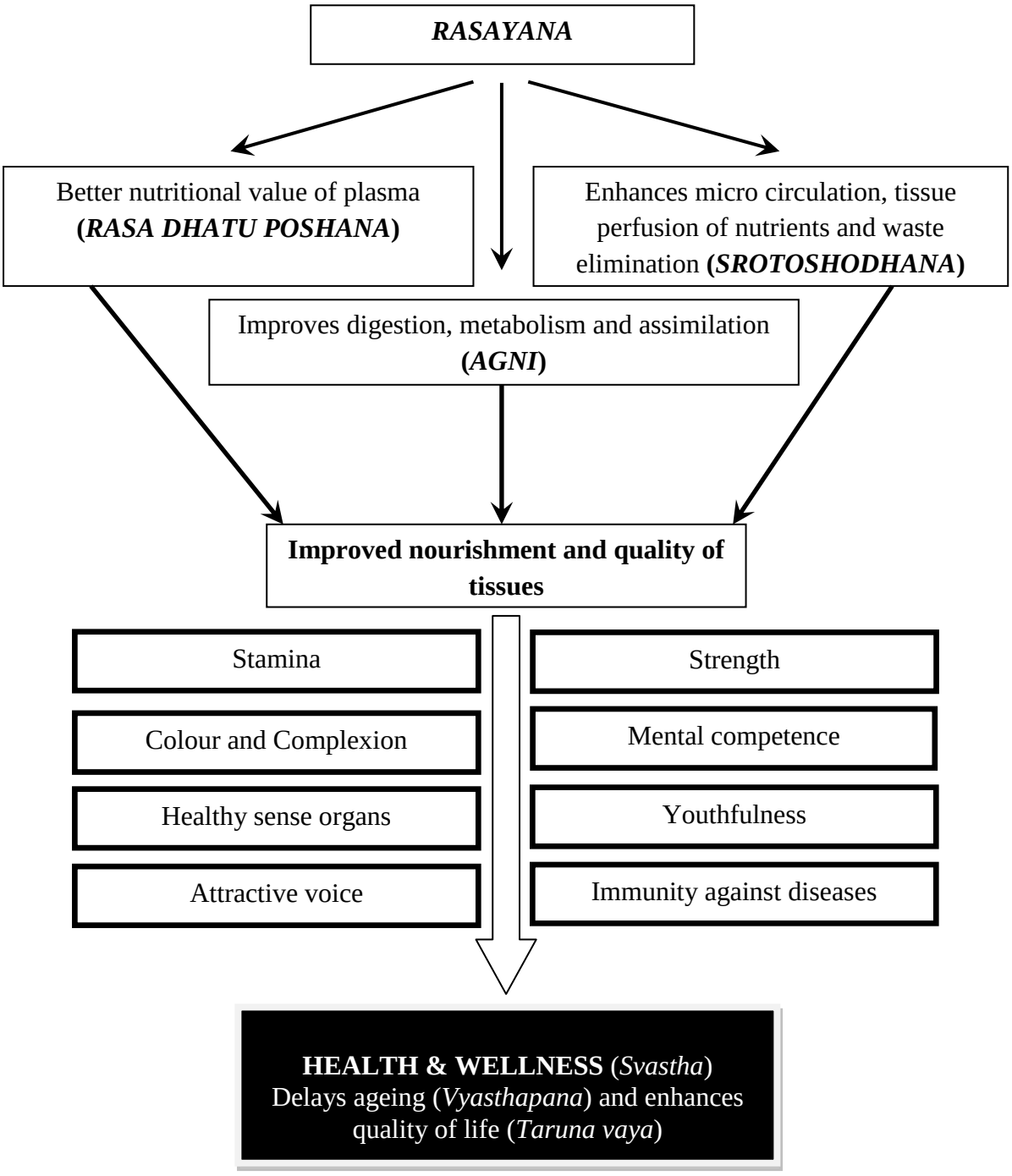

Figure 4.1: A flowchart to show the probable mode of action of *Rasayanas*

Rasayanas also aim at giving strength to the sense organs, mind and intellect. Hence *Rasayana* or rejuvenation is a form of treatment in which all the tissues are nourished and enhanced. The nourishment of vital tissues and the removal of wastes helps in delaying ageing. Thus, *Rasayana* therapies are aimed at bringing a state of equilibrium in the systemic functioning of human body, along with spiritual and mental well-being (figure 4.1). This leads to enhancement of quality life span, increased intellect and enhanced physical strength. Besides promotion of mental and physical health and rejuvenation potential, *Rasayana* therapy offers a preventive role against diseases through improved immunity or resistance to diseases (*vyadhiksamatva*) and other optimized physiological functions (Sastry, 1997). Thus, *Rasayana* is also considered as Ayurvedic geriatrics (*jara chikitsa*).

4.3.1 Classification of *Rasayanas*

The below table (figure 4.2) describes the classification of *Rasayanas* based on different categories.

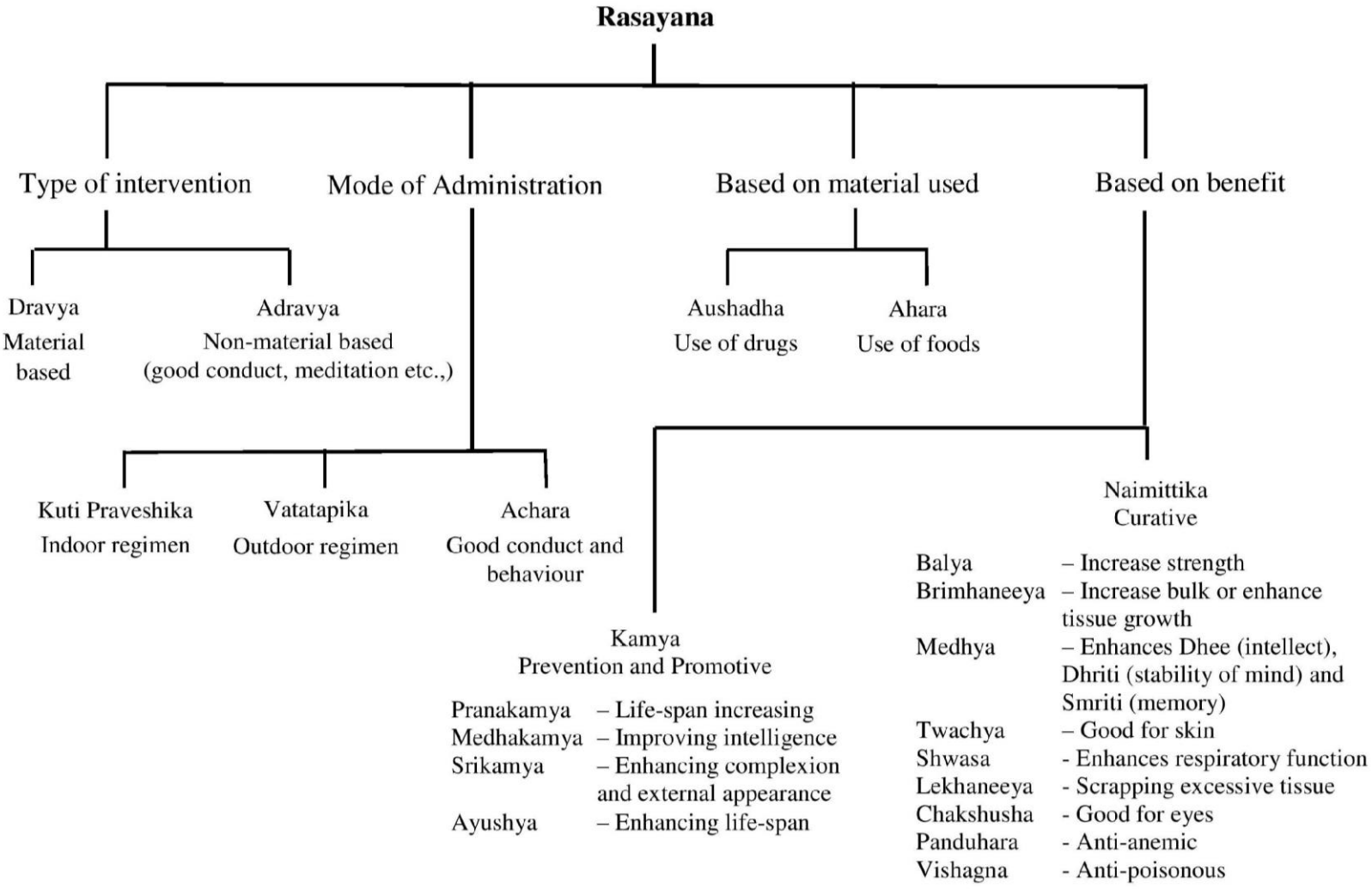

Figure 4.2: Classification of *Rasayanas* (adapted from Acharya, 1992; Udupa, 2004)

4.3.2 Mode of Action of *Rasayanas* (Sharma, 1992)

Ayurvedic literature indicates that *Rasayanas* can function by one or more of the following mechanisms:

i. By improving *Rasa* quality and acting directly by 'nourishing' the plasma.

ii. By improving the level of *agni* and enhancing the enzymatic systems of the body for better digestive and metabolic functions.

iii. At the level of *srotas*, i.e., microcirculatory and macrocirculatory channels, by inducing a *srotaprasadana* or by improving the competence of the inner transport system, microcirculation and tissue perfusion of nutrients.

Ayurvedic understanding of *rasa*, *agni* and *srotas* and their importance in maintenance of human physiology is described in the subsequent sections.

4.3.2.1 *Rasa* enhancing or direct nourishment

The essence derived from the digested nutrient portion of *ahara* (food) is considered as *rasa*. The structural components of the body (tissues) or *dhatus* are formed by *rasa*. Ayurveda texts indicate that, *rasa* forms the first *dhatu*, *rasa dhatu* or plasma. This *rasa dhatu* will aid in subsequent formation of the other six tissues namely, *rakta* (blood), *mamsa* (muscle), *meda* (fat), *asthi* (bone), *majja* (bone marrow) and *sukra* (reproductive tissues) of the body (Murthy, 2002). Quality food provides quality *rasa* and that in turn leads to a succession of events leading to the formation of seven tissues (*saptadhatu*) and maintenance of the health of the individual (figure 4.3) (Dwarakanath, 2003).

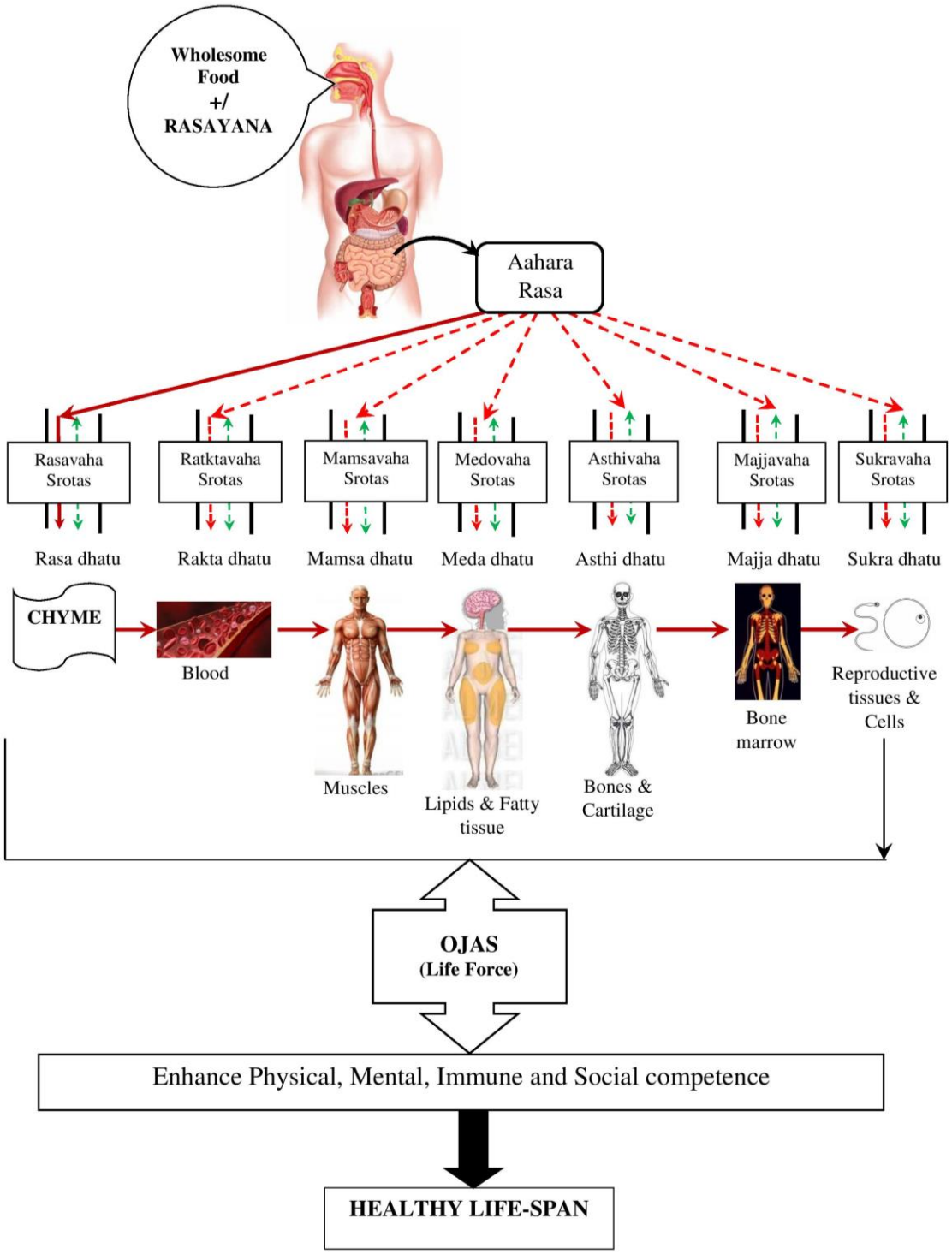

Figure 4.3: Current perspective of *Rasayana* and its role in the formation and maintenance of tissues for a healthy life-span

The process of food forming *rasa* and subsequently forming *saptadhatus* is collectively known as '*Ahara Parinamakara Bhavas*' (Sastry, 1997). *Rasa* enhancing *Rasayanas* provide essential nutrition to maintain optimal quality and quantity of body tissues. They contribute by forming quality *rasadhatu* and enriching it. When *rasa dhatu* is depleted, water content in the body is reduced, skin becomes dry and rough, dryness in the bowels produces constipation and dries mucous membranes, individuals lose their ability to withstand stress and become susceptible to disease. In addition, the secretion of breast milk is diminished in nursing mothers and menstrual flow becomes scanty (Murthy, 2002). Some of the examples of herbs enhancing the quality of *rasa* are *amlaki* (*Emblica officinalis*), *ashwagandha* (*Withania somnifera*) and *shatavari* (*Asperagus recemosus*) (Chunekar, 2004)

4.3.2.2 Ayurvedic theories on nutrition, formation of tissues and maintenance

There is a direct link between *Rasayana*, nutrition and tissue metabolism or *dhathupaka* (figure 4.3). The effect of *Rasayana* is achieved mainly through formation of good quality tissues. The process by which *rasa* aids in the formation of the *dhatus* (tissues) has been described through three different theories in Ayurveda. They are:

i. **Ksiradhadhi nyaya**: The logic of milk to ghee transformation.

   Similar to the process of continuous transformation of milk to curd, curd to butter and butter to ghee, the *rasa dhathu* gets converted into blood (*raktha*), *rakta* to muscular tissues (*mamsa*) and so on until the seventh *dhatu* (*sukra*) and then *ojas* (life force) is formed (Dwarakanath, 2003; Sastry, 1997).

ii. **Khalekapota nyaya**: The logic of corn and pigeon.

The essence of *aharasa* travels through different channels of *dhatus* and nourishes the corresponding *dhatu*. This happens like pigeons (*kapota*) picks its required food from the heap of corn (*khala*) and goes back to its nest. *Anna rasa* is the heap of corn from which each of the *dhatus* pick up its required nutrients (Dwarakanath, 2003; Sastry, 1997).

iii. **Kedarakula nyaya**:

Similar to the irrigation of paddy fields, a common pathway gets divided into many small channels to different parallel fields, thus water is served to all from a common place. *Ahara rasa* being the common source of *rasadhatu* which gets supplied to all *dhatus* and get transformed into their respective components of the *dhatus* (Dwarakanath, 2003; Sastry, 1997).

In the process of transformation of tissues, the nutrient essence (*prasada*) and waste or *mala* are formed (Dwarakanath, 2003).

The waste produced at different stage of tissue formation are listed in table 4.2. Homeostasis in the formation and elimination of waste plays a major role in maintaining health. Even though the concept of *oja* is not fully understood, it is the end product of all cellular metabolism, it is responsible for sustaining life.

Table 4.2: Essential wastes produced in the body during tissue formation (Murthy, 2001)

Body tissues (*Dhatu*)	Mala (essential by-products)
Rasa	*Kapha*
Rakta	*Pitta*
Mamsa	Waste deposit in external opening like ear wax (*kha mala*)
Meda	Sweat (*sweda*)
Asti	Nails (*nakha*), Hair (*roma*)
Majja	Lacrimal secretions (*aksisneha*), sebaceous gland secretions (*tvaksneha*), *purisasneha*
Sukra	*Oja*

Rasayana action on *agni*, *srotas* and *dhatus* are described below:

4.3.2.3 Regulators of *agni*:

In *Shaarira* (anatomy and physiology) per Ayurveda, the term '*agni*' refers to any activity which is involved in transformations like catabolism and anabolism. Ayurveda considers heart as the site of *agni*, which is responsible for maintaining the 'heat' of the whole body (Dwarakanatha, 1996). There are thirteen types of *agni* mentioned in Ayurveda at physical level (Dwarakanath, 2003). They are:

Jatharagni- one *agni* present in the stomach and duodenum. This *agni* is primarily involved in digestion and absorption process.

Bhutagni- five *agni* from five basic elements, *parthiva* (earth), *apya* (water), *tejas* (*Agni*), *vayavya* (vayu) and *nabhasa* (*akash*). This *agni* is responsible for separation of these five elements in the food and provide nourishment to the body with the respective element.

Dhatvagni- seven *agni* present, one in each of the seven *dhatus*. *Dhatvagnis* are responsible for all catabolic and anabolic functions of the respective dhatus.

Agni converts the nutrients in the food into assimilable form, generates energy and is responsible for all the vital functions of human body. Therefore, Ayurveda considers that *dehagni* is the cause of life, complexion, strength, health, nourishment, luster, *oja* (life force), *teja* (energy) and *prana* (life energy) (Agrawal et al., 2010).

Dysfunction of *agni* results in the formation of toxins (*ama*), accumulation of which leads to disease state. *Agni* is considered to be the mool (basis) of life and disappearance of *agni* indicates death of the organism. *Rasayana* drugs like ginger, *haritaki* (*Terminalia chebula*), *chitraka* (*Plumbago zeylanica), bhallataka* (*Semicarpus anacadium*) act as *agni* regulators (Chunekar, 2004; Agrawal et al., 2010).

4.3.2.4 *Agni* and *ama*

Impairment in the digestion and subsequent tissue transformation process can lead to formation of *ama*. *Ama* refers to undigested or semi-digested food entering the body. Accumulation of *ama* within the body blocks the pathways involved in nutrient and information transfer (*srotorodha*). This hinders the normal movement of nutrients and other impulse leading to weakness and disease state. Tissues have to be free from *ama* to perform the normal functions (Dwarakanath, 2003). Some of the physical targets of *ama* include the intestines, lymphatic system, nerves, arteries and veins, capillaries and genitourinary tract (Tripathi, 1994 and Dwarakanath, 2003).

4.3.2.5 *Sroto shodhaka* and its importance in physiology.

Srotas refers to channels present throughout the body which facilitate nutrient transfer, waste elimination and information passage. In brief, *sroto shodhaka* refers to enhancement of micro circulation. This includes even the "invisible" or subtle level communication within or across the cells, molecules, atoms, subatomic strata and the environment.

Ayurvedic texts describe sixteen *srotas* (Sastry, 1997 and Sharma, 1992). They are:

- Three *srotas* connect the individual to the external environment, by bringing air, food, and water into and out of the body.
- Seven *srotas* are associated with the seven bodily tissues (*sapta dhatus*).
- Another three srotas direct wastes out of the body.
- Two specifically for women (one to carry menstruum and the other for carrying milk)
- One *srotas* is associated with mind to carry thoughts, ideas, emotions and impressions.

The unimpeded flow of appropriate nutrients, energy and information through these channels is the healthy state of an individual. Excess flow, deficiency or blockage in these channels leads to diseased state (Dwarakanath, 2003).

Together with the knowledge of the *doshic* imbalances, the *dhatus* (tissues) involved, the state of the *agni* (digestive fire), and other diagnostic means, assessment of the *srotas* is one of the means in Ayurveda by which diseases can be distinguished. By

knowing which *srotas* are affected and the nature and extent of their disturbance, it is possible to understand the disease process. Certain Ayurvedic *Rasayana* drugs like *pippali* (*Piper longum*) and honey clear the channels for flow of essential tissue building components (Chunekar, 2004).

4.3.2.6 Elimination of '*Mala*' and '*Ama*'- Metabolic wastes.

Ama formed during the digestive process, essential wastes (*mala*) formed during the tissue transformation process and other metabolic wastes like urine, faeces and sweat have to be eliminated from body. Accumulation of these substances in the body is considered harmful. Proper functioning of srotas is necessary for the removal of wastes and also for nutrients or drugs to reach their targets (Murthy, 2001). As the wastes have the capability to block the srotas, purificatory measures (*sodhana*) have been suggested in Ayurvedic texts. Generally, *Rasayana* intake is prescribed only after removing the accumulation of *ama* and *malas* from the body to maximize their effect.

Homeostasis in waste generation and elimination or the concept of '*sama mala*' is one of the indicators of healthy living. The balance is very much essential in keeping the body fit. Most *Rasayana* drugs and formulations possess all three properties (*agni* normalizing, *rasa* enhancing and *srotas* clearing), at various levels in different proportion.

4.3.2.7 *Rasayanas* and *Rasayana karma*

A detailed literature survey of Ayurveda texts indicated that *Rasayanas* are capable of producing different physiological effects (*Rasayana karmas*) in organisms. Based on the types of *Rasayanas* and their biological effects, about 41 possible *Rasayana*

karmas were identified from literature (FRLHT, 2015). The list is presented in table 4.3.

4.3.2.8 Potential *Rasayana* actions (*Rasayana karma*)

Below is the list of Rasayana karmas and their physiological effect obtained through literature survey.

Table 4.3: List of potential *Rasayana karmas* and their possible physiological effect*

S. No	Potential *Rasayana* action (*Rasayana karma*)	Physiological effect
1	*Aamahara*	≈Anti-oxidants and clearing of un-metabolized substances
2	*Agnivardhaka /Agni krt*	Optimizes metabolic functions
3	*Ayuvardhana*	Lifespan enhancement
4	*Balya*	Promotes body strength/ tonic
5	*Buddhikrit*	Promotes intelligence
6	*Brimhana*	Increases body weight / bulk promoting, nourishes
7	*Chakshushya/ Netra*	Beneficial for eyes/ promoting vision
8	*DantyaDaard`hyakara*	Strengthening / beneficial to teeth.
9	*Deepana*	Promotes appetite/ induce agni / regulates metabolism
10	*Dhatuposhana*	Nourishes seven dhatus (tissues)
11	*GarbhaVriddhikara*	Supporting / promoting fetal growth
12	*Garbhakara*	Improve fertility
13	*Garbha-sthaapaka*	Improves stabilization of foetus against abortion
14	*Hridya*	Beneficial for heart
15	*Jeevana*	Promotes life
16	*Kanthya*	Beneficial for throat/ voice

17	*Kesharanjana*	Promotes hair color
18	*Keshya*	Promotes hair growth
19	*Medhya*	Promotes intellect
20	*Paachana*	Promotes digestion
21	*Preenana*	Life giving
22	*Pushtikrit*	Nourishing
23	*Rakshoghna*	Protects against unseen organisms
24	*Samjyaa Sthaapana*	Stabilizes consciousness
25	*Sandhaana*	Uniting / joining / holding
26	*Shirovirechana*	Clearing sensory pathways
27	*Shramahara*	Stress relieving
28	*Shonitaasthaapana*	Resulting stabilization of blood formation and Haemostatic
29	*Shukra Janana*	Promotes / maintain reproductive tissues
30	*Snehana*	Unctuousness, Oleation
31	*Sanjya Prabodhana*	Induce recovery from sensory malfunction / recovery of consciousness
32	*Sabnjya Sthaapana*	Retention of consciousness
33	*Santarpana*	Bulk promoting (≈*Brimhana*)
34	*Stanya Janana*	Promotes formation of breast milk/ Galactagogue
35	*Tarpana*	Refreshing
36	*Tvachya*	Beneficial for skin
37	*Varnya*	Improves skin colour, texture and complexion
38	*Vayasthaapana*	Regulating ageing process/ anti ageing
39	*Vyadhiksamatva*	Immune booster
40	*Vrana Ropana*	Wound healing
41	*Vishaghna*	Anti poisonous

*The correlations of Karma to physiological effects is as per FRLHT (2015)

4.3.3 Scientific research on *Rasayana* herbs

A detailed Pubmed literature survey indicated a total of 73 citations with *Rasayanas* until the initiation of this thesis work ie, September 2010, which included 10 review articles. About 23 *Rasayana* herbs have been extensively studied, of which *Asparagus racemosus* and *Withania somnifera* (Ashwagandha) were the most studied *Rasayana* plants.

It was observed that free-radical scavenging activity was the most studied activity of *Rasayana* plants, followed by immunomodulatory activity. Anti-cancer, aphrodisiac, CNS disorders, giardiasis, radioprotective and anti-ulcer are some of the other properties tested with *Rasayana* herbs. Even though Ayurveda has a huge database of *Rasayana* plant drugs and a variety of health benefits linked to them, published literature had only focused on limited spectrum of activities. This analysis was published as a review article (Balasubramani et al., 2011).

From October 2010 to May 2016, about 69 more publications referring to *Rasayanas* and their activities have been added to Pubmed. In this period, research on *Rasayanas* has not just focused on the usual activities as mentioned above but additionally some unique activities such as anthelmintic, improve drug bioavailability, anti-ageing and anti-anemic have been reported (table 4.4). The overall citations on the activities of Rasayanas are shown in figure 4.4.

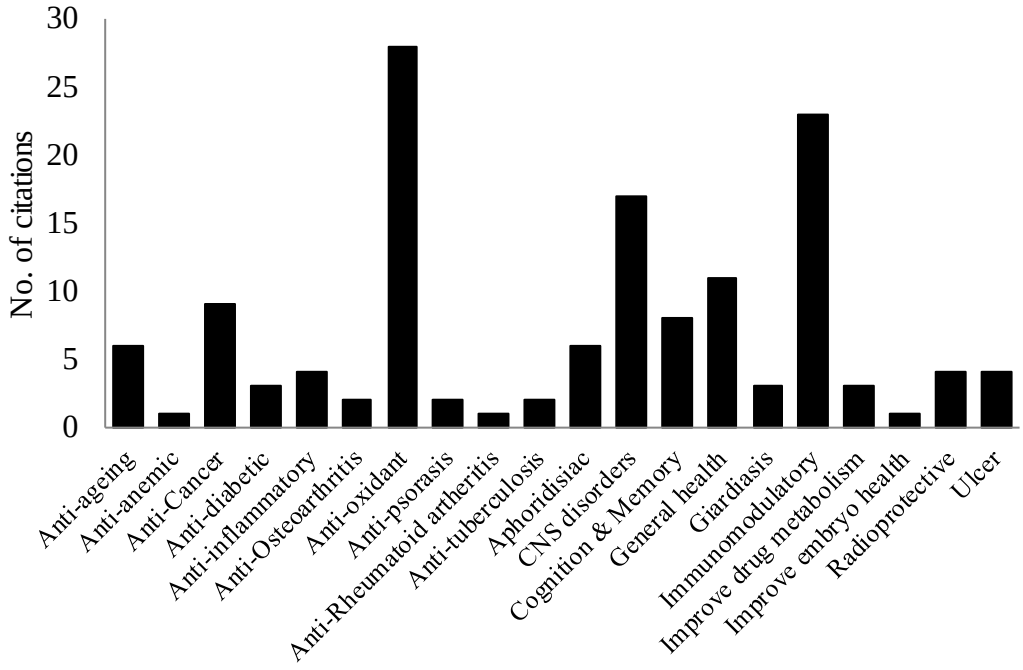

Figure 4.4: Citations on the activities of *Rasayana* plants/ formulations

Table 4.4: Some of unique *Rasayana* activities tested

Activity	Rasayana tested	Model	Reference
Anthelmintic activity	Vidanga (*Embelia ribes*)	*Caenorhabditis elegans*	Venkatasubramanian et al., 2013
Improve nutrient bioavailability	Amla (*Emblica officinalis*)	In vitro (cell free, Caco2 cells and HepG2 cells)	Venkatasubramanian et al, 2014
Improving drug bio-availability by inhibiting CYP enzymes	Triphala, Trikatu, *Asparagus racemosus* and *Gymnema sylvestre*	Rat liver microsomes	Ponnusankar et al., 2011; Harwansh et al., 2014; Borse and Kamble, 2015
Improve longevity and anti-ageing	Insect Rasayana	*Drosophila*	Priyadarshini et al., 2010
	Amalaki Rasayana and Rasa-Sindoor	*Drosophila*	Dwivedi et al., 2012; Dwivedi et al., 2015
	Brahmarasayana	Mouse	Guruprasad et al., 2012
	Pomegranate	*Drosophila*	Balasubramani et al., 2014*
	Triphala	Dermal fibroblasts and human keratinocytes	Varma et al., 2016
	Amalaki rasayana	DNA damage and repair in humans	Udupi et al., 2016
Improve embryo health	Drakshavaleha	Mouse	Kumar et al., 2013
Improve muscle strength	*Withania somnifera*	Human	Raut et al., 2012
Improve stamina and energy level	Ratnaprash	Mouse	Gupta et al., 2015
Anti-anemic	Punarnava mandura	Old age human	Pandya and Dave, 2014
	Amalaki Rasayana	IDA patients	Layeeq and Thakar, 2015
	Pomegranate	Anemic Yeast	Balasubramani et al., 2015*

*Publications from this thesis

4.4 Health and wellness in Ayurveda

सममांसप्रमाणस्तु समसंहननो नरः ।
दृढेन्द्रियो विकाराणां न बलेनाभिभूयते ॥
क्षुत्पिपासातपसहः शीतव्यायामसंसहः ।
समपक्ता समजरः सममांसचयो मतः ॥

Samamaamsapramaanastu samasamhanano narah|
drdhendriyo vikaaraanaam na balenaabhibhuyate||
ksutpipaasaatapasahah sheetavyaayaamasamsahah|
samapaktaa samajarah samamaamsacayo matah||

Charaka Samhita, Sutra Sthana, 21/18-19

Optimum quantity and quality of muscles, compactness and integrity of the body, strong sensory and motor functions, ability to resist disease, withstand hunger, thirst, physical exercises, heat and cold stress. Effective functioning digestive and assimilative processes are indicators of wellness (Sastry, 1997).

स्वस्थस्य रक्षणं कुर्यादस्वस्थस्य तु बुद्धिमान् ।

Svasthasya raksanam kuryadasvasthasya tu buddhiman|

Susruta Samhita, Sutrasthana, 15/40

The primary aim of the physician should be to maintain the wellness of his patients by attempting to establish homeostasis in the vital body functions (Acharaya, 1992).

Ayurveda considers ten factors (*dasa vidha pariksha*) to determine the state of health of an individual. They are body tissues (*dushyam*), residing location (*desham*), physical strength (*balam*), seasons/ time (*kalam*), digestive and metabolic processes (*agni* or *analam*), genetic and phenetic constitution (*prakriti*), age (*vaya*), mental strength or temperament (*satvam*), habituation (*satmyam*) and food (*ahara*)

(Payyappallimana and Venkatasubramanian, 2015). These factors are mainly used for diagnostic purpose, but they can be also applied to measure the wellbeing of an individual.

व्याधिमिन्द्रियदौर्बल्यं मरणं चाधिगच्छति ।
विरुद्धरसवीर्याणि भुञ्जानो नात्मवान्नरः ॥

Vyadhimindriyadaurbalyam maranam cadhigacchati
Viruddharasaviryani bhunjano natmavannara:

Susruta Samhita, Sutrasthana, 20/19

Ayurveda considers lack of self-discipline and consumption of incompatible food as primary causes of morbidity and mortality (Acharaya, 1992).

समदोष समाग्निश्च समधातु मलक्रियाः प्रसन्नात्मेन्द्रिय मनाः स्वस्थ इत्यभिधीयते ॥
Samadosha samaagnishcha samaadhatu malakriyaah prasanna atmendriyah manaah swastha ityabhidheeyate. Susruta Samhita, Sutrasthana, 15/41

According to Ayurveda, a person is said to be healthy when *doshas* (humors; *vata, pitta* and *kapha*), *agni* and the functions of *dhatus* and *malas* are in equilibrium. In healthy individuals' body tissues, senses and mind attain a state of self-awareness and self-contentment (Payyappallimana and Venkatasubramanian, 2015). The factors involved in making an individual healthy (*svasthya*) is presented in figure 4.5.

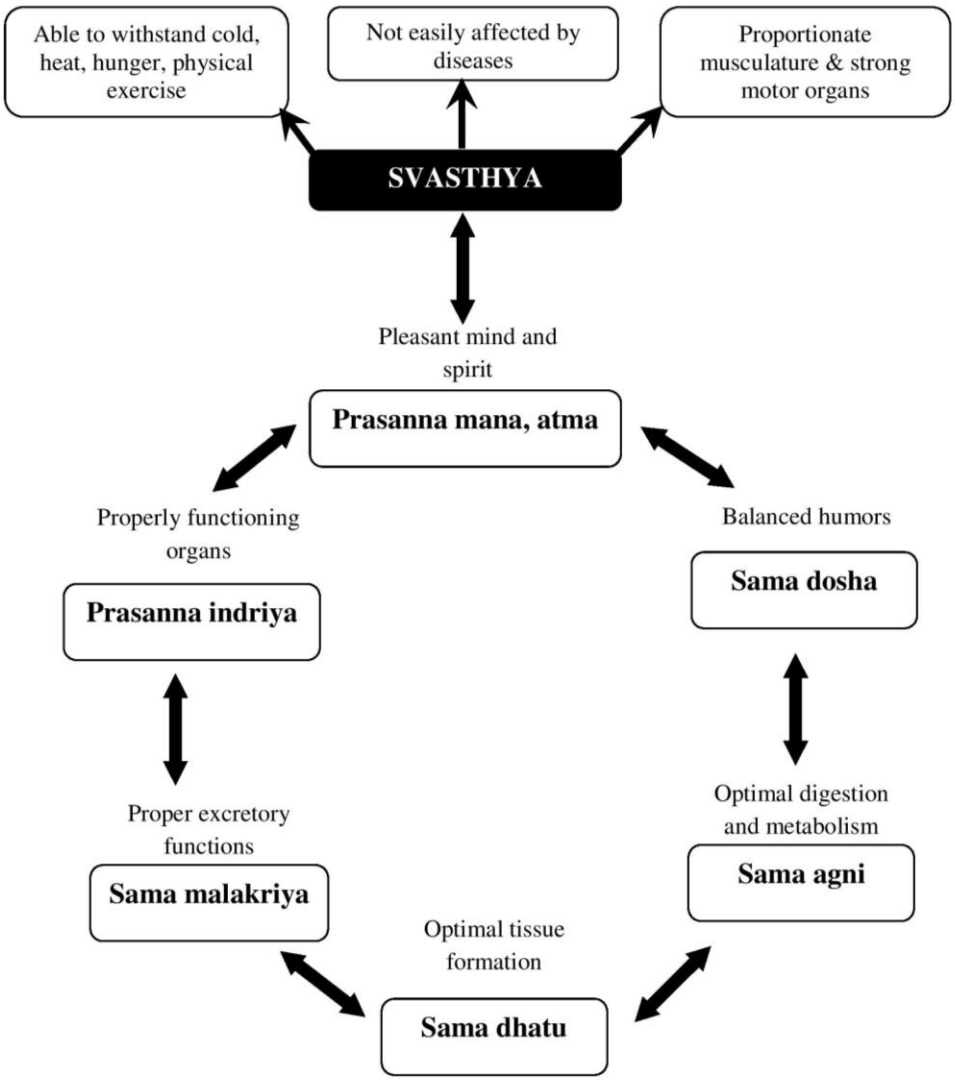

Figure 4.5: Pictorial representation of determinants of *Svasthya*

Based on the mode of action and the *Karmas* of the *Rasayanas*, it is clear that they can normalize digestion, absorption, distribution of nutrition and elimination of wastes. Homeostasis of metabolic processes is one of the hallmarks of healthy life or wellness (*Svasthya*). Thus, it can be inferred that *Rasayanas* have potential to impart health and wellness to individuals.

4.5 Pandu

In Ayurveda, '*pandu*' is considered as a specific disease characterized by pallor of body which resembles with 'Anemia' of modern biomedical science. *Rakta* (blood) has been considered as a key factor for *jeevana* (life), *varna prasadana* (complexion) and *mamsapushti* (nourishment of muscle tissue). In *pandu*, the humors (*dosha*) vitiate blood by various external or internal factors and lead to the pallor of the skin (Murthy, 2001).

4.5.1 Aetiology

Quality of food and improper digestion are considered as major determinative factors in causing *pandu*. Ayurveda considers some as compatible and non-compatible foods (*ahara*). This includes compatibility among the variety of foods consumed, as well as compatibility with the person consuming the food. Intake of incompatible or unwholesome food is said to cause *pandu*. Frequent intake of sesame oil, wine and fish also can cause *pandu*. Excessive intake of foods which are alkaline, sour, saline, corrosive and hot are also included as etiological factor for *pandu*. *Mridbhaksanajanya pandu* is caused by habitual eating clay or mud (Sastry, 1997).

Several habits like sleeping during day time, excessive exercise, excessive sexual intercourse, not following proper seasonal regimens and suppression of natural urges can lead to *pandu*. This condition can also manifest as a resultant of improperly performed *pancakarma* therapy (Murthy, 2002).

Physiological factors like passion, worry, fear, anger and grief also lead to *pandu*. Further *pandu* can also be a secondary manifestation in chronic disease conditions like *raktapitta* (bleeding disorders), *udara krimi* (worm infestation), *visamajwara*

(malaria), *sotha* (oedema: inflammatory swelling), *udra* (ascites), *grahani* (sprue) and *kamala* (jaundice) (Udupa, 2004).

4.5.2 Pathogenesis

Pathogenesis of *pandu* as per Ayurveda texts is presented in figure 4.4.

पित्तप्रधानाः कुपिता यतोक्तैः कोपनैर्मलाः ।
तत्रानिलेन बलिना क्षिप्तम् पित्तं हृदि स्थितम् ॥
धमनीर्दश संप्राप्य व्याप्नुवत् सकलां तनुम् ।
श्लेष्मत्वग्रक्तमांसानि प्रदूष्यान्तरमाश्रितम् ॥
अथातः पाण्डुरोग ... ।
त्वङ्मांसयोस्तत्कुरुते त्वचि वर्णान् पृथग्विधान् ॥
पाण्डुहारिद्रहरितान् पाण्डुत्वं तेषु चाधिकम् ।
यतोऽतः पाण्डुरित्युकतः स रोगः ॥

Pittapradhanh kupita yathoktaih kopanairmalah |

tatranilena balina kshiptam pittam hrdi sthitam ||

dhamanirdasha samprapya vyapnuvatsakalam tanum |

shleshmatvagraktamamsani pradushyantaramashritam ||

athatah panduroga .... |

tvagmamsayostatkurute tvaci varnan prthagvidhan ||

panduharidraharitan pandutvam teshu chadhikam |

yato atah pandurityuktah sa rogah ||

Ashtanga Hrdaya, Nidanashtana, 13/1-4

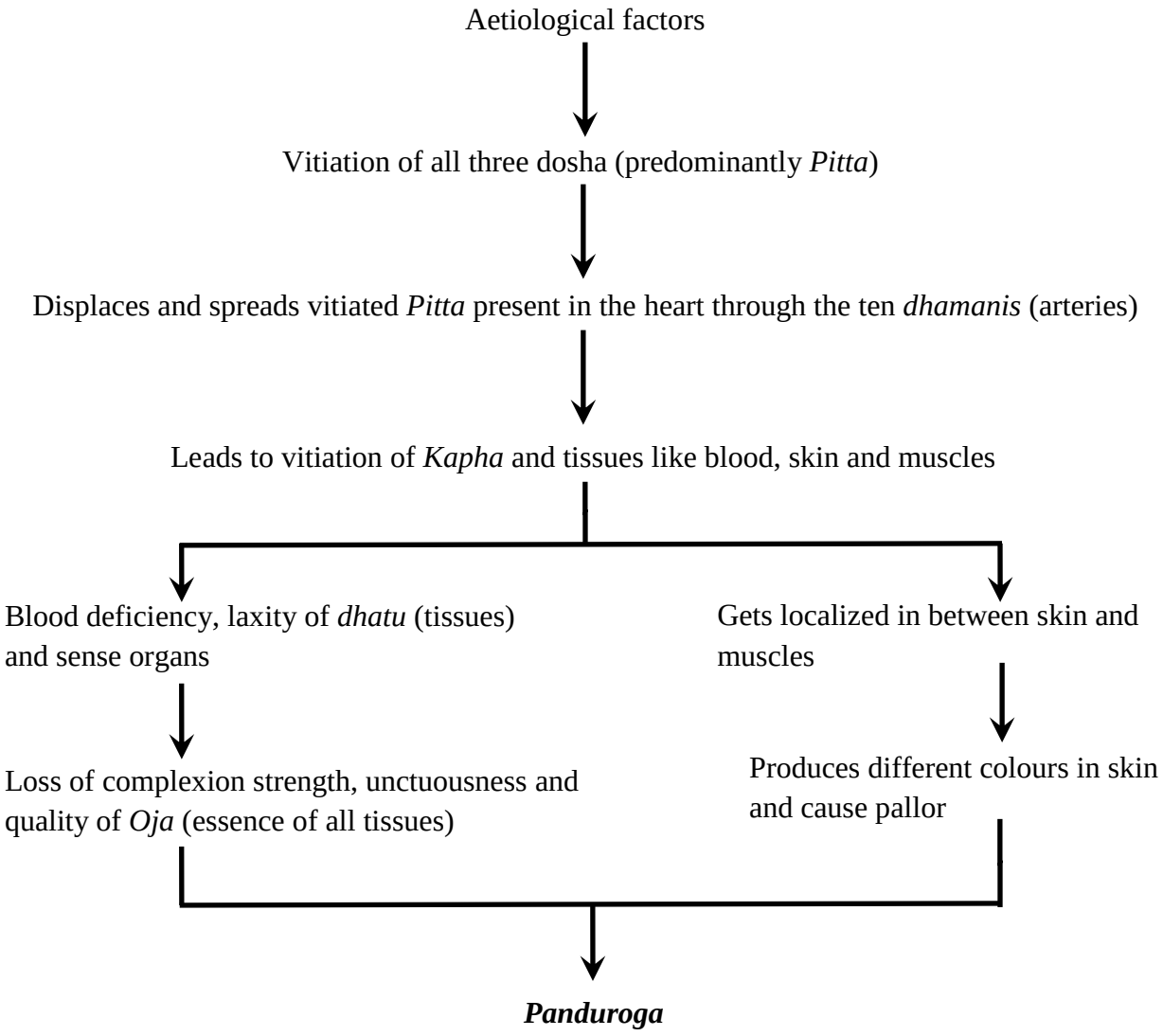

Figure 4.6: Pathogenesis of *Pandu* (derived from Udupa, 2004)

The *tridosas*, *pitta* being predominant among them, getting aggravated (increased) by their exciting cause, directed by the powerful *vata*, displaces the *pitta* present in the *hrdaya* (heart), through the ten *dhamani* (arteries / blood vessels), spreading it to all the parts of the body, then the *dosas* bring about vitiation of the *slesma* (*kapha*), skin, blood and muscles; getting localized in between the skin and muscles produce different colours in the skin, such as *pandu* (yellowish white), *haridra* (turmeric- like deep yellow), *harita* (green); of these *pandutva* (yellowish-white), being more predominant, the disease is called *panduroga* (figure 4.6).

4.5.3 Prodromal symptoms (*purvarupa*)

In susceptible individuals, before complete manifestation of *pandu*, prodromal symptoms like poor digestive activity, increased heart beat (tachycardia), dryness / roughness of skin, anorexia, yellow colour of the urine, absence of sweating, debility and fatigue is observed (Udupa, 2004).

4.5.4 General symptoms of *pandu* (Sastry, 1997; Murthy 2001)

- Poor digestive activity
- Tachycardia
- Poor quality of *rakta* (blood) and *medas* (fat)
- Weakness of tissues (*dhatus*) and sense organs (*indriyas*)
- Swelling of the eye – socketing and face
- Cold intolerance
- Fatigue and giddiness

4.5.6 Types of *pandu* (Sastry, 1997; Murthy 2001)

Five types of *pandu* are indicated in Ayurveda texts.

- Doshic - *vataja, pittaja, kaphaja* type of *pandu*
- Involvement of all *dosas* (*sannipataja pandu*)
- Caused by habitual intake of mud (*mridbhaksanajanya pandu*)

4.5.7 Principles of treatment (Sastry, 1997; Tripathi and Mishra, 1983)

Treatment strategy for *pandu* includes purification measures (*sodhana* therapy) which includes ***s**nehana* (oleation therapy), *vamana* (emesis) / *virechana* (purgation), followed by *samana* therapy (palliative treatment) with specific drugs.

4.5.8 Some important Ayurvedic formulations for *pandu*

Below are some of the single herb drugs indicated for use in pandu:

- Juice prepared from *punarnava (Boerhavia diffusa), dadima* (*Punica granatum*), *bhringaraja* (*Eclipta alba*) with honey (Mishra, 2007)
- Churna prepared with *draksha* (*Vitis vinifera*) or *amalaki* (*Emblica officinalis*) (Sastry, 1997)

Some of the herbo-mineral preparations (*rasa kalpas*) used in management of *pandu* (Sastry, 1997) is listed below:

- *Navayasa lauha*
- *Vidangadilauha*
- *Dhatrilauha*

- *Kasisa bhasma*
- *Lauha bhasma*
- *Mandura bhasma*

4.5.9 Other herbal preparations used in management of *pandu*

- Tablets (vati) like *punarnava mandura* (Sastry, 1997) and *mandura vataka* (Murthy, 2002)
- Medicated ghee preparations like *dadimadya ghrita* (Murthy, 2002), *vyosadya ghritam* (Mishra, 2007)
- Confectioneries (lehyam) like *amalaki Rasayana* (Sastry, 1997) and
- Asava and aristas like *lohasava* and *punarnavarista* are also indicated for the management of *pandu* (Mishra, 2007)

4.5.10 Dietary recommendations for *pandu* (Sastry, 1997; Murthy, 2002)

According to Ayurveda, intake of food prepared with grains and pulses like rice, barley, wheat, green gram, pigeon pea and red lentil is good in conditions like *pandu*. Milk and ghee are also considered to be effective in the management of *pandu*. Leaves of *vrsa* (*Adhatoda vasica*), *patola* (*Trichosanthes dioica*), *vetra* (*Calamus rotang*) and *parpata* (*Fumaria parviflora*) are prescribed for individuals with *pandu*. Juice of meat of animals of desert and wild animals are considered as nutritious foods for individuals suffering from *pandu*.

4.5.11 Food supplements for *pandu* (Murthy, 2002)

Ayurveda texts suggests intake of certain foods as supplements for the management of *pandu*. They include intake of fresh juice prepared from grapes (draksa), pomegranate (dadima), dates (kharjura), gooseberry (amalaki), sugarcane and butter milk (takra). These are prescribed to be taken after regular food.

4.5.12 Correlation of iron deficiency anemia (IDA) to *pandu*

An attempt was made to compare the understanding of anemia per biomedicine and *pandu* as per Ayurveda. Understanding of the clinical symptoms and management strategies indicate that IDA could be a part of *pandu*. Both the medical systems indicate similar clinical symptoms like pallor and tiredness and use of iron in the disease management. Modern biomedicine considers deficiency of iron as the causative factor for IDA, while Ayurveda considers improper digestion and incompatible foods as the major causative factors. Even though iron is an ingredient in several Ayurveda formulations used in treatment of *pandu* (eg., *dhatriloham* and *kaseesa bhasma*), no direct reference could be found in Ayurvedic texts providing iron deficiency as *pandu's* aetiology. More than iron supplementation, advice on food and food supplements formed the major treatment line in Ayurveda. Comparison on various aspects of IDA and *pandu* are given in the below table (table 4.5).

Table 4.5: Comparison of IDA and *Pandu*

Iron deficiency anemia (IDA)	**≈**	***Pandu***
Condition in which blood lacks adequate healthy red blood cells and thereby reduces oxygen carrying capacity of blood	**Definition**	Condition where pallor of skin and body is observed
• Severe blood loss due to mensuration, disease conditions like peptic ulcer, hiatal hernia, colorectal cancer, colon polyp etc. • Pregnancy • Lack of iron in diet or inhibition of iron absorption	**Etiology**	• Internal factors - Dysfunction of stomach, liver, spleen and bone marrow • External factors - improper diet, improper digestion, mal-absorption and mal-coloration
• Iron is an important co-factor for various vital enzymes and is also required for formation of hemoglobin. • Iron deficiency leads to lesser hemoglobin and reduced number of RBC's leading to lesser oxygen carrying capacity.	**Pathology**	*Pitta* gets aggravated and gets displaced from heart, through the blood vessels, spreading it to all the parts of the body. It gets localized in between the skin and muscles causing *pandutva* (yellowish-white) pallor.
Pale skin, fatigue, weakness shortness of breath, chest pain frequent infections, dizziness or lightheadedness, cold hands and feet inflammation or soreness of your tongue, brittle nails, fast heartbeat unusual cravings for eating non-nutritive substances like soil, poor appetite, restless legs syndrome	**Clinical Symptoms**	Weakness of *dhatus* (tissues), poor quality of *rakta* (blood) and *medas* (fat), lack of vigour (debility), increased rate of heart beat, swelling of the eye, aversion to food and cold, loss of hair, poor digestive activity, fatigue and giddiness
• Iron tablets and iron fortified food • Iron bioavailability enhancers like folic acid and ascorbic acid, • Stop blood loss, • Blood transfusion, • Surgery to remove tumor or polyp	**Clinical Management**	• Herbo-mineral preparations containing iron • Intake of rice, wheat, Barley and green gram • Juice of meat of desert animals, milk and ghee • Leaves of *vrsa*, *patola*, *vetra* and *parpata* • Juice of grapes, pomegranate, amla, dates and butter milk

4.6 *Dadima, a 'Nitya Rasayana'*

Several single herb preparations have been prescribed in classical Ayurveda texts for the management of *Pandu*. Consumption of pomegranate is one such suggestion. Frequency of preparations based on pomegranate indicated in management of *pandu* and also indication of pomegranate as '*Nitya Rasayana*', which can be consumed on a daily basis throughout life prompted to select pomegranate (*dadima Rasayana*) for this thesis work.

4.6.1 Pomegranate

Sanskrit Name : *Dadima* / *Phalamla* / *Raktabija* / *Raktapuspha* / *Shukavallabha*

Parts used : Whole fruit, fruit rind, Arils / seeds, tender leaves

Use of pomegranate has been recorded over thousands of years and it is a symbol of life, health, longevity, femininity, fecundity, knowledge, morality, immortality, and spirituality (Mahdihassan, 1984).

4.6.2 Varieties of *dadima*

Ayurveda texts indicate that there are two types of *dadima* (pomegranate) based on the taste (Chunekar, 2004). They are:

- *Madhura* (sweet) and
- *Amla* (Sour)

4.6.3 Ayurvedic qualities of pomegranate

मधुरं तृप्तिकरं धातुवृद्धिकरं लघु ।
तुवरं ग्राहकं स्निग्धं मेध्यं बल्यं च माधुरं ॥
पथ्यं त्रिदोषतृड्दाहज्वरहृद्रोगनाशनम् ।
मुखरोगं कण्ठरोगं नाशयेदिति कीर्तितम् ॥

Madhuram truptikaram dhaatuvrddhikaram laghu |

Tuvaram graahakam snigdham medhyam balyam cha madhuram ||

Pathyam tridoshatrid daaha jwarahrdroganaashanam |

Mukharogam kantharogam naashayediti keertitam || (Nighantu Ratnakara)

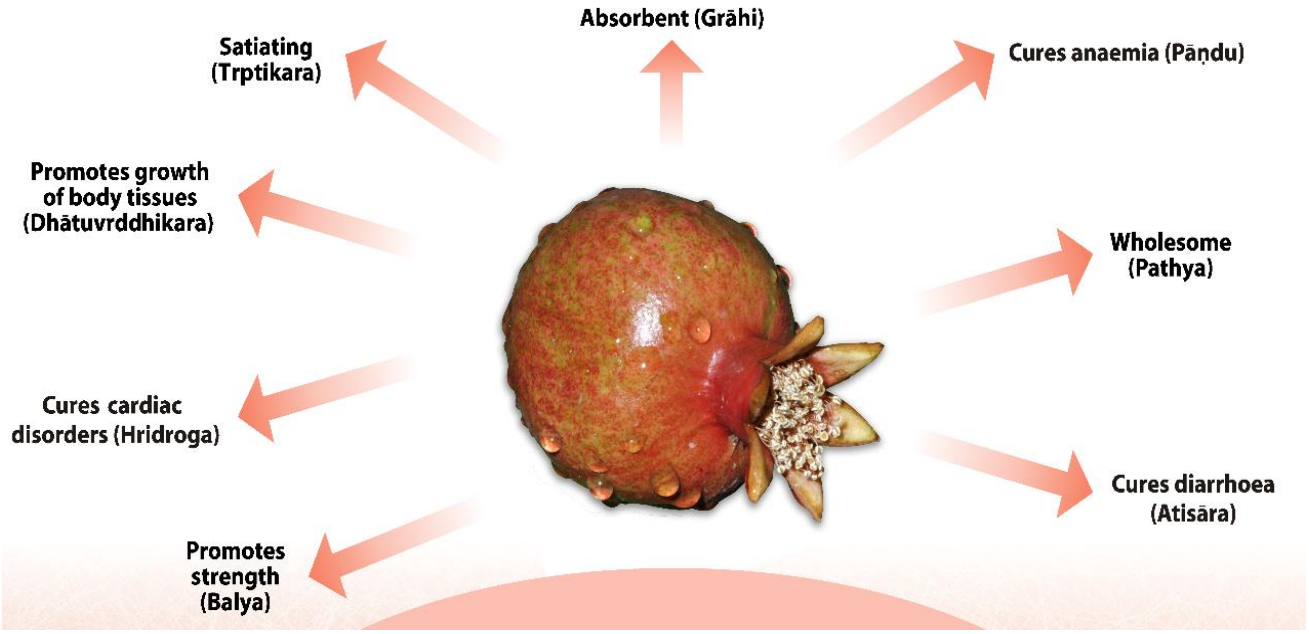

Figure 4.7: Major *Rasayana karmas* of pomegranate

(adapted from Chunekar, 2004; Tripathi, 2006; Dash, 1994)

Dadima has sweet and astringent taste and becomes sweet after digestion. It is unctuous and light to digest and prevents excess water loss from the body. *Dadima* is wholesome and suitable to all, satiating and promotes tissue growth. It is a promoter of intellect and body strength. Pacifies all three *doshas* and cures burning sensation, fever, and diseases of heart, mouth and throat (figure 4.7).

4.6.4 Functional properties of pomegranate and their respective pharmacological actions

The functional properties (*drvyaguna*) of *dadima* (pomegranate) according to Ayurveda is presented in table 4.6.

Table 4.6: Functional properties of pomegranate as per Ayurveda (Chunekar, 2004; Tripathi, 2006; Dash, 1994)

***Drvyaguna* (functional properties)**		**Pharmacological actions of listed functional properties**
Dosha- Karma (Action on Doshas)	*Tridosaghna* (Pacifies all three doshas)	Maintains homeostasis, Nourishment (complementary food in any disease treatment)
***Rasa* (taste)**	*Madhura* (sweet), *Amla* (sour), *Kashaya* (astringent)	Tissue building, Nourishing, healing lesions
***Guna* (properties)**	*Snigdha* (unctuous) and *Laghu* (light)	Lubrication, ease of digestion
***Virya* (potency)**	*Ushna* (hot)	Appetizer, digestive, purgation
***Vipaka* (taste after digestion)**	*Madhura* (sweet)	Tissue building, Nourishing

4.6.5 *Karmas* of pomegranate (potent pharmacological actions):

Ayurvedic literature lists several *Rasayana karma's* for pomegranate (figure 4.5). They include *hrdya* (good for heart), *pandughna* (cures anemia), *dipanam* (increase digestion), *rocanam* (increases taste), *srama hara* (physical/mental stress reliver), *balya* (strength giving), *chhardi nigrahaniya* (stops vomiting/ nausea), *graahee* (absorbing water content from both intra/extracellular), *tarpana* (nourishing) (Sastry, 1997; Acharaya, 1992; Chunekar, 2004; Tripathi, 2006).

4.7 Conclusion

In spite of Ayurvedic texts indicating several physiological effects of *Rasayana* herbs both in imparting wellness (*svasthya*) and in the management of chronic diseases, research has been focusing on only a few activities like anti-cancer, anti-oxidant and immunomodulatory. The literature survey indicates that *Rasayanas* act by normalizing digestive process and enhance absorption of nutrients. A better nutrition can impart resistance to stress and diseases, thereby improve wellness.

Ageing and IDA are two of the major public health issues which have multifactorial etiology. Scientific studies indicate that both of these conditions can be managed by improving nutrition intake. Thus, the focus of this thesis was to, identify or develop appropriate models to study some of the *Rasayana karmas* like *agnivardhaka* (improves digestion and metabolism), *ayuvardhana* (lifespan enhancement), *vayasthapana* (anti-ageing), *svasthya* (healthy living), *vyadhiksamatva* (immune enhancer), *dhatuposhana* (tissue nourishment) and *panduhara* (anti-anemic). The *Rasayana karmas* of pomegranate indicate that it has potential for use in management of *pandu* (IDA) and also for general wellbeing. So, pomegranate was selected to study the above mentioned biological activities (*Rasayana karmas*) using *in vitro* and *in vivo* models.

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Chapter 5

PHYTOCHEMICAL CHARACTERISATION OF POMEGRANATE JUICE

5.1 Introduction

Ayurveda preparations whether single herb or complex formulations always contain a concoction of compounds. This might have advantage in imposing a systemic effect (Chandran et al., 2015). Several single herb preparations have been indicated in Ayurveda for daily intake or as drug for specific condition. In the management of *Pandu* (~IDA), Ayurvedic texts suggest use of fresh juice made from grapes, pomegranate, dates and amla (Murthy, 2009). Juice prepared from amla has been recently shown to enhance iron bioavailability and uptake (Venkatasubramanian et al., 2014). The same study also reported that the iron bioavailability enhancing potential of amla is not just because of its ascorbic acid content. These reinforce that multiple chemical entities might play a role in bringing out a better biological activity.

Pomegranate juice (PJ) has been reported to have several bioactive phytochemical constituents. A summary of phytochemical constituents reported in PJ and their potential biological activities is summarized in the review of literature (chapter 3). In the current study, in order to have a quality controlled PJ for use in bioactivity related experiments, qualitative and quantitative characterization of phytochemical constituents in PJ was performed. Also, the phyto-constituents play a major role in determining the bioactivity of the herbal preparation.

Qualitative phytochemical screening was performed to detect the presence of specific classes of secondary metabolites like alkaloids, carbohydrates, glycosides, saponins,

phytosterols, fixed oils and fats, resins, phenolic acids and tannins, proteins and amino acids, flavonoids and gums and mucilage. Phenolics form the major proportion of biologically active compounds present in PJ (Wang et al., 2010). The total phenolics content has been used as the marker for standardization of fruit juices including PJ (Martin et al., 2009). Further, total inorganic and organic content of the PJ was estimated as the TDS (total dissolved solids) and sugar content in the juice was estimated as TSS (total soluble solids). PJ has been reported to have micronutrients (Elfalleh et al., 2011).

Iron content of PJ was estimated to have a control on the contribution of Fe by PJ in the subsequent bioactivity related experiments. Organic acids including ascorbic acid (vitamin C) has been reported as iron bioavailability enhancers (Cook and Reddy, 2001). Vitamin C also plays a major role in the anti-oxidant property of PJ (Vegara et al., 2014). Vitamin C content was quantified so as to describe the quantity of PJ in terms of ascorbic acid (AA) equivalent. Quality control of PJ is required for avoiding batch to batch variation in phytochemical profile and also for reproducibility of bioactivity studies.

5.2 Materials and Methods

The methods followed for phytochemical characterization of PJ is described below.

5.2.1 Collection & authentication of pomegranate samples

Fresh red variety pomegranate fruits were purchased from the local fruit market. Details of purchase, quantity and authentication were entered into the Sample data sheet (SDS) and a laboratory accession number was provided for each lot purchased.

5.2.2 Preparation and storage of pomegranate juice (PJ)

PJ was prepared by hand crushing the arils of fresh fruits through sterile muslin cloth. The juice was passed through a sterile syringe filter (0.2µm; Millex, Millipore, Germany) and stored as aliquots of different volume (5, 15, and 50 ml) in screw capped tubes at -80°C until use.

5.2.3 Qualitative phytochemical screening

PJ was subjected to tests for the presence of alkaloids, carbohydrates, glycosides, saponins, phytosterols, fixed oils and fats, resins, phenolic acids and tannins, proteins and amino acids, flavonoids and gums and mucilage.

5.2.3.1 Detection of alkaloids (Raaman, 2006)

A few ml of PJ was stirred well with a few drops of dilute hydrochloric acid and filtered. The filtrate was used to test for the presence of alkaloids by the following tests:

a) Mayer's test: To a few ml of filtrate, a drop of Mayer's reagent was added along the sides of the test tube. Appearance of a white or creamy precipitate indicates the presence of alkaloids.

b) Wagner's test: To a few ml of the filtrate, a drop of Wagner's reagent was added along the sides of the test tube. Appearance of a reddish-brown precipitate indicates the presence of alkaloids.

c) Hager's test: A drop of Hager's reagent was added along the sides of the test tube containing a few ml of the filtrate. Formation of a yellow precipitate indicates the presence of alkaloids.

d) Dragendorff's test: To a test tube containing a few ml of filtrate, Dragendorff's reagent was added drop by drop along the sides. Appearance of a prominent yellow precipitate indicated presence of alkaloids.

5.2.3.2 Detection of glycosides (Raaman, 2006)

a) Borntrager's test: About 3 ml of chloroform was added to 2 ml of filtrate and shaken. Then the chloroform layer was separated and equal amount of ammonia (10%) was added to it. Appearance of pink colour indicates the presence of anthraquinone glycosides.

b) Legal's test: To 2 ml of filtrate, one drop of pyridine and 1 ml of sodium nitroprusside solution was added. This mixture was made alkaline using sodium hydroxide (10%). Formation of pink to blood red colour indicates the presence of glycosides.

c) Liebermann-Burchard's test: About 3 ml of chloroform was added to 2 ml filtrate followed by addition of a few drops of acetic anhydride. This mixture was boiled and cooled in an ice bath. To this mixture, 2 ml concentrated sulphuric acid was added along the sides of the test tube. Formation of a brown ring at the junction of the two layers, along with formation of violet/green/blue colour indicates the presence of steroidal glycosides.

5.2.3.3 Detection of Phytosterols (Shah and Seth, 2010)

a) Salkowski's Test: PJ was treated with equal volume of chloroform and filtered. A few drops of concentrated sulphuric acid was added to the filtrate, shaken and allowed to stand. Appearance of golden yellow colour indicates the presence of sterols.

b) Tshugajeu test: PJ was treated with equal volume of chloroform and filtered. To the filtrate, excess acetyl chloride and a pinch of zinc chloride was added, kept aside for 10 minutes and then warmed on a water bath. Appearance of eosin red colour indicates the presence of sterols.

5.2.3.4 Detection of carbohydrates (Raaman, 2006)

PJ was mixed and shaken with 5 ml of water and filtered. The filtrate was further used to test for the presence of carbohydrates.

a) Molish's test: Filtrate was treated with 2 drops of alcoholic α-naphthol solution in a test tube. Concentrated sulphuric acid (2 ml) was added to the mixture along the sides of the test tube. Formation of violet ring at the junction of two solutions indicates the presence of carbohydrates.

b) Benedict's Test: 1 ml of filtrate was treated with 1 ml of Benedict's reagent and heated on a boiling water bath. Formation of orange red precipitate indicates the presence of reducing sugars.

c) Fehling's Test: About 1 ml of filtrate was boiled on water bath with 1 ml of each of Fehling's solutions A and B. Formation of a red precipitate indicates presence of reducing sugars.

d) Barfoed's test: To 1 ml of filtrate, 1 ml Barfoed's reagent was added and heated on a boiling water bath. Appearance of red precipitate indicates presence of reducing sugars.

5.2.3.5 Detection of fixed oils & fats (Raaman, 2006)

Stain test: Small quantity of PJ was pressed between two layers of filter paper. Formation of oil stain on the paper indicates presence of fixed oils.

5.2.3.6 Detection of saponins (Raaman, 2006)

a) Foam Test: Small amount of PJ was shaken with water in a test tube. Formation and persistence of foam for ten minutes indicates the presence of saponins.

b) Froth Test: PJ was diluted with distilled water and shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam indicates the presence of saponins.

5.2.3.7 Detection of phenolics and tannins (Raaman, 2006)

A small portion of PJ was shaken with a few ml of water-methanol and filtered. Filtrate was further tested for presence of phenolics and tannins

a) Ferric chloride test: To 2 ml of filtrate, a few drops of neutral 5% ferric chloride were added. Formation of dark green colour indicates the presence of tannins.

b) Gelatin test: To 2 ml of filtrate, 2 ml of a 1% gelatin solution containing 10% sodium chloride was added. A white precipitate indicates the presence of tannins.

c) Lead acetate test: To 2 ml of filtrate, 3 ml of 10% lead acetate solution was added. Formation of a white precipitate indicates the presence of phenolic compounds.

5.2.3.8 Detection of flavonoids (Trease and Evans, 1996)

A small portion of PJ was shaken with a few ml of water-methanol and filtered. Filtrate was further tested for presence of flavonoids.

a) Alkaline reagent test: About 2 ml of filtrate was treated with 2 ml of 10% ammonia. Formation of a yellow fluorescence indicates the presence of flavonoids.

b) Magnesium and HCl reduction (Shinoda test): To 2 ml of filtrate, 1 ml of methanol, a few fragments of magnesium ribbon and concentrated hydrochloric acid were added. Appearance of pink to crimson colour indicates the presence of flavanols.

c) Lead acetate Test: About 2ml of filtrate was treated with a few drops of lead acetate solution. Formation of yellow precipitate indicates the presence of flavonoids.

5.2.3.9 Detection of proteins & amino acids (Raaman, 2006)

a) Millon's test: A few drops of Millon's reagent was added to 2 ml of PJ. Formation of a white precipitate indicates presence of proteins.

b) Biuret test: About 2 ml of PJ was treated with 1 drop of 2% copper sulphate solution and 1 ml of 95% ethanol and excess of potassium hydroxide (KOH) pellets. Formation of pink colour in the ethanolic layer indicates presence of proteins.

c) Ninhydrin test: To 2 ml of filtrate, 2 drops of ninhydrin solution was added. A purple colour indicates presence of amino acids.

5.2.3.10 Detection of gum and mucilages (Raaman, 2006)

Alcoholic precipitation test: About 25ml of absolute alcohol was slowly added to PJ with constant stirring. Formation of precipitate indicates the presence of gums and mucilages.

5.2.3.11 Detection of resins

Acetone-water Test: Small amount of PJ was treated with acetone and then small amount of water was added and shaken. Appearance of turbidity indicates the presence of resins.

5.2.4 Quantitative phytochemical estimation

Quantitative estimation of total dissolved solids, phenolics, ascorbic acid and iron is performed as described below.

5.2.4.1 Estimation of Total Dissolved Solids (TDS)

The two principal methods followed for measuring total dissolved solids are gravimetry and conductivity. Gravimetric methods are the most accurate and involve evaporating the liquid solvent to leave a residue that can subsequently be weighed with a precision analytical balance (Eaton et al., 2005).

TDS is a measure of the combined content of all inorganic and organic substances in PJ. It refers to the material residue left in the vessel after evaporation of the juice and subsequent drying at high temperature. It constitutes the portion of the total solids that passes through the filter of about 0.2 µm normal pore size, drying sample at 180 ºC yields values for dissolved substances since at this temperature residue loose almost all mechanically occluded water (Bradley, 2010).

Method described by Eaton et al., (2005) was followed for estimation of TDS in PJ. A dry empty porcelain dish was taken and its empty weight (in g) was noted in electronic balance (Shimadzu, Kyoto, Japan) (A). About 10 ml of PJ was transferred into the dish and evaporated to dryness in hot air oven at 180 ºC ± 2 ºC. After cooling, weight of the dish was noted again (B). TDS was estimated using the following formula:

$$\text{TDS in g/l} = \frac{(B - A)\ X\ 1000}{\text{sample volume (ml)}}$$

A= Initial weight of the dish (g)

B= Final weight of dish (g)

5.2.4.2 Estimation of Total Soluble Solids (TSS)

TSS estimation by refractometer is widely used during fruit and vegetable processing to determine the concentration of sugar in the products. Sugar concentration is expressed in degrees Brix. At 20°C, the ºBrix is usually considered equivalent to the percentage of sugar content in the solution.

A drop of PJ was placed on the prism of the refractometer (Advanced Technocracy Inc., Ambala, India). The reading was observed through the eyepiece. The critical angle (the angle beyond which light is totally reflected back into the sample) is a function of the refractive index. The reading was noted at the critical angle where a dark-bright boundary falls on an engraved scale.

5.2.4.3 Determination of Total Phenolics in PJ

Total phenolics present in PJ was estimated by colorimetry using Folin–Ciocalteu (F–C) reagent. The F–C assay relies on the transfer of electrons in alkaline medium from phenolic compounds to phosphomolybdic/ phosphotungstic acid complexes to form blue complexes that are determined spectroscopically at approximately 735 nm (Singleton et al., 1965; Slinkard and Singleton, 1977).

About 10 µl of extract and 160 µl Folin-Ciocalteu reagent were mixed well and this reaction mixture was allowed to stand for 5 min followed by addition of 130 µl of 1M sodium carbonate solution and mixed well in a 96 well microtitre plate. This was kept in the dark at room temperature for 30 min and then the absorbance was measured at 735 nm with a microplate absorbance spectrophotometer (Biorad, CA, USA). Gallic acid was used as a standard (20, 40, 60, 80, 100 and 200 mg/l) and the calibration

equation was Y= 0.003X + 0.007 (R^2= 0.999), where X is the gallic acid concentration in mg/l and Y is the absorbance read at 735 nm. All the analysis were done in triplicate and results (mean) were expressed as mg gallic acid equivalent per liter of juice. Distilled water was used as blank.

5.2.4.4 Determination of total acids in PJ (AOAC, 2000; AOAC 2005)

The total organic acid content of PJ was estimated as follows.

Sample preparation

About 10 ml of PJ mixed with 50ml distilled water was used for estimation of total acids.

Measurement of total acids

NaOH was standardized using potassium hydrogen phthalate. Standardized 0.1M NaOH solution was taken in a burette and slowly titrated into the juice (10 ml) with continuous swirling. Titration was continued until the NaOH-PJ mix reached pH12, which was monitored using a digital pH meter (Micropro Labmate, Servewell, Bangalore, India). Graph was plotted on the volume of NaOH added against raise in the pH. The percentage of acid present (expressed in terms of equivalent to citric acid) in the juice/water extract was calculated using the following formula.

$$Percentage\ acid = \frac{\text{Titre x acid factor x 100}}{\text{10 (ml juice)}}$$

Titre was determined as the point at which the ratio between the NaOH added and pH change was maximum. Acid factor for citric acid standard was considered as 0.0064.

5.2.4.5 Quantitative estimation of L-Ascorbic acid

The method described by Mitic et al., (2011) was followed for quantitative estimation of ascorbic acid in PJ.

Preparation of Standard Ascorbic acid: Standard L-Ascorbic acid (Sigma, MO, USA) stock solution of 1mg/ml was prepared in water. For preparing the linear graph working range, 0.025mg to 0.175 mg/ml ascorbic acid was used.

PJ was filtered through 0.22µm syringe filter and used for the analysis. The high performance liquid chromatographic system consisted of a Shimadzu HPLC (Kyoto, Japan) fitted with a Purospher, Hiber RP18 (250x4.60 mm). Elution was carried out with phosphate buffer (10mM KH_2PO_4 in HPLC grade water; pH 2.25 adjusted with orthophosphoric acid) and flow rate was 1 ml/min, isocratic program. Detection was at wavelength of 245 nm using a photodiode array detector and analysed using LCsolution software (Shimadzu, Kyoto, Japan).

5.2.5 Total iron estimation in PJ

5.2.5.1 Preparation of PJ

Total iron in PJ was estimated using the standard AOAC method (Helrich, 1990). Briefly, 5g of PJ was weighed in crucibles, carbonized in flame and ashed in muffle furnace (Servewell, Bangalore, India) at 600°C for 10hrs. Freshly prepared Aquaregia (3: 1; HCl: HNO3) was added and evaporated to dryness. This was repeated 3 times. Then evaporated to dryness with 4ml of concentrated HCl. After cooling, 2ml of 3% HCl was added to the crucibles and warmed. Volume was made up to 10 ml with 3% HCl.

5.2.5.2 Iron estimation

Total iron was estimated following the protocol described by Nilsson et al., (2002).

Stock Iron solution (10µg/ml): 1ml of Iron standard reference solution (1mg/ml) purchased from Fluka Analytical (Buchs, Switzerland) was dissolved in 100ml distilled water (10µg/ml). Different concentrations (5 µg, 2.5 µg, 1.25 µg, 0.625 µg, 0.31 µg and 0 µg) of iron standard solution were prepared by serial dilution in distilled water. Distilled water was used as blank.

Chromogen: 250 mg/l Bathophenanthroline di-sulfonate (Sigma, MO, USA) in 2 M sodium acetate, 0.25% Ascorbic acid and incubate for 1 hr in dark.

Estimation of iron

About 200µl of the sample was transferred to 96 well microplate in triplicates. 100µl of chromogen solution was added and incubated at room temperature for 10 minutes. Absorbance was read at 533nm against blank in a microplate reader (Biorad, CA, USA). Iron content was estimated from the standard graph prepared with different known concentrations of iron.

5.3 Results and discussion

For all experiments in this study, red variety pomegranate was used (figure 5.1). The lab ID's of different batches are: L/11/10/009, L/12/08/005 and L/14/11/012. Arils constituted approximately 50% of the weight of fresh fruits. About 750 ml of juice was obtained from 1kg arils.

The results of various qualitative and quantitative phytochemical estimation studies done with PJ used in this study are summarized below. The phytochemical analysis performed with all the three different accessions of PJ did not show any significant variation for about 6 months when stored in -80 ºC.

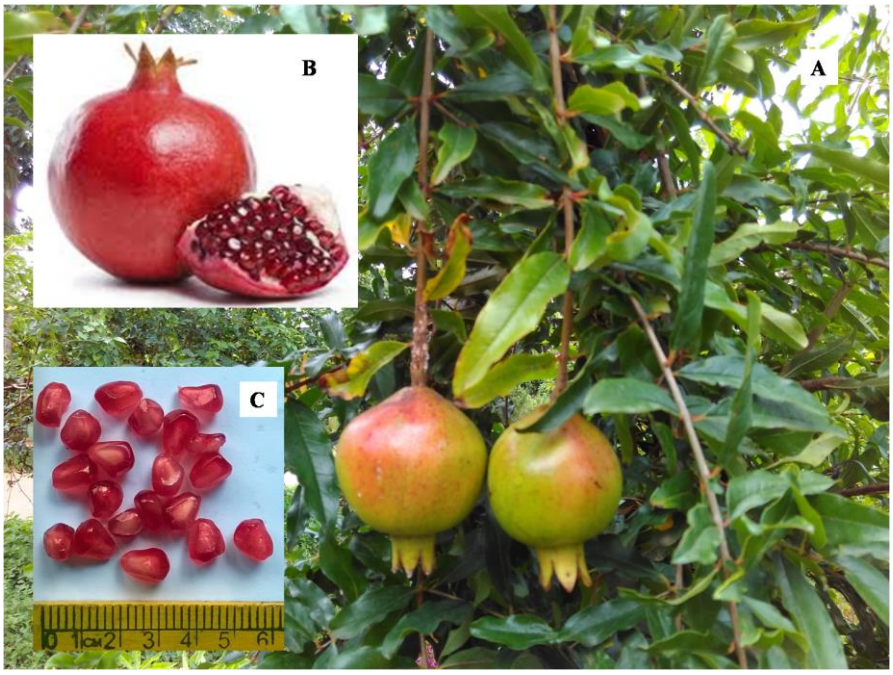

Figure 5.1: Pomegranate fruit and arils A. Unripen pomegranate fruits in the tree; B. Ripened pomegranate fruit and a portion of rind with arils; C. Arils of pomegranate fruit

5.3.1 Qualitative phytochemical analysis of PJ

Qualitative phytochemical analysis of PJ indicated the presence and absence of specific phytochemicals as summarized in table 5.1.

Table 5.1: Qualitative phytochemical analysis of PJ

Presence (+)	Absence (-)
Carbohydrates	Alkaloids
Fixed oils	Gums & mucilage
Flavonoids	Saponins
Glycosides	
Phenolic compounds & tannins	
Phyto-sterols	
Proteins & amino acids	
Resins	

5.3.2 Quantitative phytochemical analysis

The results of quantitative phytochemical analysis of PJ are summarized in table 5.2.

Table 5.2: Quantitative phytochemical analysis and iron estimation in PJ

S. No	Physico-chemical	Concentration
1	TDS	140 ± 3.4 g/l
2	TSS	13.96 – 15.08 ºBrix
3	Total phenolics	1.48 ± 0.008 g/l
4	Total organic acids	3.0 - 3.4 % w/w
5	Ascorbic acid (Vitamin C)	10.12 – 13.8 mg/100 ml
6	Total iron	0.7 – 0.9 mg/100 g

Literature indicates that, TSS in PJ ranges from 13.68 – 15.18 ºBrix (Al Said et al., 2009). Fructose and glucose are the major sugars present in PJ, while a minor quantity of sucrose is also reported (Ozgen et al., 2008).

5.3.2.1 Total Phenolics in PJ

Total phenolics in PJ was estimated by Folin-Ciocalteu reagent based method with known concentration of gallic acid as standard (figure 5.2). It was found to be 1.48 ± 0.008 g/l (gallic acid equivalent) (table 5.2). Gadže et al., (2012) found a variation from 0.777 g/l to 1.447 g/l, in the phenolic content in different samples of pomegranate.

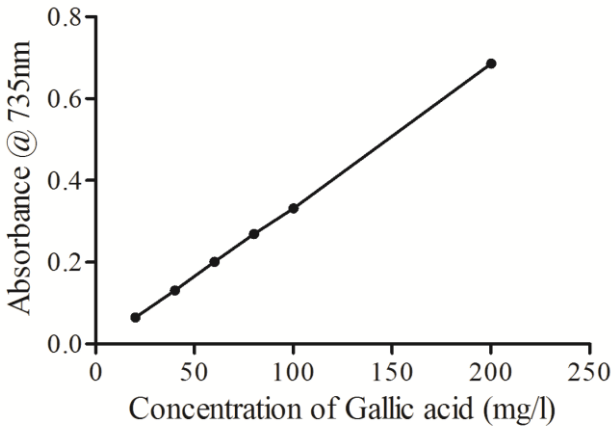

Figure 5.2: Calibration curve for estimation of total phenolics in PJ

PJ has hydrolysable tannins like ellagitanins and gallotannins. Gallic acid and ellagic acid are the major tannins present in PJ. Flavones, flavanols, anthocyanidins and flavan-3-ols are the common flavonoids of *P. granatum.* Major flavonoids present in the juice are catechin, catechol, cyanidin-3-glucoside, cyanidin-3,5-diglucoside, cyanidin-3-rutinoside, delphinidin-3-glucoside, delphinidin 3, 5-diglucoside,

pelargonidin 3-glucoside, pelargonidin 3,5-diglucoside, epicatechin, epigallocatechin-o-gallate, flavan-3-ol, quercitin and epiquercitin (Wang et al., 2010).

5.3.2.2 Total organic acids in PJ

Pomegranate juice mainly contains organic acids like citric acid and malic acid, tartaric acid and oxalic acid (Rahimi et al., 2012). It has triterpenes like ursolic acid, oleolinic acid, punicanolic acid and steroids like stigmasterol, camesterol and beta-sitosterol (Wang et al., 2010). Polyphenols such as gallic acid, ellagitannins, gallotannins, chlorogenic acid, caffeic acid, ferulic acid, coumaric acid, and catechin and anthocyanins are attributed to be responsible factors for antioxidant activities (Tehranifer et al., 2010). In the present study, total organic acid content of the different PJ accessions was found to be in the range of 3.0 - 3.4 % w/w (table 5.2). This value was obtained by titration with standardized NaOH (figure 5.3).

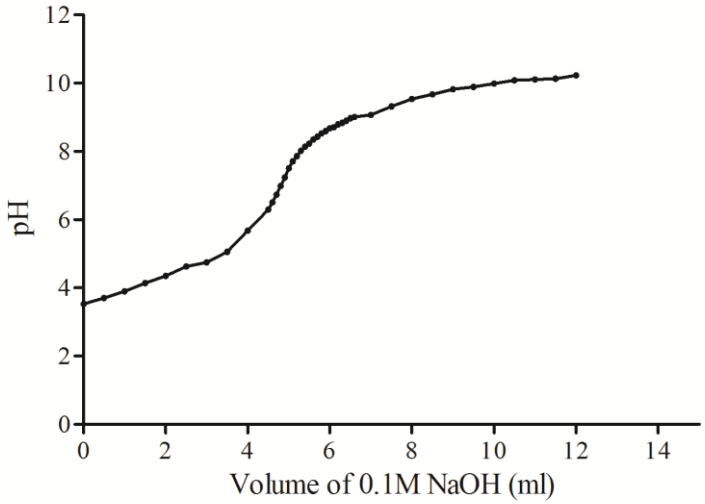

Figure 5.3: Volumetric analysis for estimation of total acids in PJ

5.3.2.3 Ascorbic acid content of PJ

In the present experimental conditions for HPLC based estimation of ascorbic acid, the standard L- ascorbic acid showed the retention time at 5.5 minutes. On overlaying PJ used in the study also had a peak at the same retention time (figure 5.4), indicating the presence of ascorbic acid. The calibration curve prepared with different known concentrations of ascorbic acid gave R^2 value of 0.99 and calibration equation of y=5E+07x+156 (figure 5.5). Amount of ascorbic acid in PJ was estimated to be in the range of 10.12 to13.8 mg/100 ml (table 5.2). Earlier reports have shown that the AA content of PJ can vary from 4.21 – 19.8 mg/100 ml (Akbarpour et al., 2009; Paul and Ghosh, 2012).

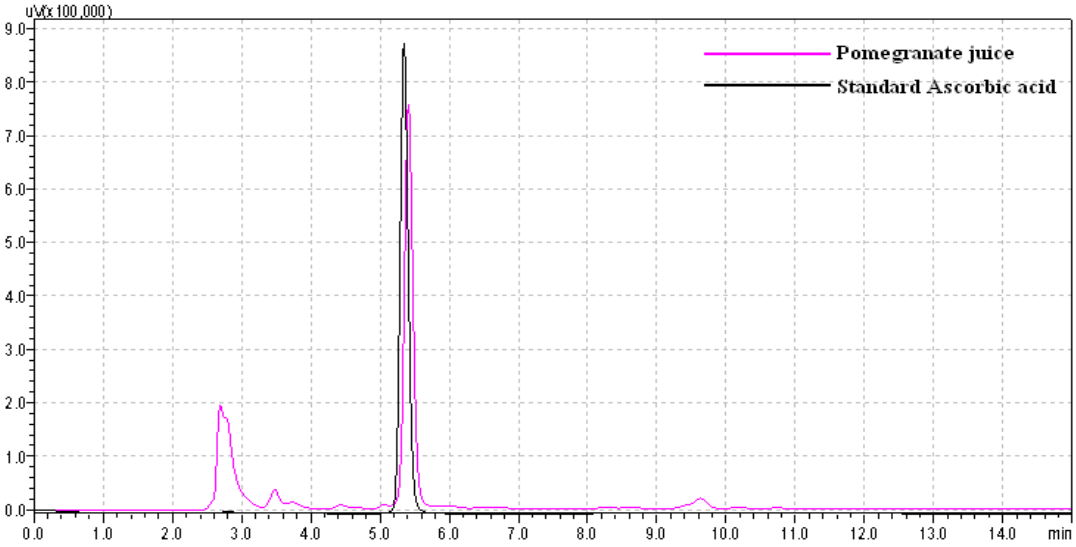

Figure 5.4: HPLC chromatogram of standard Ascorbic acid and Pomegranate juice under UV 245nm.

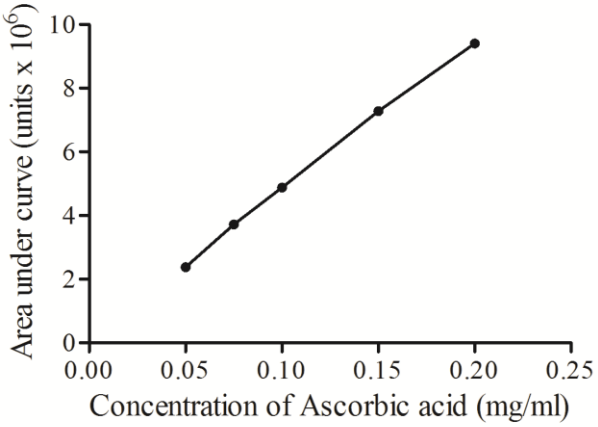

Figure 5.5: Calibration curve for estimation of ascorbic acid in PJ

5.3.2.4 Iron content of PJ

The calibration curve prepared with known concentrations on iron gave R^2 value 0.999 and the calibration equation was Y=0.043x+0.003 (figure 5.6). The iron content of PJ was estimated to be in the range of 0.7 – 0.9 mg/100g (table 5.2). Morton (1987) has reported that pomegranate has iron content in the range of 0.3 – 1.2mg/100g. Sodium, potassium, calcium, magnesium, copper, zinc and manganese are also present in pomegranate (Elfalleh et al., 2011).

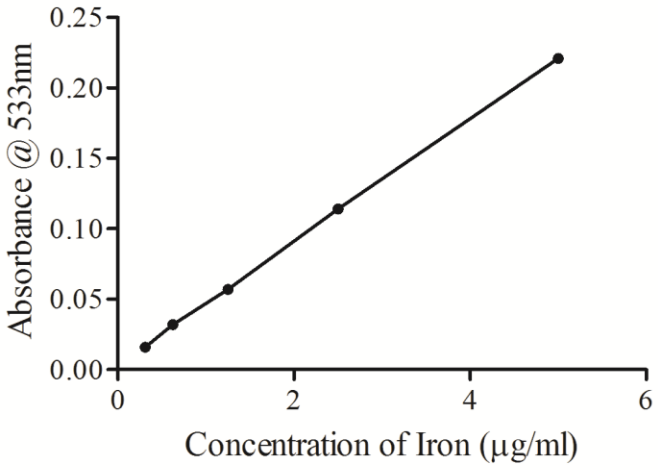

Figure 5.6: Calibration curve for estimation of total iron in PJ

5.4 Conclusion

The observations indicate that PJ has phenolics as major bioactive molecules. Organic acids including ascorbic acid is also present in PJ. Organic acids play a role in improving the digestion and bioavailability of nutrients. While the secondary metabolites are innate products, iron content of pomegranate is dependent on the soil it grows. All the phytochemicals tested and iron content might be responsible for the health benefits imparted by PJ. The ascorbic acid content of the different batches of the PJ was used as a standard for quantitative expression of PJ used in bioactivity experiments.

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Chapter 6

POMEGRANATE JUICE INCREASES IRON BIOAVAILABILITY AND UPTAKE IN *IN VITRO* MODELS

6.1 Introduction

Ayurveda literature indicates that, '*Dadima*' (pomegranate) has *Panduhara* (anti-anemic) property (Dash, 1991; Chunekar, 2004). '*Deepana*' or improving the digestive potential is also one of the *karma's* (functions) of pomegranate (Dash, 1991). Based on these Ayurvedic indications, it was hypothesized that, pomegranate can improve digestion, enhance iron bioavailability and uptake.

Phenolics, sugars and organic acids are the major class of compounds reported from pomegranate juice (Wang et al., 2010). While, ascorbic acid and sugars have ability to enhance iron bioavailability (Teucher et al., 2004; Christides and Sharp, 2013), plant polyphenolics have been shown to have both enhancing and inhibitory effect (Ma et al., 2011; Hart et al., 2015). Polyphenols like epicatechin, gallic acid and catechin have ability to enhance iron absorption while tannins and phytates inhibit (Hart et al., 2015).

Testing for iron bioavailability in humans is expensive and resource consuming, so several *in vitro, ex vivo* and *in vivo* models have been developed to study iron bioavailability (Garcia and Diaz-Castro, 2013). Animal models such as rats offer a useful alternative, but their response may differ from that of humans (Reddy and Cook, 1991). Alternatively, *in vitro* methods provide an attractive, rapid and low cost option for initial screening of iron bioavailability. A combination of *in vitro* digestion and iron uptake by enterocyte (Caco-2) or hepatocyte (HepG2) is considered to be a

unique approach to study digestion and absorption in human intestine (Miller et al., 1981; Yun et al., 2004; Christides and Sharp, 2013).

The cell-free *in vitro* dialysis method described by Miller et al., (1981) is a two step process. In first step, gastric digestion of the test food with iron is performed with pepsin and HCl at physiological temperature (37ºC) and with pH2. The second step simulates intestinal phase, where digestion in physiological temperature is performed with pancreatin and bile salts at pH7. The digestate is then passed thru a dialysis membrane and the dialysate is then tested for iron dialysability. To further study the effect of test food on iron uptake, the dialysate is then passed thru cell lines to study iron uptake. Several studies have used cultured Caco-2 cells as a surrogate for enterocytes of the small intestine or HepG2 hepatoma cells to study iron uptake (García and Díaz-Castro, 2013). Quantification of iron and ferritin, the storage form iron can be performed to understand the role of bioavailability enhancer.

Raisins (Yeung et al., 2003), organic acids (Teucher et al., 2004), *Phyllanthus emblica* (amla, Indian gooseberry) (Venkatasubramanian et al., 2014), Dang-Gui-Bu-Xue-Tang, a Chinese herbal decoction made using *Angelicae sinensis radix* and *Astragali radix* (Huang et al., 2016) are some of the iron bioavailability enhancers identified using the *in vitro* methods. The aim of this study was to understand the role of pomegranate juice in increasing the bioavailability, uptake and assimilation of iron using *in vitro* models.

6.2 Materials and Methods

Pomegranate juice (PJ) preparation and quantitative estimation of ascorbic acid in PJ was performed as described in the previous chapter (Chapter 5). The protocol followed for *in vitro* digestion, uptake and assimilation of iron in the presence of PJ is shown in figure 6.1.

6.2.1 Chemicals

Ascorbic acid (AA) was purchased from HiMedia (Mumbai, India); trypan blue, sulphorhodomine B (SRB), digestive enzymes porcine pepsin (800-2,500 units/mg protein), pancreatin (activity equivalent to 4 x USP specifications), bathophenanthroline di-sulfonic acid (BPS), epidermal growth factor (EGF), triiodothyronine (T3), sodium selenite, insulin, hydrocortisone, PIPES, trichloroacetic acid, chelex-100, phosphate buffered saline (PBS), 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p`-disulfonic acid (Ferrozine), Bradford reagent were purchased from Sigma Chemicals (St. Louis, Mo., U.S.A.); bile salt (sodium tauro-glycocholate) was purchased from Loba Chemie (Mumbai, India); sulphanilamide and N-(1-naphthyl) ethylenediamine di-HCl were purchased from Spectrochem (Mumbai, India); hydroxylamine hydrochloride and sodium hydrogen carbonate were purchased from Fisher Scientific, Mumbai, India. Minimum essential medium (MEM), 0.25% trypsin-EDTA, Pen-Strep and foetal bovine serum (FBS) were purchased from GIBCO, Auckland, New Zealand. All other chemicals, reagents and solvents were of analytical grade and purchased locally. Water used in the preparation of reagents was double deionized.

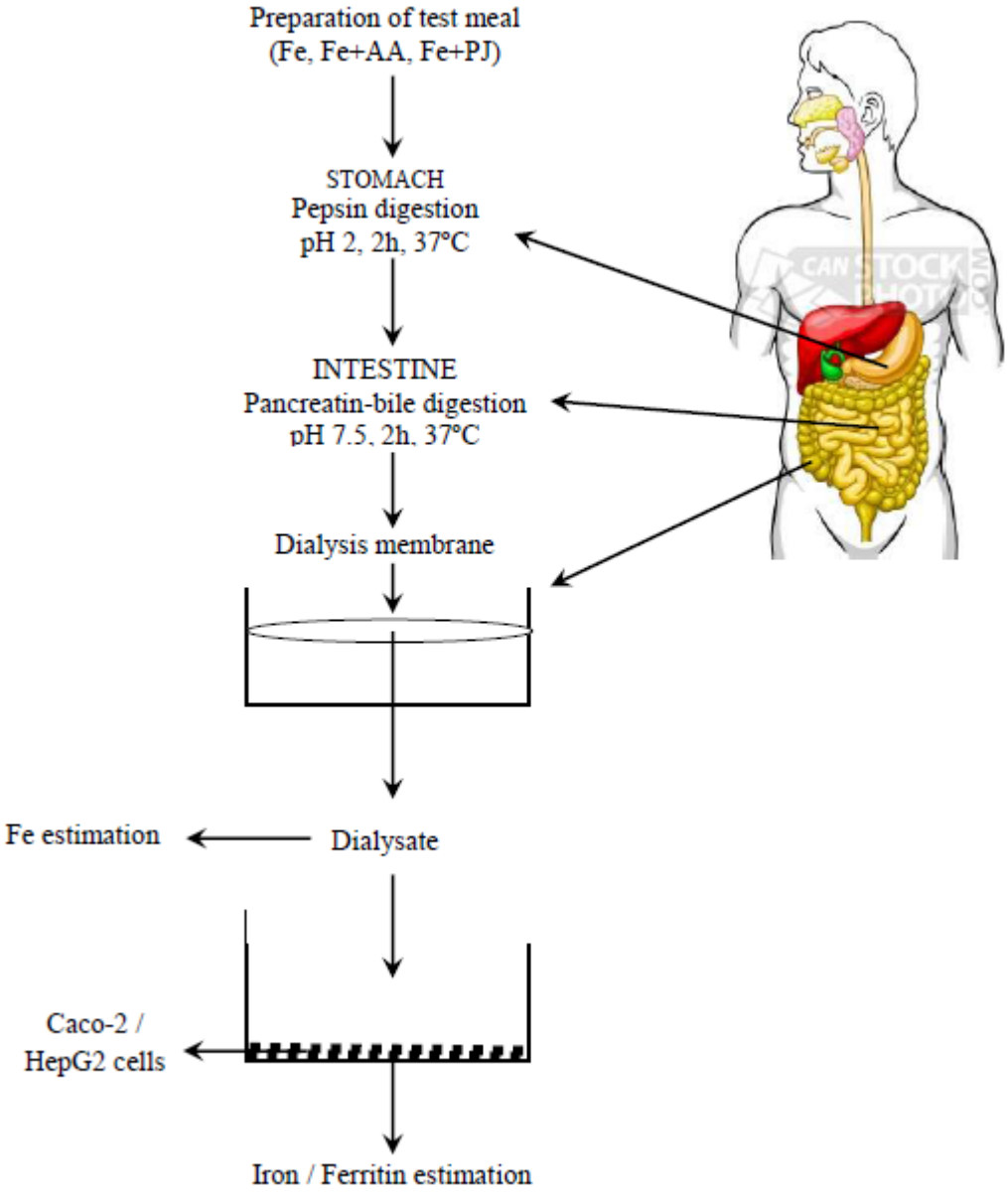

Figure 6.1: Flowchart showing protocol followed for *in vitro* digestion, uptake and assimilation of iron in the presence of PJ.

6.2.2 Iron stock

A 5 mg/ml stock solution of $FeSO_4.7H_2O$ (with 2 mg/ml Fe equivalent) was prepared in 0.01 N HCl and used as the iron source.

6.2.3 Test and control groups

Blank contained only 0.01N HCl; Fe control group contained $FeSO_4$ (equivalent to 2 mg Fe); AA control group contained AA equivalent of PJ dose. Dose response of PJ was done with different molar ratios of PJ (represented as AA equivalent) with 2 mg Fe. Fe+AA mixture contained 2 mg Fe and AA equivalent of PJ. Fe+PJ mixture contained 2 mg Fe and PJ. AA or PJ in the test mixtures was added immediately prior to the start of digestion to avoid oxidation due to subsequent processing. The blank, controls and test samples were prepared in 50 ml screw cap Falcon tubes so that each weighed 20 g in 0.01N HCl.

6.2.4 Cell-free *in vitro* digestion

In vitro digestion was performed according to the cell-free model described by Miller et al. (1981) with modification as described by Venkatasubramanian et al., (2014) to suit 6-well plates. Dose response of PJ was performed with different molar ratios (1:0 to 1:4.5) of $FeSO_4$: PJ (Fe equivalent: AA equivalent). On identifying the optimum molar ratio which showed maximum iron bioavailability, the effect of PJ on dialyzable iron was compared with the PJ equivalent AA.

6.2.4.1 Pepsin digestion

The pH of each of the mixtures was adjusted to 2.0 using 6M HCl / 1M $NaHCO_3$ (Sodium bicarbonate) and was incubated in shaking water-bath at 37℃ for 10 min. The digestive enzymes were prepared fresh before use. Pepsin solution, 0.64 ml (1.60 g of pepsin made up to 10 ml with 0.1N HCI) was added to each of the samples and was incubated in shaking water bath for 2 hrs at 37℃ with 200 rpm.

6.2.4.2 Intestinal digestion

Pepsin digests of the different samples obtained from the above procedure were divided into three aliquots of each 2 g. To the first aliquot of pepsin digest taken in 15 ml screw cap Falcon tubes, 0.5 ml pancreatin-bile salt mixture (4 g of pancreatin and 25 g bile suspended in 1 l of 0.1M $NaHCO_3$) was added and titrated against 1M $NaHCO_3$ till pH increased to 7.5. The volume of titratable 1M $NaHCO_3$ was recorded. The other two aliquots were used as duplicates. Dialysis tubing, 5 cm long (nominal MW cut-off 6,000 – 8,000 Da, Fisher Scientific, Pittsburgh, PA) pre-soaked in distilled water was cut open from one side. The opened dialysis membrane was fixed to the base of glass inserts using latex elastic system and placed back in distilled water.

6.2.4.3 Dialysis

Dialysis was carried out in 6-well plate (Becton Dickinson, USA). Volume of 1 M $NaHCO_3$ equivalent to that of titratable acidity was added to each well and made up to 2.5 ml with distilled water. The pepsin digest (2 gm) obtained from the above step

was taken on the upper chamber of the insert and was placed on the well with the membrane just immersed in the 1M $NaHCO_3$. The plates were then incubated on shaking water bath at 37℃ for 30 min. When the pH of the digest increased to 5, pancreatin-bile salt mixture (0.5 ml) was added to the samples and the incubation was continued for 2 h in shaking water bath at 37℃. At the end of this incubation period, the insert with the digest was removed. The dialysate was collected from the well and made up to 2.5 ml with distilled water and stored at -80℃ until further use.

6.2.4.4 Iron estimation

The colorimetric method described by Kapsokefalou and Miller (1991) using ferrozine was adopted for estimation of iron content in the dialysate. Briefly, 250 µl of the dialysate was aliquoted and made up to 1 ml with distilled water. About 800 µl of the supernatant was collected after a brief centrifugation. About 100 µl of reducing solution (5% hydroxylamine hydrochloride in 10% HCl) was added to the collected supernatant and allowed to stand at room temperature for 1 hour. Finally, 100 µl ferrozine solution (5 mg/ml) was added and dispensed into 96-well plate. The absorbance was read at 562 nm after 15 minutes in a microplate spectrophotometer (Biorad, USA). A standard graph prepared with known concentrations of iron standard (Fluka Analytical, Buchs, Switzerland) was used to estimate the iron concentration in the dialysates. Total dialyzable iron was calculated using the following formula:

$$\text{Total dialyzable Iron (\%)} = \frac{(\text{Fe in dialyzate µg/g} \times \text{Total dialyzate g})}{\text{Test mixture µg/g} \times 2\text{ g}} \times 100$$

6.2.5 Iron uptake studies in cell-based models

The Caco-2 (human colorectal adenocarcinoma cell line) and HepG2 (human liver hepatocellular carcinoma cell line) were obtained from NCCS, Pune, India. Both the cell lines were maintained in MEM with 10% FBS and 1% antibiotic-antimycotic solution at 37°C in 5% CO_2. The test (Fe + PJ) and control dialysates (Fe/ AA/ PJ alone and Blank) were used to study the iron uptake in Caco-2 and HepG2 cells. The protocol described by Glahn et al., (1998) with modifications as described by Venkatasubramanian et al., (2014) was followed for iron uptake studies.

For iron uptake experiments, Caco-2 (passages 35–45) and HepG2 (passages 22–33) cells were seeded in 6-well plates at a density of about 1.0×10^5 and 6.0×10^4 per well respectively. The medium was changed every two days. After 5 days, the medium was removed and constituted with MEM medium containing growth factors (10 mmol/l PIPES, 4mg/l hydrocortisone, 5mg/l insulin, 5µg/l sodium selenite, 34µg/l T3, 20µg/l EGF) and incubated further for 10 days. After the cells reached 90–100% confluency, the cell layers were used for iron uptake experiments. The medium was removed and cells were washed twice with PBS. MEM medium containing growth factors (1 ml) and 1 ml of the filter sterilized (0.2 µm; Pall life sciences, NY, USA) control or test dialysates were added directly to individual wells containing cells. The cells were then incubated at 37°C under 5% CO_2 - 95% atmospheric O_2 for 18-20 hours.

Subsequent to incubation, the medium was removed from the wells and cells were washed with ice cold saline (0.9% NaCl). Cells were then washed three times with 500 µl volumes of stop solution (140 mmol/l NaCl and 10 mmol PIPES – pH 6.7, 4°C).

This was followed by washing with 500 µl of removal solution (140 mmol/l NaCl, 10 mmol/l PIPES, 5 mmol/l bathophenanthroline disulfonic acid - pH 6.7). Cells were then solubilized in 0.5 mol/l NaOH (500 µl per each well) and scrapped. The scrapped cell lysate was collected in 2 ml microcentrifuge tube. Total proteins in the cell lysate were estimated by Bradford assay (Bradford, 1976).

6.2.5.1 Iron estimation

Iron was estimated in cell lysate using ferrozine as described by Fish (1988). Briefly, 100 µl cell lysate was mixed with 100 µl distilled water and 100 µl iron-releasing agent (1.4 M HCl and 4.5% w/v $KMnO_4$ in water and freshly mixed in 1:1 ratio). The mixture was then incubated at 60^{o}C for 2 h. It was then cooled to room temperature and 30 µl iron detection reagent (6.5 mM ferrozine, 2.5 mM ammonium acetate and 1 M ascorbic acid in water) was added. Absorbance was measured at 550 nm after 30 minutes' incubation at room temperature. Iron uptake by the cells was expressed as µg iron/mg total protein.

6.2.5.2 Estimation of ferritin in cell lysate

The amount of ferritin in cell lysate was estimated by Spectroferritin kit (Ramco laboratories, TX, USA) following kit protocol. Briefly, 10 µl of cell lysate taken in anti-ferrritin antibody coated micro wells and is mixed with 200 µl of conjugated anti-human ferritin and incubated on a shaker, set at 180-200 rpm, for 2 hours at room temperature. After incubation, the wells were washed with deionized water and shaken before decanting. After washing 200 µl of the substrate solution was added to each well and incubated for 30 minutes at room temperature. The color was developed by adding 100ul of the 0.24% potassium ferricyanide to each micro well and the OD

was measured at 500 nm in a microplate reader (Biorad, USA). Amount of ferritin was calculated using a standard graph prepared with known concentrations of ferritin and expressed in ng/ml.

6.2.6 Statistical analysis

Students't test was used to compare the two means. P valve <0.05 was considered to be statistically significant.

6.3 Results

Ascorbic acid (AA) concentration of the PJ used in this study was found to be 11.96 ± 1.84 mg/100ml (details in the previous chapter, section 5.3.2.3). Quantity of PJ used for the *in vitro* experiments was calculated in terms of AA equivalent.

6.3.1 PJ enhances iron dialysability in cell free model

PJ enhanced iron dialysability in a concentration dependent manner when added in the molar ratio from 1:0.001 until 1:0.45 of Fe: PJ (AA equivalent). In the present experimental set up, Fe bioavailability was 4%, this was enhanced to 13%, ie., 3.25 fold when the Fe: PJ (AA equivalent) was added in the ratio of 1: 0.45 (figure 6.2).

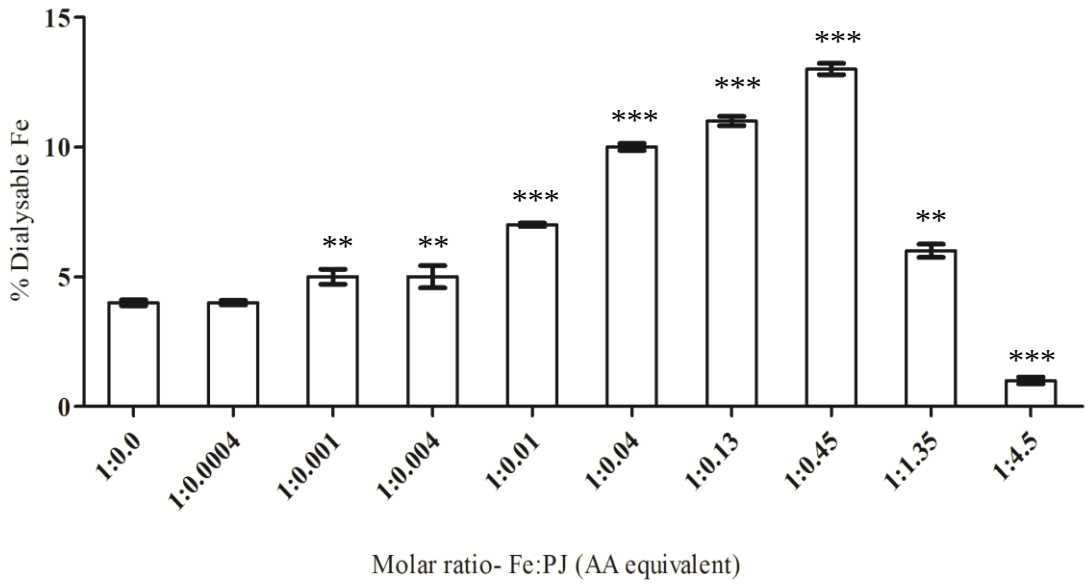

Figure 6.2: Dose response of iron (Fe) dialysability to PJ in cell free model. Fe: PJ (AA equivalent) in the molar ratio 1:0.45 showed the maximum of 3.25-fold enhancement in iron dialysability ($p<0.0005$). Error bars represent standard deviation of mean values of the respective groups. (** $p<0.005$; ***$p<0.0005$).

At lower molar ratio (1: 0.0004) it did not have any effect, while at higher ratios tested (1: 1.35 and 1: 4.5) PJ showed an inhibitory effect (figure 6.2). Thus 1: 0.45 (Fe: PJ, AA equivalent) was selected as the optimum dose for studying the effect of PJ on iron bioavailability. When, the same molar ratio of AA was tested, it was able to enhance the iron dialysability only by 1.6 fold (figure 6.3), which is 50% lesser than its PJ equivalent. Even though PJ used in the current study had iron content of about 0.9 mg/100 ml, it could not be detected in the dialysate.

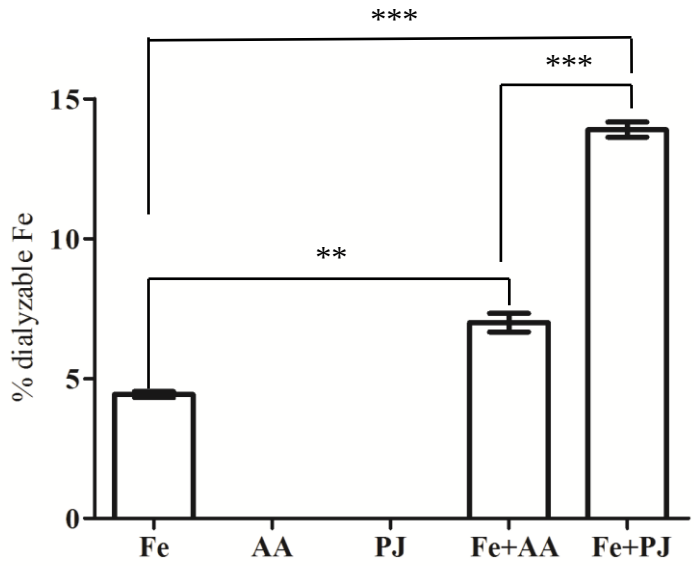

Figure 6.3: PJ improves iron bioavailability in cell free *in vitro* model. Dialysable iron in the presence of Fe: PJ (1 molar Fe: 0.45 molar AA equivalent PJ) was found to be significantly enhanced when compared with the same molar ratio of Fe: AA. Error bars represent standard deviation of mean values of the respective groups. (** $p<0.005$; ***$p<0.0005$).

6.3.2 PJ improves iron uptake and assimilation by Caco-2 cells

A dose response study was performed to identify the concentration of PJ which can enhance maximum iron uptake in Caco-2 cells. Molar ratio of Fe: PJ (AA equivalent) from 1:0 to 1:4.5 was tested for this purpose. At 1: 1.35 molar ratio Fe: PJ showed a significant ($p<0.0005$) enhancement in iron uptake when compared with only Fe group (figure 6.4).

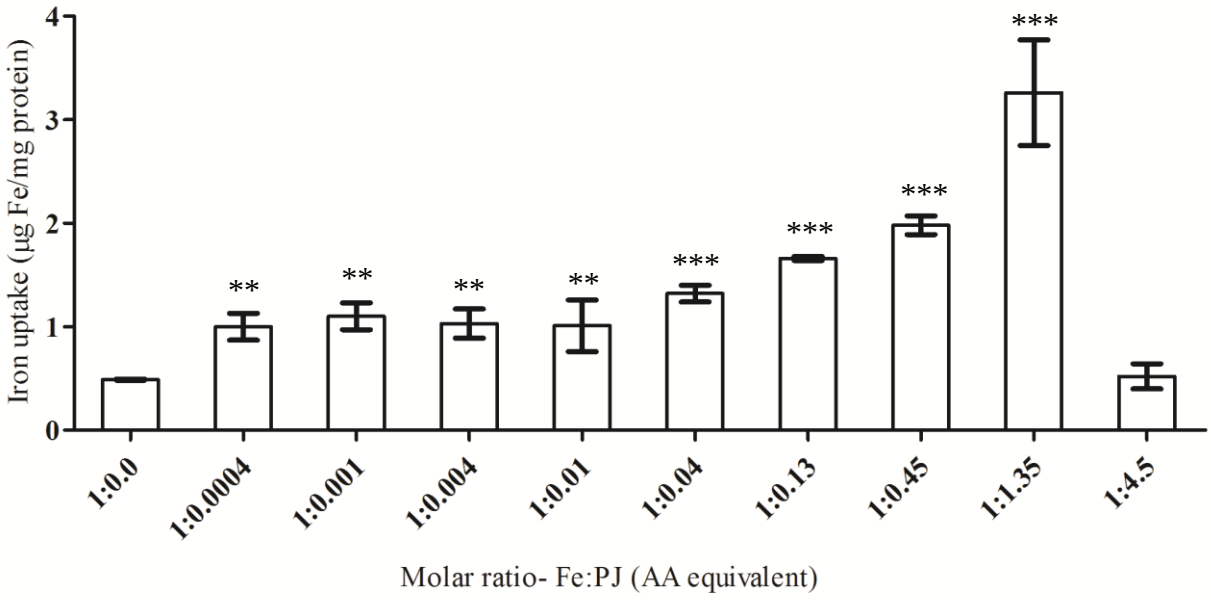

Figure 6.4: Dose response of Fe uptake to PJ in Caco-2 cells. The experimental group with 1:1.35 molar ratio showed maximum iron uptake ($p<0.0005$). Error bars represent standard deviation of mean values of the respective groups. (** $p<0.005$; ***$p<0.0005$).

When compared with the same molar ratio of Fe: AA, PJ had a six-fold increase ($p<0.0005$) in iron uptake by Caco-2 cells (figure 6.5). Further, at this concentration AA showed a mild (20%) reduction in the iron content of Caco-2 cells, it was a significant reduction ($p<0.05$) when compared to only Fe group (Figure 5.5). The increase in the iron uptake was reflecting in the iron assimilation as well, in terms of ferritin, storage iron content. Fe: PJ (AA equivalent) had a significantly ($p<0.0005$) higher ferritin content then the Fe and Fe: AA groups (figure 6.6). Amount of ferritin in the Fe: AA group was 30% lesser then the AA equivalent PJ group.

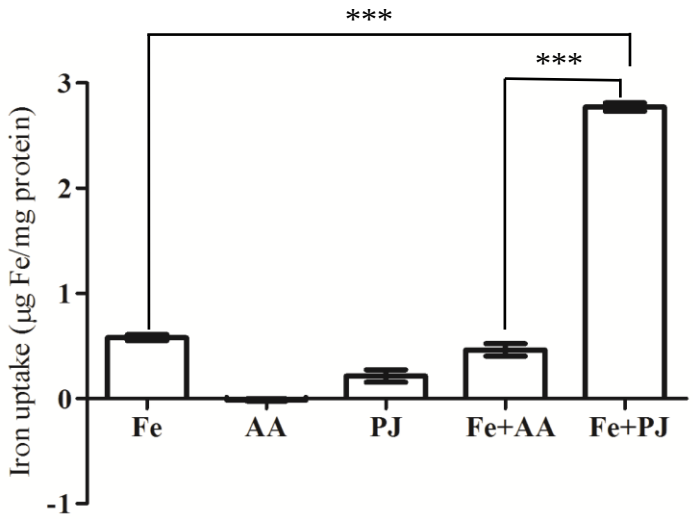

Figure 6.5: PJ improves iron uptake by Caco-2 cells. Iron uptake by Caco-2 cells in the presence of Fe: PJ (AA equivalent) in the molar ratio 1:1.35 was found to be significantly ($p<0.0005$) enhanced when compared to only Fe and Fe: AA groups. Error bars represent standard deviation of mean values of the respective groups. (***$p<0.0005$).

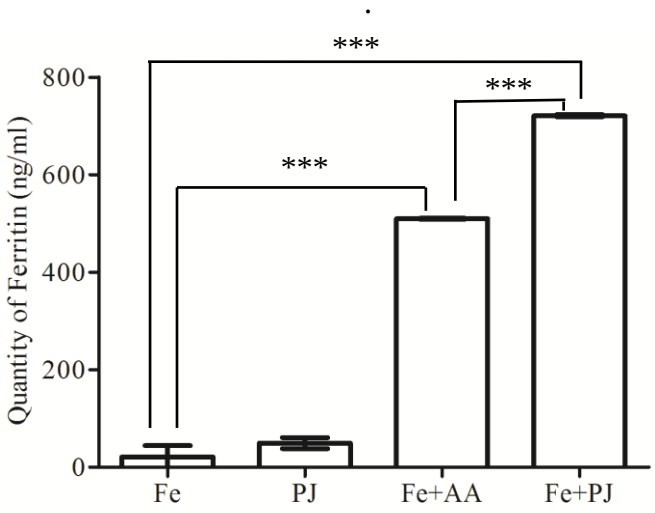

Figure 6.6: PJ improves ferritin content in Caco-2 cells. At the molar ratio 1:1.35 (Fe: PJ in terms of AA equivalent) Caco-2 cells had significantly ($p<0.0005$) higher quantity of ferritin. Fe: AA in the same molar ratio also had a significant ($p<0.0005$) increase in ferritin content, but it was 30% lesser than the Fe: PJ group. Error bars represent standard deviation of mean values of the respective groups. (***$p<0.0005$).

6.3.3 PJ enhances iron uptake and assimilation in HepG2 cells

A dose response study performed with different molar ratios of Fe: PJ (AA equivalent) from 1:0 to 1:4.5 to identify the concentration of PJ at which maximum iron uptake occurs in HepG2 cells. At 1: 1.35 molar ratio Fe: PJ showed a significant ($p<0.0005$) enhancement in iron uptake when compared with only Fe (figure 6.7).

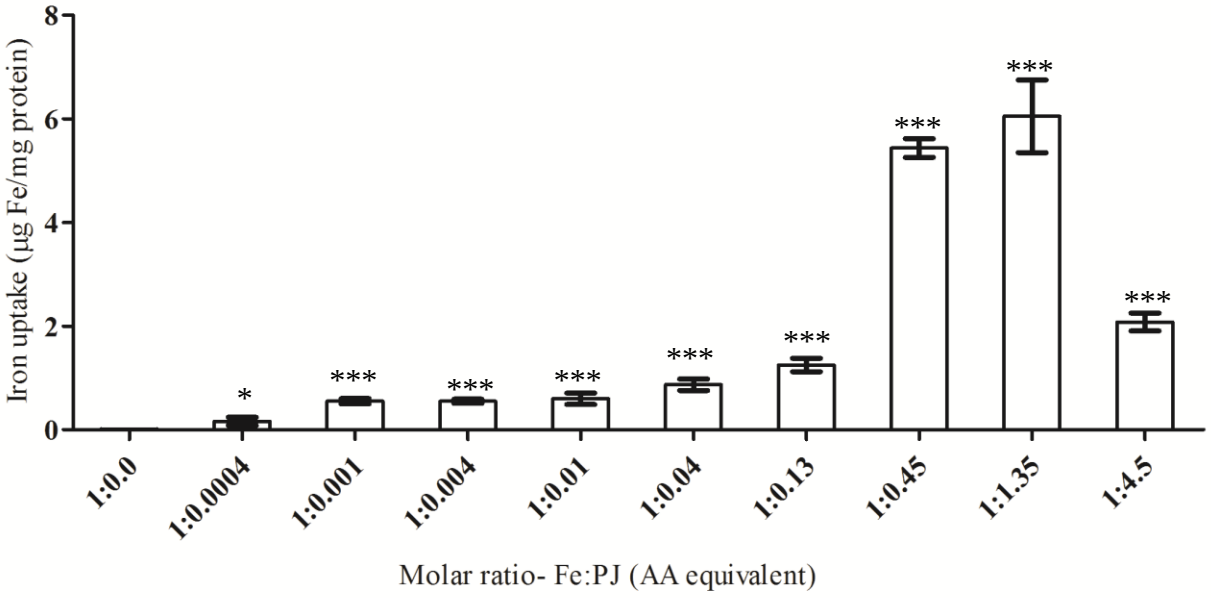

Figure 6.7: Dose response of Fe uptake to PJ in HepG2 cells. The experimental group with 1:1.35 molar ratio showed maximum iron uptake ($p<0.0005$). Error bars represent standard deviation of mean values of the respective groups. (* $p<0.05$; ***$p<0.0005$).

At the same molar ratio, AA showed 60% lesser iron uptake than the AA equivalent PJ (figure 6.8). Further, at this concentration only AA showed a reduction in the iron content of HepG2 cells, which was similar to that of the observation with Caco-2 cells. The increase in the iron uptake was reflecting in the ferritin content as well. Fe: PJ (AA equivalent) had a significantly ($p<0.0005$) higher ferritin content then the Fe and Fe: AA groups (figure 6.9). Amount of ferritin in the Fe: PJ group was twice that of the Fe: AA group.

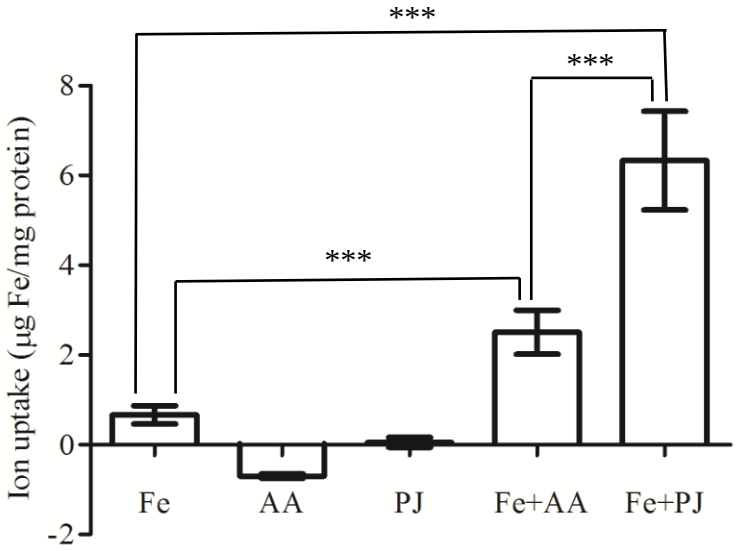

Figure 6.8. PJ improves iron uptake by HepG2 cells. Fe: PJ (AA equivalent) in the molar ratio 1:1.35 was found to significantly ($p<0.0005$) enhance iron uptake by HepG2 cells *in vitro*. AA in the same molar ratio also enhanced iron uptake ($p<0.0005$) but it was 60% lesser in Fe: PJ group. Error bars represent standard deviation of mean values of the respective groups. (***$p<0.0005$).

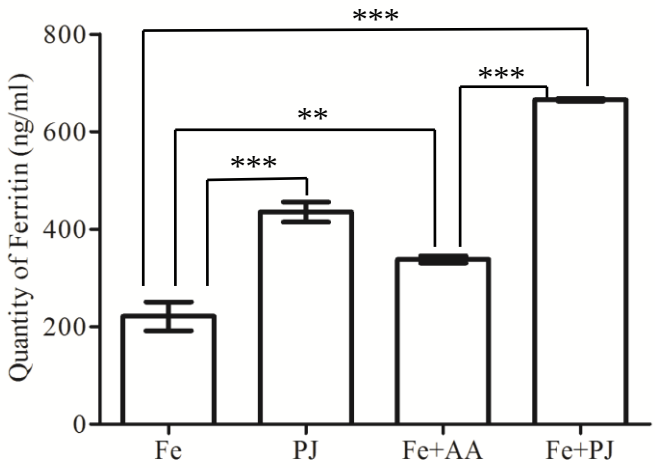

Figure 6.9. PJ improves ferritin content in HepG2 cells. At the molar ratio 1:1.35 (Fe: PJ in terms of AA equivalent) ferritin content was significantly ($p<0.0005$) higher when compared only with Fe group and Fe: AA groups. Just addition of PJ was also found to significantly ($p<0.0005$) enhance ferritin production in the HepG2 cells. Error bars represent standard deviation of mean values of the respective groups. (**$p<0.005$; ***$p<0.0005$).

6.4 Discussion

In vitro methods can be simple, rapid, low in cost, easy, fast, straightforward and may provide insights which not achievable in whole animal studies (Miller et al., 1981). Besides that, it reduces the usage of experimental animals and easier to investigate a large number of samples and allowing replication. Most of the *in vitro* digestion models describe a two-step (stomach and small intestine) or three-step procedure (mouth, stomach, small intestine) (Omar et al., 2013). Simulation of large intestine is not mandatory because human food digestion and absorption mainly takes place in the small intestine (Oomen et al., 2003).

Scientific understanding of traditional medical systems can impart us different perspective into disease diagnosis, etiology and management. Ayurveda considers improper digestion as the major cause for any disease including *Pandu* (~IDA). This may be due to derangement in the digestive and absorptive 'fire' (*jataragni*) in the body due to which the nutrients are not absorbed. *Jataragni,* in turn is balanced by *pachakapitta,* the *pitta* that drives metabolic processes in the proximal gastrointestinal tract. *Pitta* is one of the three humors that control all metabolic transformations in the body (Dwarakantha, 1996). Undigested or partially digested food by the deranged *agni* causes the production of *ama* (~undigested materials) that blocks natural assimilative and eliminative processes, which is considered to be the root cause of most diseases (Kumar et al., 2008).

Improper functioning of *agni* can also lead to insufficient absorption of essential nutrients from the food to the body (Dwarakantha, 1996). Correcting *agni* might be a strategy for management of diseases due to nutrition deficiency like IDA. Adapting

the human body to absorb and assimilate the required amount of iron from food itself would help in regaining the iron status. Current food practices have a larger proportion of inhibitors rather than facilitators of iron absorption (Nair and Iyengar, 2009). Based on the mechanisms of action of *Rasayanas* it can be understood that, they can help in normalizing *agni* and can improve the absorption and distribution of nutrients (Udupa, 2004).

Pomegranate is one of the *rasa* enhancing (improved quality dialysate) *Rasayanas*, which has been indicated for its *Dhatuvrddhikara* (improved tissue quality) property (Dash, 1991). According to Ayurvedic theory of tissue formation the dialysate or the *rasa* gets converted into *rasadhatu* and subsequently forms the seven tissues of the body, including the blood (*raktha dhatu*) (Dwarakantha, 1996). Probably this might be the reason behind the Ayurvedic recommendation for use of pomegranate in management of IDA. Several formulations containing pomegranate has been indicated for use in management of IDA in Ayurvedic texts (Chunekar, 2004).

Higher molar ratios of Fe: PJ (AA equivalent) like 1:1.35 and 1:1.45 used in this study were found to be reducing the iron dialysability. This might have been due to the increasing concentrations of polyphenols. Higher concentrations of polyphenols have been indicated to inhibit iron bioavailability in Caco-2 model (Hurrell et al., 1999) and as well as in humans (Ma et al., 2011).

Thus, PJ was found to enhance iron dialysability, uptake and assimilation by Caco-2 and HepG2 cells. Forbes et al., (1989) have shown that the results obtained through *in vitro* tests are comparable to the *in vivo* assays and clinical observations to determine iron bioavailability. From the cell free and cell based *in vitro* experiments it is evident

that PJ has ability to enhance iron dialysability, uptake and assimilation. This study provides an experimental proof for the '*dipana*' or digestion enhancing potential of pomegranate. Enhancement of digestion could be a resultant of 'agni' normalizing effect by pomegranate. The increase in the iron bioavailability in the presence of PJ is not just because of its AA content. Other organic acids, sugars and polyphenols present in PJ (Wang et al., 2010) might as well have a role in enhancement of iron bioavailability.

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Chapter 7

POMEGRANATE JUICE IMPROVES IRON STATUS AND AMELIORATES IRON DEFICIENCY INDUCED CELLULAR CHANGES IN *SACCHAROMYCES CEREVISIAE*

7.1 Introduction

Iron is an essential micronutrient involved in a variety of biological processes in cells and plays a role in oxygen transfer (Munoz et al., 2009). Iron deficiency occurs when there is insufficient intake, iron loss or the bio-available iron is inadequate to meet the body iron demands (Formanowicz and Formanowicz, 2011), resulting in low haem concentrations and lead to iron deficiency anemia (IDA) (Nagababu et al., 2008).

Interventions guided by traditional knowledge on dietary supplements to manage IDA may provide ecosystem specific natural materials that are cost-effective and culturally acceptable. Ayurvedic texts have indicated several herbs and fruits that can be used in the management of '*Pandu*', a disease condition correlated to IDA (Murthy 2009). Pomegranate is one of such prescriptions with 'wholesome' (*Pathya*) properties, abilities to induce tissue generation, development (*Dhatu vrddhikara*), strength promotion (*Balya*) and anemic conditions (Dash, 1991; Chunekar, 2004). Rasayanas are rejuvenating methods that improve digestion, metabolism and tissue perfusion of nutrients (Udupa, 2004; Balasubramani et al., 2011).

Iron is a transition metal and it exists in two readily reversible redox states, the reduced ferrous (Fe^{2+}) and oxidized ferric (Fe^{3+}) forms. At physiological oxygen concentrations, the stable state of iron in most of its biological complexes is the Fe^{3+} form (Lesuisse et al., 1987). Reduction reactions play a crucial role in iron metabolism, because only reduced iron can be transported across the membrane, load

and release iron from ferritin and contribute to heme synthesis (Lesuisse et al., 1987; Munoz et al., 2009).

Several iron bioavailability enhancers have been identified, which include ascorbates (Hallberg et al., 1986), citrates and folic acid (Hartman-Craven et al., 2009). While these are pure molecules, prescribed as iron-complexes in the form of tablets and consumed as drugs, researchers are also attempting to demonstrate the use of greens (Rao, 2014), herbs (Venkatasubramanian et al., 2014), fruits (Shah et al., 2003; Monárrez-Espinoet al., 2011; Nair et al., 2013), vegetables as food and beverage to support IDA management (Takatera et al., 2012). Several in vitro, in vivo and ex vivo models have been developed to study iron uptake, bioavailability and metabolism under laboratory conditions (Garcia and Diaz-Castro, 2013). Even though in vitro assays give considerable understanding on the iron uptake and bioavailability but studying the same at the organism level gives a holistic picture.

The current chapter describes the study conducted to understand the role of pomegranate juice in ameliorating iron deficiency induced cellular changes in *S. cerevisiae* model. The hypothesis was that pomegranate juice can improve the iron status and cellular physiology in experimentally generated anemic yeast cells.

7.2 Materials and Methods

7.2.1 *Saccharomyces cerevisiae* strain

Wild type *S. cerevisiae* strain (BY4742; Genotype: MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0) obtained as a kind gift from Prof. Mathew's lab, National Centre for Biological Sciences (NCBS), Bangalore was used for the study.

7.2.1.1 Generation of mitochondrial smGFP *S. cerevisiae* cells

Yeast expression vector (p426 GPD) with GFP linked to the mitochondrial localization sequence, isovaleryl-CoA-dehydrogenase (IVD) was obtained by sub-cloning the encoding cDNA (IVD-*GFP) from a plant expression vector (Forner and Binder, 2007). Full length IVD-GFP was cut by using Xba I and EcoRI enzymes and was cloned. The constructs were transformed to BY4742 yeast strain and the clones were selected for growth on a minimal media lacking Uracil. Soluble modified GFP (smGFP) positive cells were used for mitochondrial morphology assessment.

7.2.1.2 Growth Conditions

Cells were routinely grown in the synthetic defined (SD) medium that composed of yeast nitrogen base (YNB, 1.7 g/l; Himedia, Mumbai) and complete supplement mixture (CSM, 0.79 g/l; Himedia, Mumbai). All cells were grown by incubating at 28ºC in a shaking incubator (Remi, Mumbai) at 180 rpm for 12 to 14 h.

7.2.1.3 Induction of iron deficiency

Iron deficiency was induced by growing the *S. cerevisiae* cells in synthetic defined (SD) medium without iron (calling it D medium) and also by incorporating 100µm bathophenanthroline di-sulfonate (BPS; Sigma-Aldrich, MO, USA) (Minear et al., 2011; Jo et al., 2009).

7.2.2 Experimental outlay

The dose response with incorporation of pomegranate juice (5% to 50% v/v) in medium on the growth profile of cells in SD media was studied. A concentration of 10% PJ was selected for further experiments based on the growth curve, generation time and viable cell count in complete SD medium, iron-free medium and uracil-free medium.

A loop full of yeast cells from SD agar plate was inoculated in 100 ml each of SD medium containing normal (200 µg/l as $FeCl_3$) levels of iron (N) and iron deficient (D) medium (ie., SD medium without iron and containing 100µm BPS) in 250 ml conical flasks until the cells reached mid-log phase (A_{600} of 0.35).

In order to study the iron deficiency reversal potential of pomegranate, the ID cells were further grown in different media:

i. D medium: iron deficient SD medium to obtain IDD cells

ii. P medium: D medium supplemented with 10% PJ (90µg/100ml Fe content) to obtain IDP cells

iii. F medium: D medium with 90µg/100ml Fe (iron content equivalent of that in 10% PJ) as $FeCl_3$ to obtain IDF cells

iv. N medium: Normal SD medium (20µg/100ml Fe content) to obtain IDN cells

Cells were grown by incubating at 28ºC in a rotary shaking incubator at 180 rpm for 14 h, following which they were harvested and analyzed for various parameters.

7.2.2.1 Cell viability

Viable cells were counted as colony forming units (CFU) using spot plating method (Minear et al., 2011). Briefly, 10µl of each culture was aseptically diluted to obtain 10^{-2}, 10^{-4} and 10^{-6} dilutions. About 10µl of each dilution was spotted individually on SD agar plates with 4% agar in triplicates and kept upright until the inoculum got absorbed into the medium. The plates were incubated inverted at 28ºC for 48 h. Distinct individual colonies on each spot were counted and CFU/ml was calculated accounting for the dilution factor. Generation time / doubling time was calculated using online software tool 'Doubling Time' (http://www.doubling-time.com/compute.php) and was expressed in minutes.

7.2.2.2 Cell size

Method described by Tibayrenc et al., (2010) was used with modifications. Briefly, 10µl of the *S. cerevisiae* broth culture (14 h) was placed on a clean glass slide and focused under 100x of Olympus microscope (BX41, Tokyo) and image captured with Olympus digital camera (DP72, Tokyo) fitted to the microscope. The cell size was determined as the average diameter of 50 individual cells using calibrated Image Pro Express 6.0 software and represented as µm ± standard deviation.

7.2.2.3 Dry biomass

About 1 ml of broth culture containing 10^6 cells/ml was aliquoted into pre-weighed micro centrifuge tube, pelleted at 6000 rpm for 10 minutes and washed with sterile distilled water. After removing traces of the liquid, the pellet was dried at 60°C until constant weight. The difference in the pre and post weight of the tube was taken as the dry weight (mg) per 10^6 cells.

7.2.2.4 Total RNA and Protein

Total RNA and protein from yeast cells were extracted using TRI reagent (Sigma, MO) following manufacturer's instruction. Briefly, $5x10^6$cells were lysed with TRI reagent (1 ml) by repeated pipetting and vortexing followed by addition of chloroform (200 µl). After vigorous shaking and incubation at room temperature for 15 min, the samples were centrifuged at 10650 rpm for 15 min at 4°C (Eppendorf, Hamburg, Germany). The upper aqueous phase and lower organic phase were used to precipitate RNA and protein respectively with iso-propanol. The pellet containing total RNA was dried and suspended in DEPC (0.1% diethylpyrocarbonate) treated MilliQ water. Protein pellet was dissolved in 1% SDS (w/v) and centrifuged at 9700 rpm for 10 min at 4°C. The supernatant contained total proteins. The quantity of RNA and protein was estimated using nano-quantity spectrophotometer (Thermo Scientific, DE) at 260 and 280 nm respectively.

7.2.2.5 Lipid content

Lipid extraction was performed as per the method described by Folch et al., (1957) with some modification. Overnight cultured cells were vigorously vortexed with 20

volumes of chloroform: methanol (2:1) (100 mg in 2 ml of solvent mixture). The homogenate was filtered using Whatman filter paper no.1 to recover the liquid phase. The liquid collected was washed with 0.2 volume 0.9% (w/v) NaCl solution. After vortexing, the mixture was centrifuged at 2000 rpm for 4 minutes at room temperature to separate the two phases. The lower chloroform phase containing lipids was collected in a pre-weighed (A) micro-centrifuge tube. Chloroform was evaporated by keeping the tubes open in an incubator set at 65°C, until a constant dry weight (B) was obtained. The difference between B and A was considered as the quantity of total lipids and expressed as mg lipids per g dry weight of yeast cells.

7.2.2.6 Iron content

Iron content in the cells was estimated using colorimetric assay kit (Catalogue no: K390 – 100; Biovision, CA) following manufacturer's instruction. Cells were lysed with 4 volumes of assay buffer and centrifuged at 12300 rpm for 10 minutes to remove the debris. The supernatant was used for estimation of iron. About 25 µl of the supernatant was diluted to 100 µl in individual wells of a 96 well microtitre plate with assay buffer. Iron probe (100 µl) was added, mixed and incubated at 25 °C for 1 hour in darkness. Absorbance at 593 nm was measured in a microplate spectrophotometer (Biorad, USA) to quantify ferrous ions (Fe^{2+}). The same procedure was repeated after adding 5 µl of iron reducer to estimate the total iron content. The difference between the total iron and Fe^{2+} content was considered as the amount of Fe^{3+}. Iron content in the cells was estimated using a calibration curve with known concentrations of iron standard provided with the kit. All experiments were performed in triplicates with three different sets of samples.

7.2.2.7 Heme content

Heme content was estimated using Hemin assay kit (Catalogue No: MAK036; Sigma-Aldrich, MO) following manufacturer's instruction. About $2x10^6$ cells were vigorously vortexed in 4 volumes of cold hemin assay buffer and centrifuged at 11100 rpm for 10 minutes at 4°C. The supernatant containing heme was collected in a fresh tube and diluted 100 times before heme estimation. About 50 µl of the diluted sample was mixed with 50 µl of reaction mix containing peroxidase enzyme, hemin substrate, hemin assay buffer and hemin probe in a 96 well microtitre plate. A sample blank was also maintained without the enzyme mix. After 1 hour incubation at room temperature in dark, absorbance at 570 nm was measured using a microplate spectrophotometer (Biorad, USA). Heme content in the sample was calculated from a calibration curve prepared with known concentrations of hemin supplied with the kit. The experiment was performed in triplicates with two different sets of samples.

7.2.2.8 ATP content

Total ATP was extracted and estimated using Enliten bioluminescence ATP detection kit (Catalogue No: FF2000; Promega, WI). Cells were pelleted by centrifuging at 3000 rpm for 5 min at 4°C. Pellet was washed with sterile distilled water to remove media. Then the cells were suspended in 1ml of 5% w/v Trichloroacetic acid (TCA) prepared in citrate phosphate buffer (0.1M citric acid, 0.2M Na_2HPO_4; pH 6.0). The suspension was vortexed for 10 min and centrifuged at 3000 rpm for 4 minutes. The supernatant containing ATP was collected in a fresh 2ml tube and diluted 50 times using 1M tris-acetate buffer (pH 7.75), before estimation. About 10 µl of sample was mixed with 100 µl rL/L (rLuciferase/Luciferin) reagent in a white 96 well microtitre

plate (Thermo Fischer Scientific, MA, USA). Relative Light Units (RLU) was measured in a microplate luminometer (Thermo Fischer Scientific, MA, USA) with 2s delay followed by 10s measurement period. ATP concentration was estimated using a calibration curve plotted with known concentrations of ATP. The experiment was performed in triplicates.

7.2.2.9 Assessment of mitochondrial structure

Iron deficient smGFP cells were prepared by culturing the GFP cells in D medium and subsequently the ID cells were grown in different media as mentioned in the experimental outlay. The morphology of the mitochondria was assessed at 12 and 24 hours using a fluorescence microscope (BX41, Olympus, Tokyo) fitted with a digital camera (DP72, Olympus, Tokyo) by random image capturing of about 100 cells per each sample. Each cell was manually scored by visual observation under microscope for the presence of reticulate, fragmented or clumped mitochondria (Seo et al., 2010). The percentage of cells with each form of mitochondria per group was tabulated. The experiment was performed in triplicates with two different sets of samples.

7.2.3 Statistical analysis

Student's t test was used to compare the averages obtained in each of two groups. Values were considered significant if $p < 0.05$ at 95% confidence limits.

7.3 Results

Pomegranate juice was found to ameliorate iron deficiency induced cellular changes in the *S. cerevisiae.* Detailed observations are summarized in the following sections.

7.3.1 Iron deficiency did not alter cell viability

The observations from the present study (table 7.1) indicate that culturing *S. cerevisiae* cells in iron deficient medium with 100µm BPS did not alter the cell viability and generation time. A 2% reduction in the cell size was observed (table 7.1), however, this was not statistically significant.

Table 7.1: Physiological and biochemical parameters in IN and ID *S. cerevisiae* cells.

Parameter	IN	ID	% Reduction
Viability (CFU/ml)	1×10^6	1×10^6	0
Generation time (min)	97.18	97.18	0
Cell size (µm)	1.49 ± 0.08	1.46 ± 0.14	2
Dry weight (mg/10^6)	1.75 ± 0.09	1.44 ± 0.03	18*
RNA (µg/mg DW)	126.57 ± 6.69	86.25 ± 5.90	32*
Protein (mg/g DW)	401 ± 10	398 ± 10	0.75
Lipid (mg/g DW)	137 ± 5	103 ± 6	25*
Total iron (ng/mg DW)	9.71 ± 0.22	1.041 ± 0.13	89*
Ferrous iron (ng/mg DW)	4.8 ± 0.11	1.04 ± 0.13	78*
Heme (ng/mg DW)	3.99 ± 0.55	2.44 ± 0.08	39*
ATP (ng/mg DW)	117.95 ± 27.24	83.12 ± 33.10	30

DW – Dry weight; * - Significant ($p<0.01$)

7.3.2 Iron deficiency reduced the biomass, RNA, heme and lipid content

About 18% reduction in the biomass (as dry weight) was observed in the ID cells (table 7.1). The iron content in ID cells was 1.041 ± 0.13 ng/mg dry weight, which was 89% lower ($p<0.0001$) than that that in IN cells (table 7.1). It was also observed that the ID cells mainly had ferrous (Fe^{2+}) form of iron, the storage or ferric (Fe^{3+}) form was not detected in the ID cells. Further, the heme content in the ID cells was 2.44 ± 0.08 ng/mg DW, which was 39% lower than that in IN cells (table 7.1).

RNA content in the ID cells was estimated to be 86.25 ± 5.90 µg/mg DW, which was 32% less than that in the IN cells ($p<0.0001$). However, reduction in the RNA content did not correlate with the total protein content reduction significantly in the ID cells, but led to 25% reduction ($p<0.01$) in their lipid content.

7.3.3 Iron deficiency altered mitochondrial structure and function

The percentage of cells with reticulate or healthy mitochondria was significantly ($p<0.001$) lower in the ID cells when compared to IN cells (figures 7.1 and 7.2). It was also observed that the proportion of cells with fragmented and clumped (degenerating) mitochondria was higher in the ID cells than in the IN cells (figure 7.2). This trend was observed in both 12 h and 24 h cultures even though the difference was much pronounced after 24 h. ATP levels in the ID cells, was 30% lower than that detected in IN cells (table 7.1).

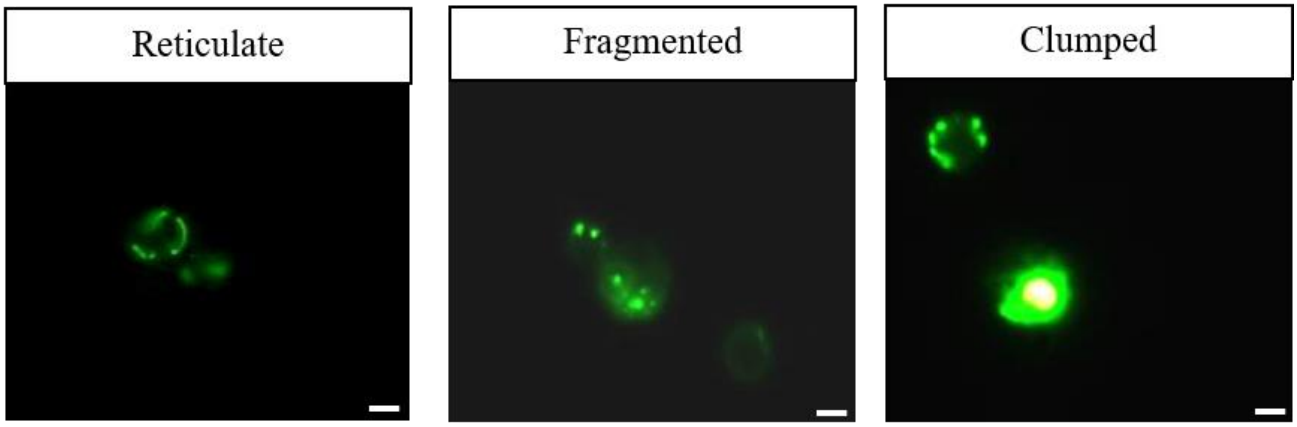

Figure 7.1: Representative images of reticulate, fragmented and clumped mitochondria of *S. cerevisiae* cells (scale - 1µm)

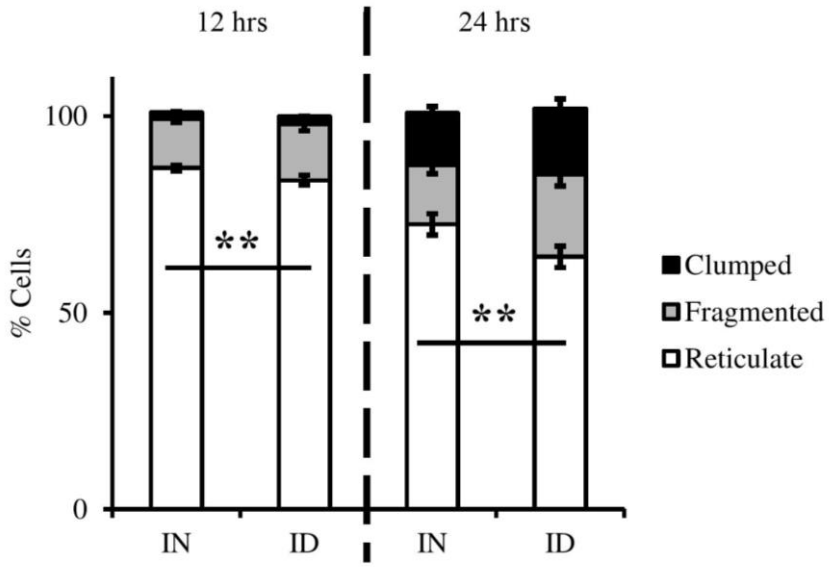

Figure 7.2: Percentage of IN and ID cells with different forms of mitochondria at 12 h and 24 h. Percentage of cells with reticulate mitochondria was significantly (**p<0.001) lower in ID cells when compared to IN cells, both at 12 and 24 h lesser percentage of cells with reticulate mitochondria than the IN cells at both 12 hrs and 24 hrs. Also, the proportion of cells with fragmented and clumped (degenerating) mitochondria was higher in the ID cells than in the IN cells.

7.3.4 PJ supplementation improved the viability of ID *S. cerevisiae* cells

Culturing the ID cells in iron deficient (D) medium for 14 h reduced the number of viable cells and increased the generation time (table 7.2), while it remained unchanged when the ID cells were cultured in D medium with 10% PJ substitution or in the iron normal (N) medium. Direct substitution of iron at the concentration present on 10% PJ (IDF cells) showed >50% reduced number of viable cells and 14% increase in generation time when compared to IDN cells (table 7.2). The cell size remained significantly unchanged when cultured in different media.

Table 7.2: Culture characteristics of ID *S. cerevisiae* cells cultured in different media

Group	IDD	IDP	IDF	IDN
Viability (CFU/ml)	7.6×10^5	1.0×10^6	4.8×10^5	1.0×10^6
Generation time (Min)	101.84	97.18	110.75	97.18
Cell size (µm)	1.43±0.20	1.41±0.12	1.42±0.12	1.48±0.28

7.3.5 PJ ameliorates ID induced cellular changes

The dry weight of IDP cells was significantly ($p<0.0001$) higher when compared to that of IDD cells (figure 7.3a). The dry weight of IDP cells was almost equivalent to that of the IDF and IDN cells. IDD, IDF and IDN cells had significantly ($p<0.01$) higher RNA content than the IDP cells (figure 7.3b). However, IDP cells had significantly ($p<0.0001$) higher protein content than all the other groups tested (figure

7.3c). PJ supplementation significantly ($p<0.0001$) increased the total lipid content of ID cells when compared to IDD cells (figure 7.3d), but was lower ($p<0.01$) than that found in IDF and IDN cells.

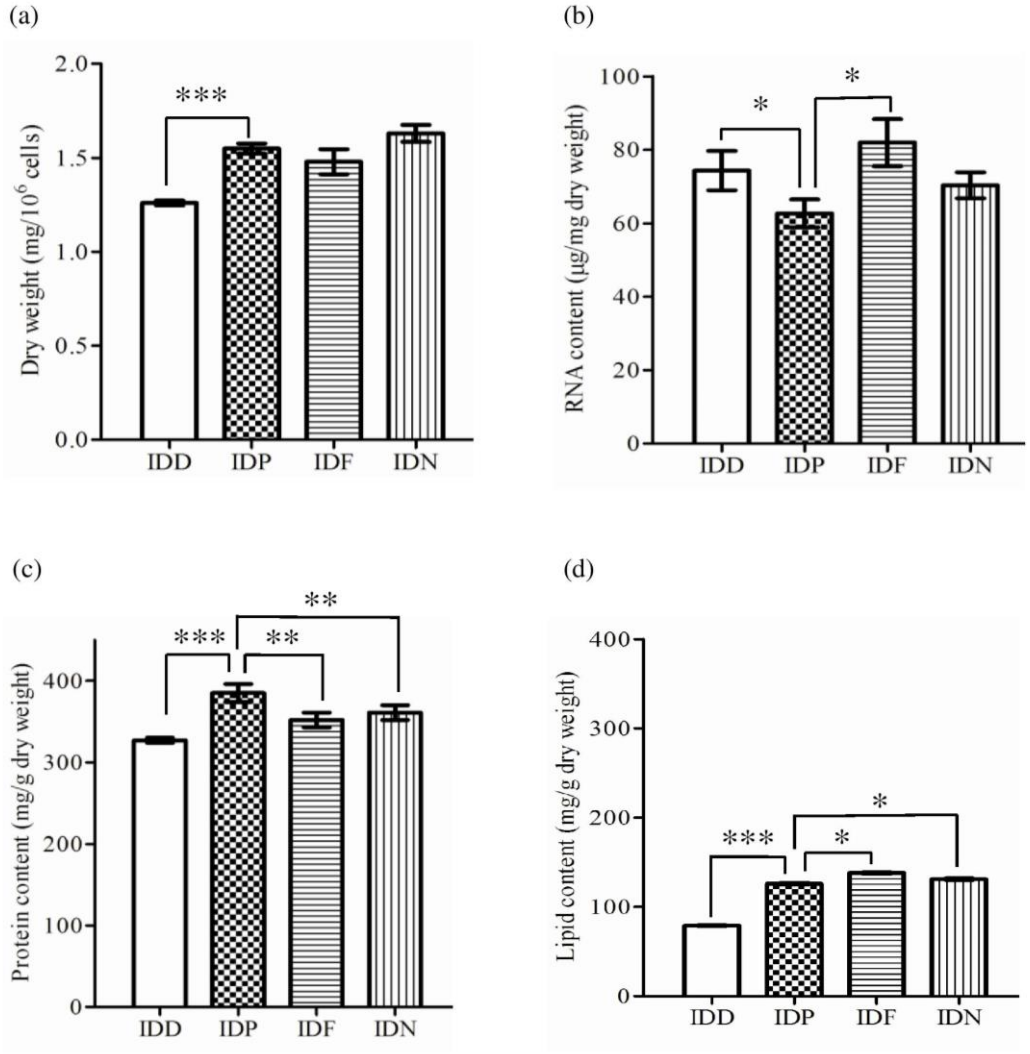

Figure 7.3: PJ ameliorates ID induced cellular changes in *S. cerevisiae* cells cultured in different media. (a) The dry weight of IDP cells was significantly higher than that of IDD cells and was almost equivalent to that of the IDF and IDN cells (b) IDP cells had significantly lesser RNA content than the IDD, IDF and IDN cells (c) IDP cells had significantly higher protein content than all the other groups tested (d) Significant increase in the lipid content of IDP cells was observed when compared to IDD cells. But was lower than that found in IDF and IDN cells. (***$p<0.0001$; **$p<0.001$; *$p<0.01$)

7.3.6 PJ supplementation improves the iron status of ID *S. cerevisiae* cells

IDP cells had 7-fold higher (0.63 ± 0.007 ng/mg dry weight) iron content than IDD cells (figure 7.4). However, unlike in the IDN cells which contained ferrous (Fe^{2+}), ferric (Fe^{3+}) and heme forms of iron, only Fe^{2+} and heme forms could be detected in IDP cells. IDN and IDF cells had significantly ($p<0.0001$) higher total iron content, in both the ferric (Fe^{3+}) and ferrous (Fe^{2+}) forms (figure 7.4), when compared to IDD or IDP. However, the heme content in the IDP cells was the highest, being significantly ($p<0.0001$) higher than that in the cells from any of the other experimental groups (figure 7.5).

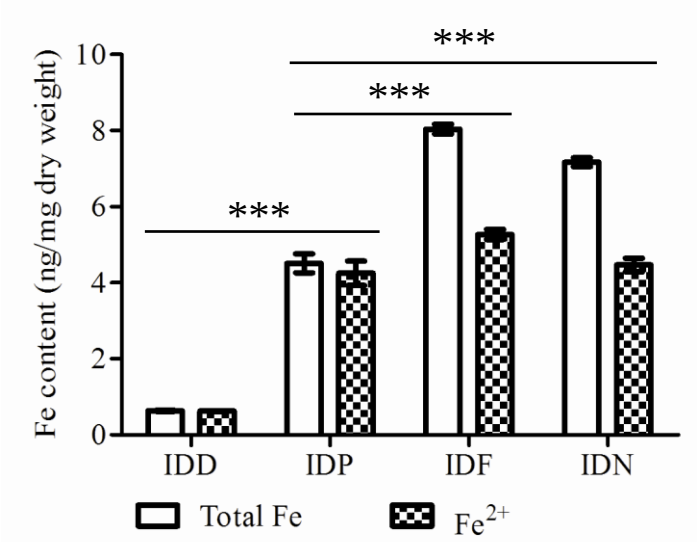

Figure 7.4: Estimation of total iron and Fe^{2+} in the ID *S. cerevisiae* cells cultured in different media. IDP cells had 7-fold higher iron content than IDD cells (***$p<0.0001$). Further, it was observed that IDP cells had only ferrous (Fe^{2+}) iron, while IDF and IDN cells had both ferrous and ferric form.

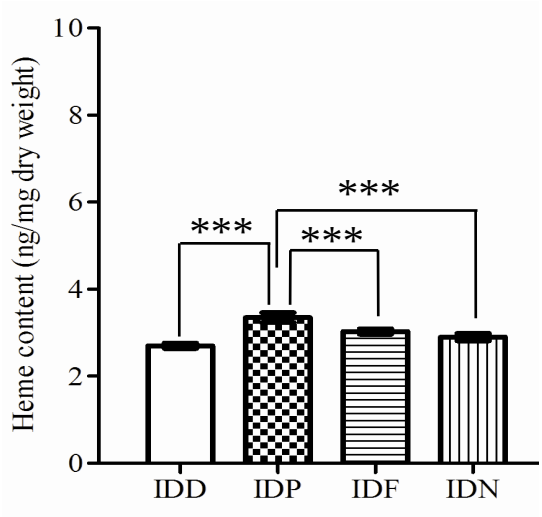

Figure 7.5: Estimation of heme content in the ID *S. cerevisiae* cells cultured in different media. IDP cells were found to have significantly (***p<0.0001) higher heme content than that of the cells from other experimental groups.

7.3.7 PJ improves mitochondrial structural composition and ATP synthesis

When the mitochondrial structure of the cells grown in the different media was analyzed, IDP cells had the highest percentage of cells with healthy reticulate mitochondria at both 12 and 24 hrs (figure 7.6). The difference was significant ($p<0.0001$) at both the time points when compared to IDD cells. While IDN cells also showed a similar trend like IDP, IDF had significantly ($p<0.005$) lower percentage of reticulate mitochondria than the IDP cells at both 12 and 24 h (figure 7.6). Even though the ATP content in the IDP cells was found to be higher than the cells of other experimental groups (figure 7.7), the difference was not statistically significant.

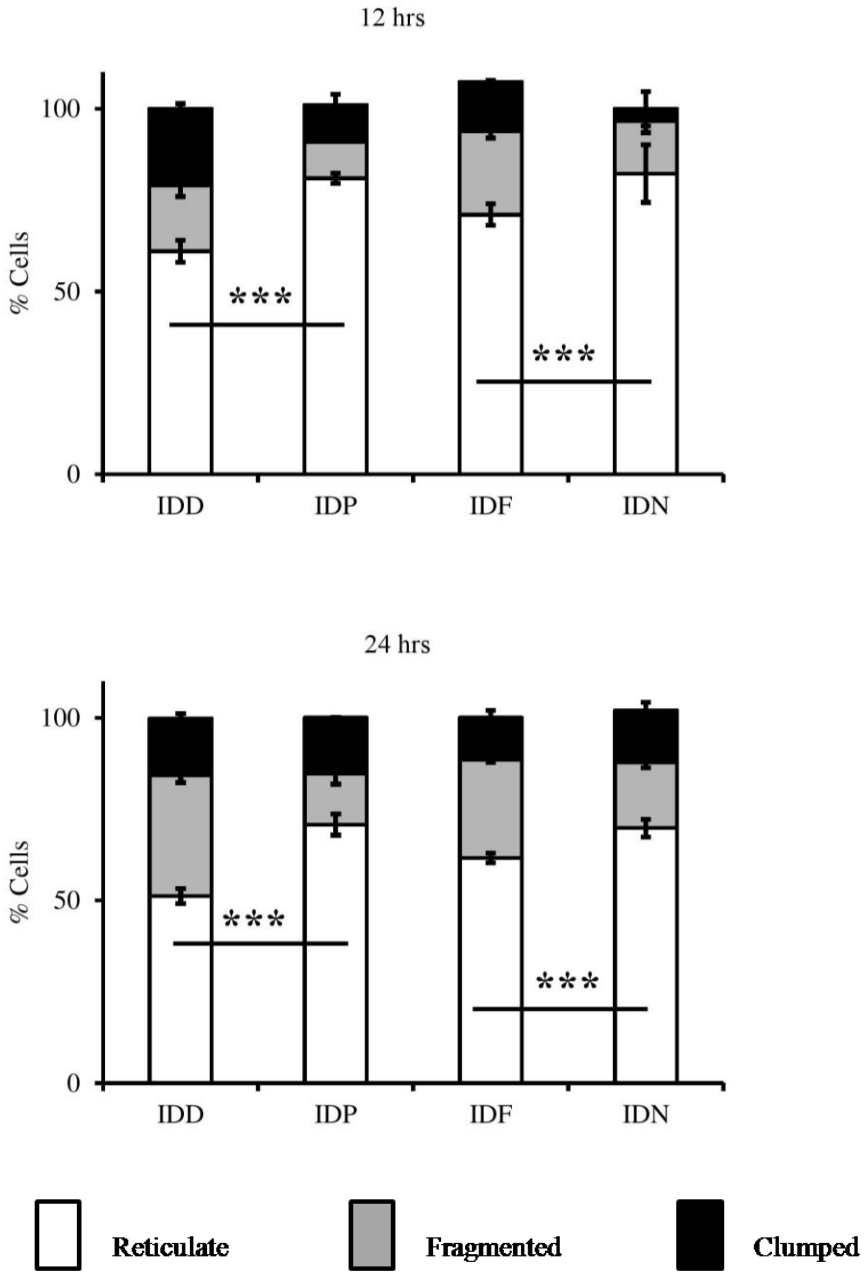

Figure 7.6: Effect of PJ substitution on mitochondrial structure of ID *S. cerevisiae* cells. The proportion of cells with healthy reticulate mitochondria was significantly (***$p<0.0001$) high in the IDP cells at both 12 and 24 hrs.

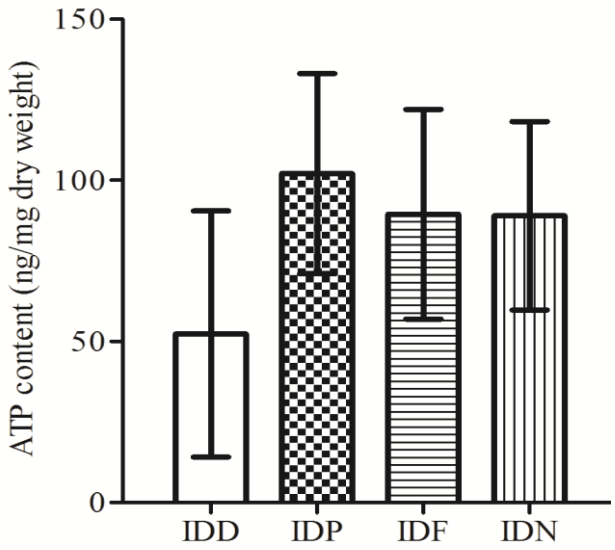

Figure 7.7: Estimation of ATP content in the ID *S. cerevisiae* cells cultured in different media. Even though quantity of ATP in the IDP cells was found to be high, it did not have any statistical significance with the cells of other experimental groups.

7.4 Discussion

IDA is a serious global problem despite ongoing national or international programs for decades with Fe-Fol (iron-folic acid) tablets (WHO, 2011). As per Ayurveda, an Indian traditional medicine, one of the main causes of '*Pandu*' (~IDA), is mal-absorption due to '*mandagni*' (low digestive fire) (Sharma, 2001). Several fruits, vegetables and herbs are prescribed as dietary supplements in the management of *Pandu*, which are said to correct problems of digestion and absorption (Murthy, 2009); pomegranate being one such fruit (Sharma, 2001). In this study, the effect of PJ on reverting IDA-like condition was simulated in yeast model. With similarities in the iron metabolism pathways of humans, the single cell eukaryote, yeast has been unraveling the response of cells to availability and uptake of iron (Philpott and Protchenko, 2008). PJ induced changes in the iron deficienct cells as observed in the current study have been summarized in figure 7.8.

7.4.1 Iron deficiency impairs mitochondrial function

Iron is an important co-factor required in several enzymatic processes including for the conserved oxidative phosphorylation and energy (ATP) production (Munoz et al., 2009). Iron deficient cells had lesser heme content reduced ATP levels. Dysfunction of iron homeostasis also damages structure of cellular organelles and affects normal metabolism (Shakoury-Elizeh et al., 2010). Reduction in the healthy reticulate form of mitochondria in the iron deficient cells that was observed in the current study may be due to iron deficiency induced damage. Walter et al., (2002) has reported that iron deficiency or iron overload damages mitochondria structure and mitochondrial DNA

in rat model. However previous studies have not looked at the mitochondrial form and ATP synthesis in iron deficient conditions.

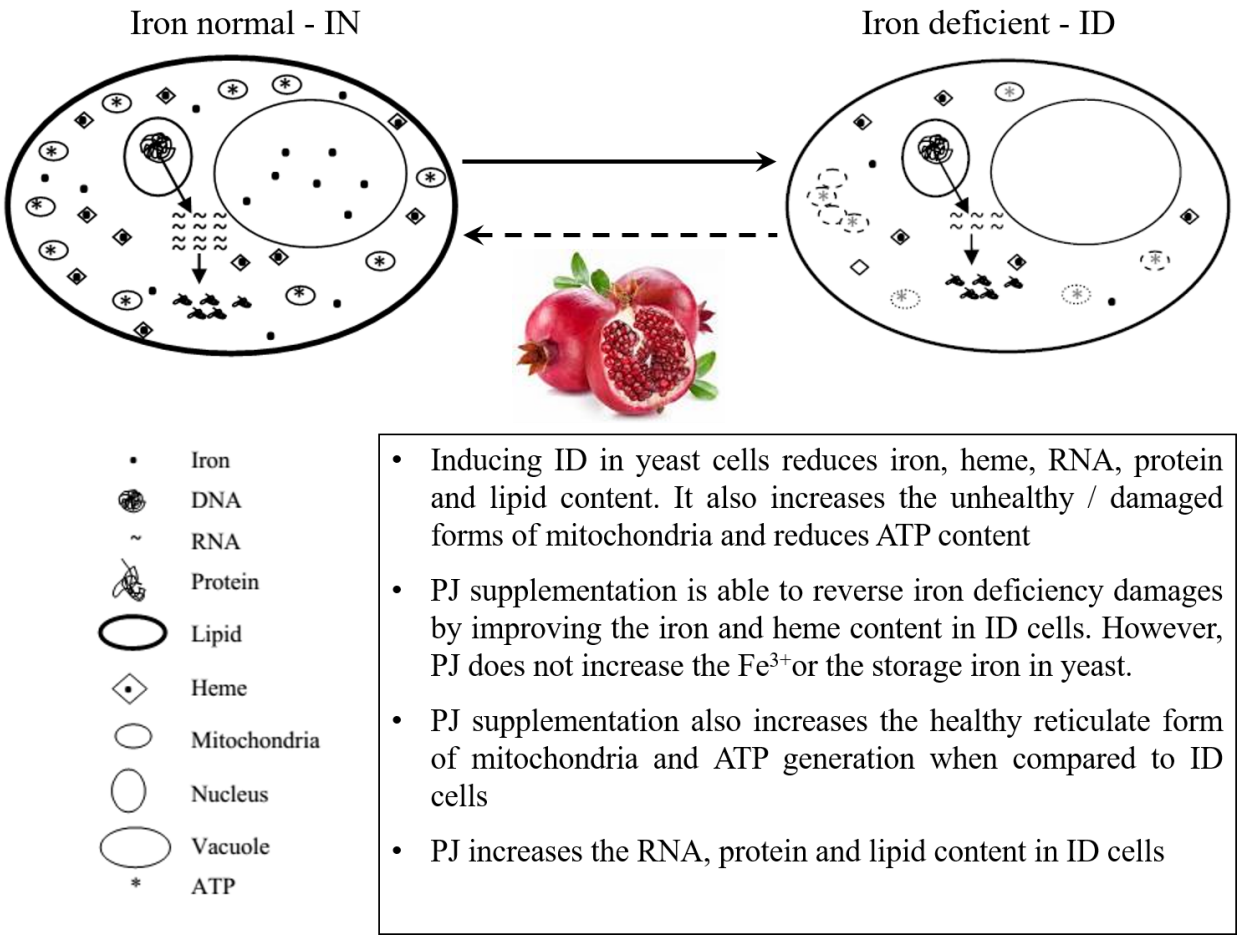

Figure 7.8: Diagrammatic representation of iron deficiency induced cellular changes in *S. cerevisiae*, and its amelioration by pomegranate juice (PJ).

7.4.2 PJ improves mitochondrial structure and function

Continuous fusion and fission shape the morphological forms of mitochondrial network and play a role in maintaining the integrity of mitochondria. They contribute to healthy survival of the cell (Okamoto and Shaw, 2005). Increase in the clumped mitochondria is an indication of cells entering apoptosis (Bossy-Wetzel et al., 2003). This study has shown that, iron deficiency reduces the proportion of cells with healthy reticulate mitochondria, while replenishing it with normal quantity of iron or PJ supplementation was able to revert and sustain the mitochondrial health. But, the *S. cerevisiae* grown with iron content equivalent to 10% PJ, which was 4.5 fold higher than the iron normal media had comparatively lesser percentage of cells with reticulate mitochondria and a higher percentage of cells with fragmented mitochondria. Probably, reduction in the mitochondrial integrity could also have contributed the lesser (4.8×10^5 CFU/ml) viable cells in this group. This shows that, even though, cells can tolerate a higher iron content (about 4.5 fold) in the media, their physiological status deteriorates. Also, it indicates either the iron content of PJ are not be toxic to cells or secondary metabolites present in PJ reduce the toxic effect of excess iron. The impact of mitochondrial integrity was also observed in its ability to synthesize ATP. ATP content cells grown in the presence of PJ was higher when compared to that of other groups, however it was not statistically significant.

7.4.3 PJ maintains functional iron concentration of cell

While iron supplementation in the medium itself reversed the iron deficient status of cells, PJ improved the iron status substantially in IDP cells, particularly of the Fe^{2+} form rather than the stored Fe^{3+} form. Normally, the iron obtained is primarily used

for physiological purposes in the ferrous form, excess iron is stored in vacuoles in ferric form (Raguzzi et al., 1988). While the N media had the required optimal iron concentration (20 µg/100ml), the P and F media contained 4.5 fold higher iron content (90 µg/100ml). Experimental observations from this study indicate that the storage iron (Fe^{3+}) improved only in the IDN and IDF cells and not in the cells grown in the presence of PJ. The fact that the IDP cells showed the presence of only ferrous iron could be due to the reducing environment in the presence of PJ (Faria and Calhau, 2011). However, the bio-assimilated heme form of iron was significantly higher in IDP than that in other experimental groups. Increase in the heme content would have positive implications in iron uptake (Huang et al., 2011). PJ appears to enhance heme production, as observed by the higher heme levels in IDP cells when compared to that in IDD, IDF or IDN cells. Heme nourishes the cells and tissues by supplying oxygen. Perhaps, the *dhatuphoshana* property of PJ might have contributed the heme production in cells. Even though there was significant increase in both functional and storage iron in the IDF cell, viability of these cells decreased drastically by 50%.

7.4.4 PJ's role in protein and lipid metabolism requires further study

Puig et al., (2005) have reported that iron deficiency nessiciates metabolic reprogramming in cells. This mechanism helps the cells to survive with altered physiology. Iron deficiency down regulates genes of several mitochondrial metabolic pathways (Ihrig et al., 2010). The decrease observed in the RNA content in the IDP cells and the increased RNA content of IDF cells can be due to the cellular response for iron deficient or iron excess condition. It was also observed that *S. cerevisiae* cells

grown in the presence of PJ had elevated protein content and lesser lipid content than the other test groups. Reasons for this are not clear.

7.4.5 Conclusion

Saccharomyces cerevisiae cells can be a good model to screen interventions to reverse IDA, particularly when testing herbal preparations. From the current study, it can be understood that PJ has the potential to be of use in managing IDA (*panduhara* – anti anemic) and it can be further tested in higher organisms. Organic acids, sugars and other classes of phytochemicals present in PJ are known to be bioavailability enhancers but individual components with hematinic activity have not yet been identified. This could be worth exploring. Future, studies on the global genome expression and proteomics might give a better understanding of the mechanisms involved in modulation of iron metabolism and cellular physiology by PJ. Scientific exploration of traditional medicines can also provide culturally relevant, sustainable healthcare solutions for managing public health conditions like IDA.

7.5 References

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Chapter 8

POMEGRANATE JUICE ENHANCES HEALTHY LIFESPAN IN *DROSOPHILA MELANOGASTER*

8.1 Introduction

Traditional literature describes *Rasayanas* as methods to "reverse naturally occurring senility" and delays death (Sharma, 2004). Apart from delaying ageing, *Rasayanas* are also claimed to increase immunity against diseases, enhance physical functions, improve mental competence, vitality and lustre of the body (Udupa, 2004). Validation of the healthy life promoting ability of commonly available *Rasayanas* like pomegranate and understanding the mode of action can help establish holistic and simple ways to wellness through diet. Several studies have indicated that eating well is prerequisite for ageing gracefully (Jong et al., 2014). Epidemiological evidence demonstrates a role for dietary intervention in the primary prevention of age related chronic diseases (Qureshi et al., 2002; Pennings et al., 2011; Kanzaki et al., 2012). Always people wish, not only to 'live longer' but to 'live better' and to maintain optimal quality of life during old age.

Ageing is defined as a progressive, irreversible, endogenous and deleterious process that occurs post-maturation (Strehler, 1962). It is a complex physiological phenomenon as it manifests over a wide range of biological systems, tissues, and functions. This turns young healthy adults into older, more frail individuals, increasingly susceptible to environmental challenges, diseases and at increase risk of death (Buffenstein et al., 2008). Better efficiency, being healthy, resist diseases and meet emergency situations are considered as markers of wellness (Grewal et al., 2006). Body composition, flexibility, endurance, strength, balance, coordination, speed, power and reaction time are some of the parameters used in describing

wellness (Bowling and Iliffe, 2006). The fundamental biology of cells, regulation of metabolism and mechanisms of ageing are conserved across a variety of widely separated species. Ageing pathways are highly conserved across species including *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Drosophila melanogaster*, rodents (Iliadi et al., 2012) and humans (Kim, 2007; Feng et al., 2011).

Pomegranate is indicated in Ayurveda as a *Rasayana*. The objective of this trans-disciplinary research study was to revalidate the *Rasayana* properties of pomegranate using *Drosophila melanogaster* model system. Experiments were designed to understand the effect of pomegranate juice (PJ) supplementation on the lifespan (*dheerghayu*) and health-span (*svasthya*). Health status of the fly was quantified in terms of its lifespan extension (*ayuvardhana*), reproductive ability (*vrshya*), immunity (*vyadhikshamatva*) and delay ageing (*vayasthapana*).

8.2 Materials and methods

The experimental approach followed to study the healthy lifespan enhancing property of PJ in *Drosophila* model is described below.

8.2.1 *Drosophila* strain, media and culture conditions

Drosophila melanogaster wild type, *Canton-S* strain, was used for this study. The basal media for culturing the flies (modified from Graf et al., 1992) consisted of corn flour (60 g), sugar (20 g), D-glucose (20 g), dry yeast (15 g) and agar (8 g) per litre of water. After cooking, media was allowed to cool to room temperature and then propionic acid (4 ml), benzoic acid (0.7 g in 5 ml of ethanol), orthophosphoric acid (0.6 ml) were added and mixed thoroughly. Media cooked as slurry was poured into the bottles or vials and was allowed to solidify and cool down before transfer of adult flies or pupae. Stock flies were maintained in bottles while culture vials were used for maintenance of smaller populations or experimental groups. Flies were allowed to feed *ad libitum* and grown at 25°C with 60% humidity in 12-hour day night cycle.

8.2.2 Transferring flies from one vial to another

Stock flies were transferred to fresh media every 10 to 14 days while flies in vials were transferred every 3rd day. To transfer, flies were gently tapped down by softly tamping the old bottle or vial on a soft surface, to bring the flies to the bottom. Then quickly the plug off the bottle or vial was removed, inverted into the fresh media and plugged with cotton.

8.2.3 Etherisation

To examine and count flies it is necessary to anesthetise them with a light dose of ether. This was done by using a special etherising bottle. Briefly, ether was added to the cotton pad in the bottom of etherising jar. Flies were transferred to a 50 ml tube with minute holes in the bottom (etherising tube) after placing it inside the etherising jar. A funnel was used while transferring the flies. After removing the funnel, etherising tube was tapped a few times gently to the cotton pad on the bottom of the etherising jar. Ether, being heavier than air, will flow from the etherising jar into the tube through the tiny holes in its bottom. Etherising tube was separated from the jar, after about a minute of etherisation the flies will stop moving; then flies were collected on the stage of the binocular stereo-microscope.

Flies that are dead due to excess ether dose extend their wings and legs at right angles to their bodies. Dead flies if any were removed and discarded into "morgue" (a bowl of mineral oil or water containing a household detergent). Flies usually remain etherised for 5 to 10 minutes. Re-etherisation was done only if very necessary because frequent re-etherisation will kill the flies.

8.2.4 Preparation and standardisation of Pomegranate juice (PJ) and Resveratrol stock

Pomegranate juice (PJ) was prepared and standardised as described in chapter 5. Resveratrol (RV) (Sigma-Aldrich, St. Louis, MO, USA) stock (100 mM) was prepared by dissolving 22.8 mg in 1 ml of ethanol. PJ or RV was added to the cooked, warm media just before dispensing into the vials.

8.2.5 Lifespan extension experiment

About 2-3 days aged flies were generated by removing all adult flies in the bottles. Once a synchronous culture was generated, sex separated flies were transferred into vials containing basal medium supplemented with 0.1, 1, 5, 10 and 15% (v/v) of PJ. Flies were transferred to fresh media every third day. Dead flies were counted and removed daily until the death of the last fly in each vial. 15% PJ could not be used since at that concentration the medium would not set.

8.2.6 Longevity and Fecundity analysis

Synchronous cultures of 2-3 day old files were obtained as described earlier and transferred into vials containing basal medium supplemented with 10% of PJ (v/v) or 200 µM RV and control. Each group including the control had 10 vials each with 14 – 20 flies per vial with equal sex ratio. Flies were transferred to fresh media every third day. Dead flies were counted and removed daily throughout the experiment.

Fecundity analysis was performed by counting the number of flies emerging in the emptied vials used for longevity studies. Numbers of emerged flies were counted daily for ten days from the day of the emergence of the first fly in each experimental group.

8.2.7 Gustatory Assay

The method described by Lee et al., (2010) was adopted for gustatory assay. Adult flies were reared for 20 or 40 days in media supplemented with 10% PJ or 200 µM RV. A basal media control was also maintained. To perform a feeding assay, after starving the flies for 2 hours, 30 male or female flies from each experimental group were transferred into the vials containing the specific diets with bromophenol blue

dye (0.05% w/v) (Sigma-Aldrich, St. Louis, MO, USA). After 10 min of feeding, the fed flies were etherized, washed with phosphate-buffered saline (PBS) and homogenized in 1 ml of distilled water. The absorbance of 100 times diluted supernatant was measured at 595 nm using a spectrophotometer (Bio-Rad, CA, USA).

8.2.8 Resistance against Hydrogen peroxide (H_2O_2) and Paraquat induced stress

The method described by Peng et al., (2011) was used for studying the fly's ability to resist free radical stress induced by H_2O_2 (Fisher Scientific, Mumbai, India) and paraquat (Sigma-Aldrich, St. Louis, MO, USA). H_2O_2 generates hydroxyl radical (OH*) while paraquat produces superoxide (O*) radicals. 20 days old male and female flies from control, RV (200 µM) and PJ (10%) fed were starved for 2 hrs and transferred to separate vials (n= 50; 10 flies / vial) containing 5% H_2O_2 or 20 mM paraquat prepared with 5% sucrose on saturated tissue paper mat. Dead flies were counted every 4 hours until the death of the last fly.

8.2.9 Infection Survival assay

The method described by Apidianakis and Rahme (2009) was followed with slight modifications. Antigen was prepared by suspending an overnight culture of *Candida albicans* SC5314 in sterile distilled water (2×10^8 cells/ml). Flies (20 days old) were anesthetized with CO_2. Infection was induced with a sterile tungsten needle (0.01 mm diameter) dipped in the *C. albicans* suspension by gently pricking in the thoracic region. Flies were returned to their respective food vials and were incubated at 25ºC with 12 h day-night cycle until 50% of flies died in any of the groups. Control flies were pricked with needle dipped in sterile distilled water.

8.2.10 Physical performance

On day 20 and 40, locomotor function of flies was assessed using the climbing or negative geotaxis assay as reported by Bahadorani and Halliker (2008) with slight modifications. In brief, 20 flies/trial were placed in the bottom of a measuring cylinder and given 20 s to climb up. At the end of each trial, the number of flies that climbed up to a vertical distance of >8 cm was recorded. Each trial was repeated three times.

8.2.11 Gene expression studies

Semi-quantitative gene expression of *AMP-activated protein kinase* (*AMPK*), *c-Jun N-terminal kinases* (*JNK*), *fork head transcription factor* (*FOXO*), *manganese superoxide dismutase* (Mn*SOD*) and *target of rapamycin* (*TOR*) was measured as previously described (Peng et al., 2012). The methodology followed is described below.

8.2.11.1 Total RNA extraction and cDNA Synthesis

Total RNA was extracted using the commercial extraction agent TRIzol (Sigma-Aldrich, St Louis, MO, USA). Fruit flies (3 homogenates of 10 flies each from control, 200µM resveratrol fed and 10% pomegranate juice fed) were homogenized in 750 µl of TRIzol solution, and then centrifuged at 12,000 g at 4°C for 10 min. The supernatant was transferred to another new tube containing 160 µl chloroform. The mixture was then subjected to centrifugation at 12,000 g at 4°C for 15 min. The upper layer was mixed with 400 µl isopropanol. After 10 min of incubation at room temperature, the samples were centrifuged at 12,000 g at 4°C for 10 min, and the pellet was saved and washed in 1 ml of 75% ethanol followed by re-centrifugation.

Finally, 20 µl DEPC (0.1% v/v) water was employed to resuspend the RNA pellet. The concentration and purity of RNA obtained were determined by measuring their absorbance at 260 nm and 280 nm using a nanoquantity spectrophotometer (Thermo Fisher, MA, USA). First strand cDNA synthesis Kit (Thermo Fischer, Waltham, MA, USA) with oligo $(dt)_{18}$ was used to construct cDNA in 10 µl volume and stored at −20°C until use.

8.2.11.2 Semi-quantitative PCR

Aliquots of cDNA (diluted 1: 20) were amplified with Thermo Scientific PCR Master mix (Waltham, MA, USA) in the presence of gene specific primers (table 8.1) and *rp49* (internal control). Primers were designed using the Primer 3 software and synthesized by Bioserve Biotechnologies (Hyderabad, India). The details of the mRNA transcript variants used for designing gene specific primers are presented in annexure I. Reactions were carried out in a Nexus gradient cycler (Eppendorf, Germany). The standard program comprised of 30 s at 95ºC, 30 s at 56ºC and 2 min at 72ºC for a number of cycles previously determined to assure that amplification was in the exponential range. The details of the primers used and expected product size are presented in table 8.1. The PCR products were analysed in a 2% agarose gel; images of the ethidium bromide stained agarose gels were acquired with a gel documentation unit (Bio-Rad, CA, USA) and quantification of the bands was performed with Image Lab software, version 3.0 (Bio-Rad, CA, USA). The intensity of the amplified products was normalized to the control values of *rp49* and expressed in terms of relative transcript level.

Table 8.1: Details of the primers used and expected product size for semi-quantitative gene expression studies

Gene	Primer sequence		Expected product size (bp)
	Forward (5' – 3')	Reverse (5' – 3')	
TOR	CTCCGCAAAAACAGAGCGAG	TTGTGGGACACCTCCGATTG	217
AMPK	GCGATGAAGGCACTCAGCTA	AAACTCCATGGTGTGGTGGC	270
JNK	GAGCAGTTGTGCCAGGTTGT	GCCGTCTTTCTCCATCGACT	237
SOD	TAAAATTTCGCAAACTGCAAGCC	TGCCAGAAGATGGTGTGGTT	273
FOXO	TGACCCACACAGATAACGGC	CCAATGGCATGCGTGATGAG	292
rp49	TTCTACCAGCTTCAAGATGAC	GTGTATTCCGACCACGTTACA	470

8.2.12 Longevity of *FOXO* and *SOD* knockdown flies

UAS- SOD RNAi (BS# 29389; Chromosome III) flies and UAS- *FOXO* RNAi (BS# 25997; Chromosome III) virgin female flies were crossed with tubulin Gal4 tubby males (BS# 62712; Chromosome III) to generate *FOXO* and Mn*SOD* knockdown flies. The flies used in this study were obtained from Bloomington Stock Centre, Indiana University, USA and National Centre for Biological Sciences (NCBS), Bangalore, India. The crosses were set-up at 18ºC. Flies with RNAi were identified by non-tubby phenotype. Eclosed adults were collected, separated in vials containing control, RV and PJ media and incubated at 29ºC for induction of tubulin GAL4/UAS RNAi. Their longevity in the pomegranate supplemented media was studied as described earlier.

8.2.13 Statistical Analysis

The day when 50% of the total flies in each of the experimental group survived was calculated as the median survival day. Online tool available at http://bioinf.wehi.edu.au/software/russell/logrank/ was used to perform Log rank test for survival analysis. Online Wilcoxon rank sum test available at

http://www.fon.hum.uva.nl/Service/Statistics/Wilcoxon_Test.html was performed for comparing the lifespan extension (Grandison et al., 2010). Student's t-test was used for comparison of means. ANOVA was employed while comparing more than two groups. P value <0.05 was considered as significant.

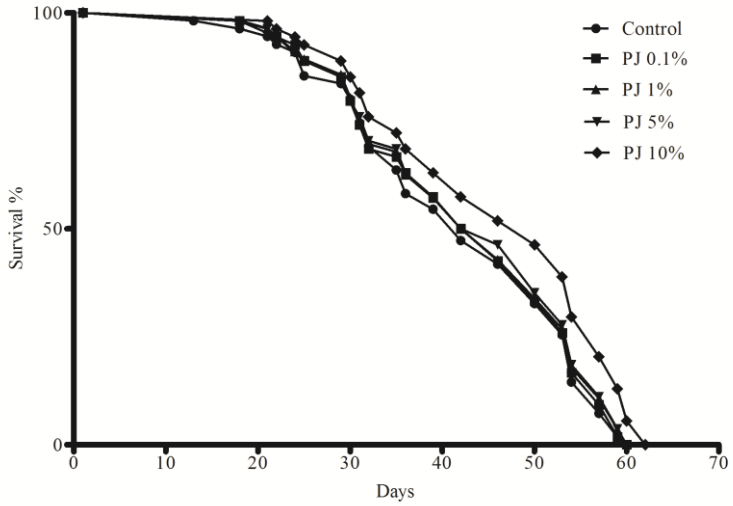
8.3 Results

The present study shows that PJ fed flies live longer, show increased fecundity and are better protected against stress and infection and are active even when aged (better health span). The details of the observations are presented in this section.

8.3.1 PJ supplementation extends lifespan in *Drosophila*

PJ supplementation with the media extends lifespan in *Drosophila* (figure 8.1). To determine the lifespan extending potential in *Drosophila*, basal media was supplemented with 0.1, 1, 5, 10 and 15 % of PJ. Substitution of the media with 10% PJ significantly ($p<0.0001$) enhanced the median lifespan by 18.51% and 8% in male and female flies respectively (figure 8.1; table 8.2). Last fly survival day was also enhanced by 2 days in the 10% PJ group. While the lower concentrations tested (0.1, 1 and 5 %) did not produce any significant change in the lifespan, 15% PJ substitution to the basal media affected solidification of the medium; therefore, this concentration was not considered. Hence, 10% pomegranate juice was used in all further experiments.

(A) Male flies



(B) Female flies

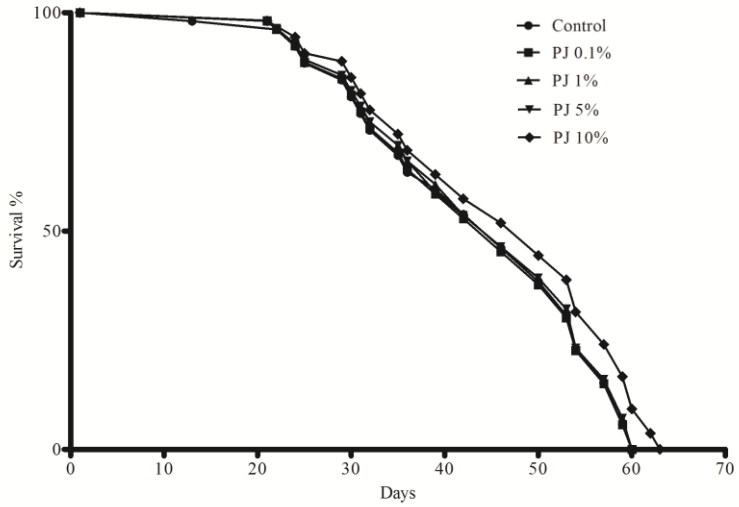


Figure 8.1: Lifespan extension of *Drosophila melanogaster* flies with different concentrations of Pomegranate Juice (PJ). 10% PJ substitution to the media significantly increased ($p<0.0001$) the survival of the flies and also extended their lifespan by 2 days. In both the sexes 0.1, 1 or 5% PJ did not show any significant enhancement in the survival when compared to the control group.

8.3.2 PJ supplementation controls the 'trade-off' between fecundity and longevity

As 10% PJ substitution to the media significantly enhanced the lifespan for both male and female flies, the effect of this concentration on the longevity and fecundity of male and female mixed sexes group was investigated. RV at 200 µM dose was used as positive control, based on the reports of Bass et al., (2007).

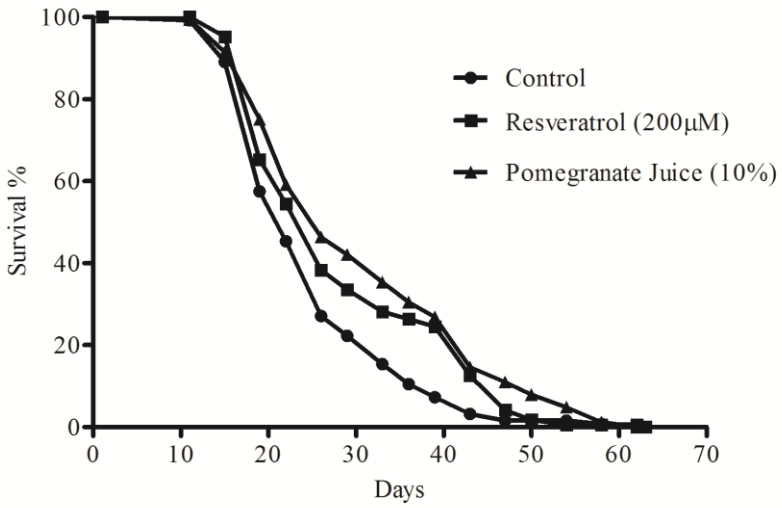


Figure 8.2: Effect of Pomegranate juice supplementation on *Drosophila* longevity. Flies were fed with 10% PJ (n= 164) or 200µM RV (n=167). A basal medium control (n=247) was also maintained. Log rank test indicated significant ($p<0.0001$) increase in the fly survival by both PJ and RV (table 2). But PJ increased the median survival by 19.23% while it was 11.05% in the RV group.

PJ enhanced the median survival of a mixed population of flies by 19.2% from 20.8 (as seen in control) to 24.8 days (PJ) ($p<0.0001$) (figure 8.2; table 8.2). RV group on the other hand enhanced the median survival to 23.1 days, which was only 11% over the control group (figure 8.2; table 8.2). The maximum lifespan observed in the control and PJ groups was 61 days while that in the RV group, the last fly survived till 62nd day.

Reproductive potential of an organism also indicates its physiological fitness, so number of flies emerging from each vial of PJ, RV and control group was monitored. The data shown in figure 8.3 indicates that PJ group produced significantly ($p<0.0001$) more number of flies throughout the reproductive phase. Also, the new fly emergence data indicated that PJ extended the reproductive viability phase (figure 8.3).

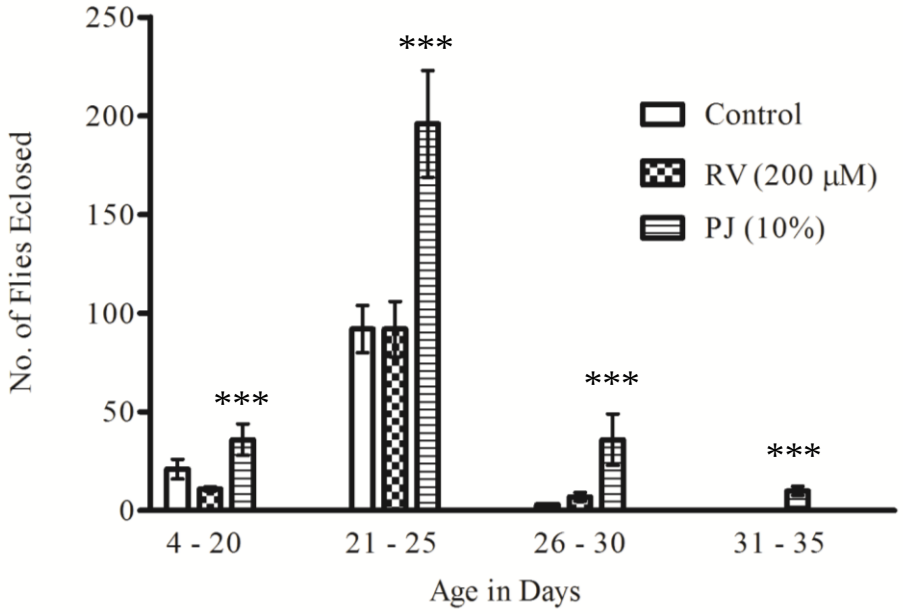


Figure 8.3. Effect of 10% Pomegranate juice supplementation on *Drosophila* fecundity at different age. PJ group showed a significant increase in the number of off springs produced (***$p<0.0001$) at every sampling period. The graph also indicates an extended reproductively viable phase in the PJ fed flies. No significant difference in fecundity was observed with RV and control groups.

8.3.3 *Drosophila* has the same feeding preference to PJ and RV supplementation

To ensure that the observed changes in the life-span were due to PJ or RV supplementation and not because of starvation or dietary restriction, gustatory assay was performed, quantifying the food intake on 20 and 40-day old flies using bromophenol blue dye.

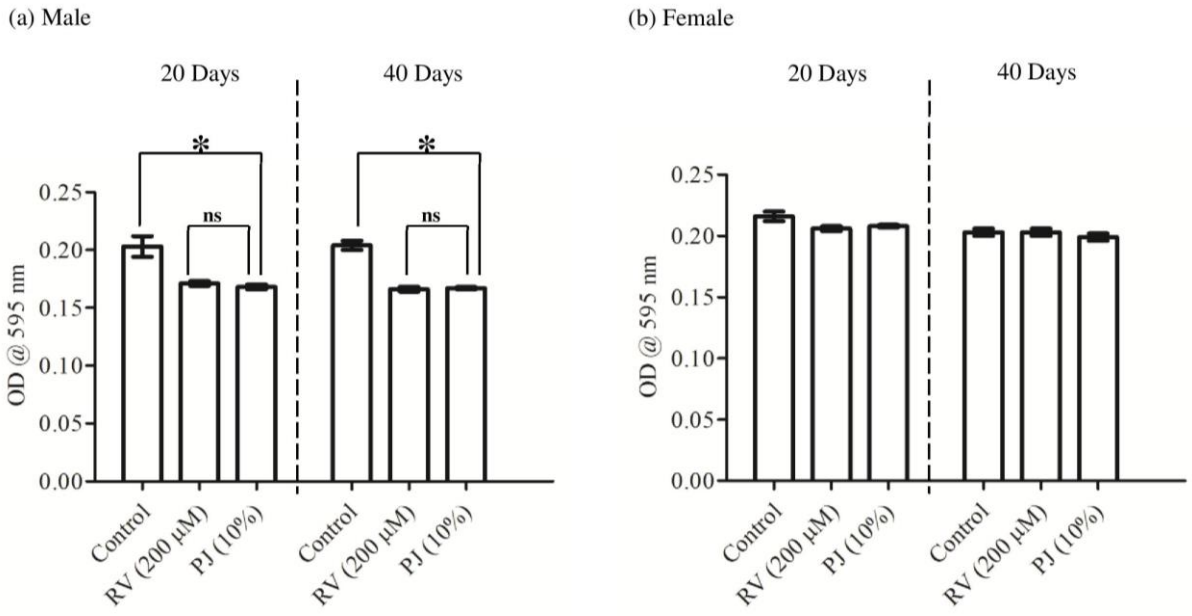


Figure 8.4: Gustatory assay with PJ (10%) or RV (200 µM) supplementation in 20 and 40 days old *Drosophila* flies. (a) Male flies in the PJ and RV group were found to have reduced feed intake when compared to control (*$p<0.05$). However, there was no difference in food intake by PJ and RV groups. (b) Female flies did not show any significant difference in the food intake on both the days tested. (ns –not significant)

While there was no significant difference observed between PJ and RV fed groups, the feed intake was lower in both these groups as compared to that in the control ($p<0.05$), particularly in the male flies (figure 8.4). This difference was not consistent in the female flies. The food intake of 40-day old female flies was similar in control, PJ and RV groups (figure 8.4b). Also, there was a significant difference ($p<0.05$) in food intake between the male and female flies in both PJ and RV groups (figure 8.4). Due to this difference, lifespan extension by feeding PJ cannot be directly attributed to dietary restriction.

8.3.4 PJ enhances tolerance to free radical induced stress

Survival of organisms in stress conditions is one of the resistance mechanisms. *Rasayanas* have been shown to have anti-oxidant and free radical scavenging activity (Balasubramani et al., 2011).

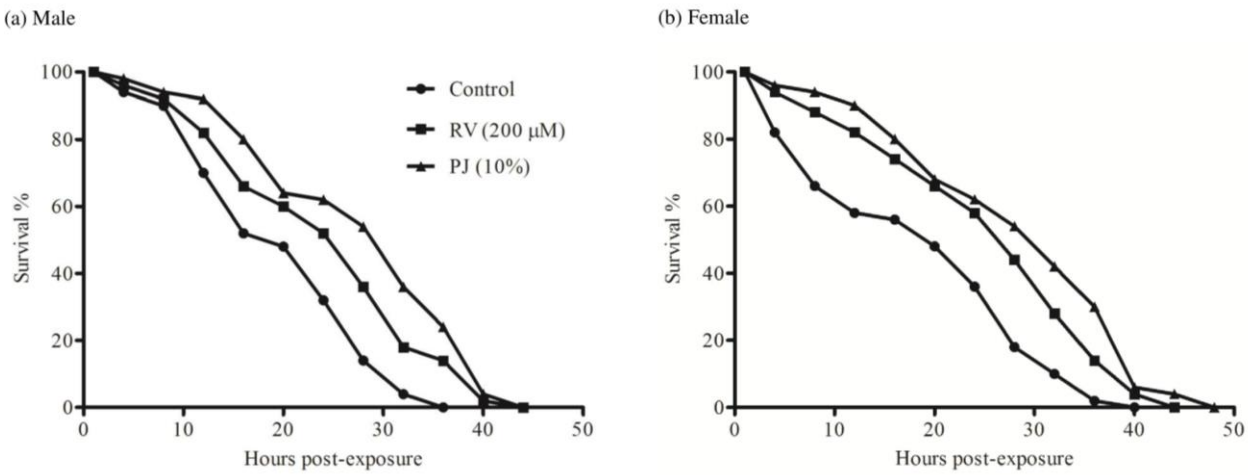


Figure 8.5: Survival against hydroxyl radical (OH*) produced by hydrogen peroxide exposure in 20 days old *Drosophila*. Both male (a) and female (b) flies fed with PJ (10%) showed approximately 50% increase in median survival ($p<0.0001$) (table 2). RV (200µM) fed flies showed only about 30 – 35 % increase in median survival.

Exposure to H_2O_2 and paraquat produce free radical stress to flies. A significant ($p<0.0001$) increase in the post H_2O_2 and paraquat exposure survival time was observed in both PJ and RV supplemented flies when compared to the control (figures 8.5 & 8.6; table 8.2). Analysis of median survival indicated an average 50% increase over the control group in both male and female flies in the PJ group. Though, RV was also found to protect against the free radical induced stress, its level of protection reflected as only about 30 – 35 % increase in survival (figures 8.5 & 8.6).

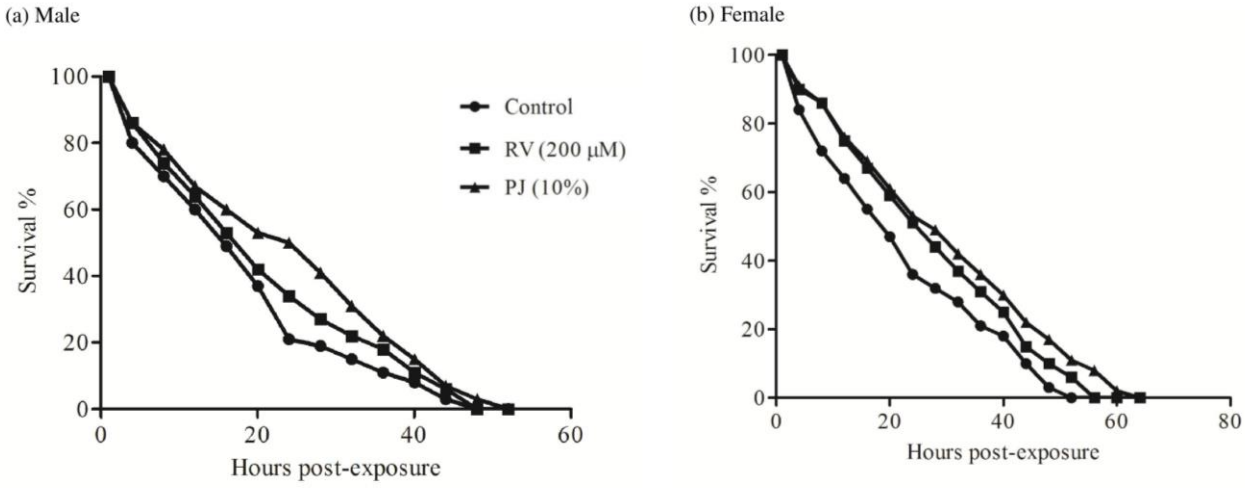


Figure 8.6: Survival against superoxide radical (O*) produced by paraquat exposure in 20 days old *Drosophila*. (a) PJ (10%) group showed 54% increase in median survival ($p<0.0001$) while RV (200µM) could increase only by 9.6% (table 2) (b) Female flies fed with PJ showed 45.9% increase in median survival ($p<0.0001$) while RV was able to increase the median survival by 32.4 %.

Table 8.2: Statistical analysis of the survival curves using Log rank and Wilcoxon rank sum test.

Experimental Group	Number of flies tested (n)	Median Survival day / hour (Day till last fly survived)	% Change	Chi square value @ 1 degree of freedom	Log rank (Survival)	Wilcoxon (Maximum life-span)
Life-spanLife-span extension						
Male						
Control	110	40.5				
PJ 0.1	108	42.0	3.7	0.7	p=0.396	p≤0.6421
PJ 1	112	42.0	3.7	1.6	p= 0.207	p≤0.5322
PJ 5	108	42.0	3.7	3.9	p=0.047	p≤0.6208
PJ 10	108	48.0	18.51	46.2	p<0.00001*	p≤0.2545
Female						
Control	104	43.5				
PJ 0.1	106	43	-1.14	0.1	p=0.699	p≤0.6861
PJ 1	112	43.5	0	0.1	p=0.720	p≤0.5084
PJ 5	112	44	1.14	0.1	p=0.758	p≤0.5392
PJ 10	108	47	8.0	19	p<0.00001*	p≤0.3439
Longevity and Fecundity						
Control	247	20.8				
RV	167	23.1	11.05	16.2	p<0.00001*	p≤ 0.6016
PJ	164	24.8	19.23	48.7	p<0.00001*	p≤ 0.3506
H_2O_2 stress survival						
Male						
Control	50	18.0				
RV	50	24.5	36.1	29.3	p<0.00001*	p≤0.4705
PJ	50	28.0	55.5	1.6	p<0.00001*	p≤0.2602
Female						
Control	50	19.0				
RV	50	26.5	39.4	22.5	p<0.00001*	p≤0.4237
PJ	50	29.5	55.2	49.4	p<0.00001*	p≤0.1824
Paraquat stress survival						
Male						
Control	50	15.5				
RV	50	17.0	9.6	7.4	p=0.0066*	p≤0.6295
PJ	50	24.0	54.8	19.2	p<0.00001*	p≤0.4907
Female						
Control	50	18.5				
RV	50	24.5	32.4	8.7	p=0.0032*	p≤0.4553
PJ	50	27.0	45.9	30.3	p<0.00001*	p≤0.2856
***FOXO* Knockdown**						
Control	60	22.3 (29)				
RV	60	27.9 (39)	25.11	36.4	P<0.0005*	P<0.0005*
PJ	60	39.8 (58)	78.47	236	p=0*	P<0.0005*
Mn*SOD* Knockdown						
Control	30	9.3 (23)				
RV	30	10.3 (18)	10.75	1.1	p=0.287	p ≤ 0.4154
PJ	30	13.2 (34)	41.93	9.2	p=0.002*	p ≤ 0.0121*

*Significant

8.3.5 PJ fed *Drosophila* flies are protected from *Candida albicans* infection

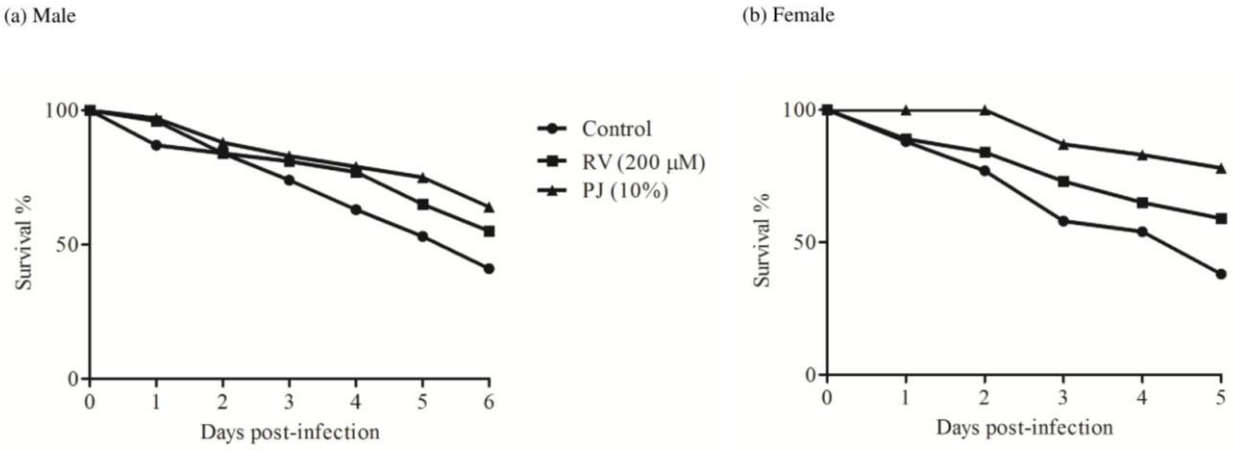


Figure 8.7: PJ fed *Drosophila* flies are protected from *Candida albicans* infection. 20 days old male (a) and female (b) flies fed with PJ, RV or control diet were infected with *Candida albicans*. In both the sexes, flies fed with PJ showed a significantly ($p<0.05$) better survival response than the control and RV fed flies.

D. melanogaster has been used as a model to study host-fungal interaction and pathogenicity (Alarco et al., 2004). Glittenberg et al., (2011) established *Drosophila* as an alternative model for investigating the pathogenicity of *Candida albicans*. Literature indicates that *Rasayanas* have immune-modulatory (*vyadhikshamatwa*) potential (Balasubramani et al., 2011). As proof of principle, PJ substitution was tested for its ability to offer protection to the flies from death due to *C. albicans* infection. The survival curve indicated a significant ($p<0.05$) protective effect of PJ until 144 hours post-infection in both male and female flies. Even though RV too protected the flies (>50%) from *C. albicans* infection, the magnitude of protection by PJ (>70%) was significantly ($p<0.05$) higher. The control flies had only 40% survival (figure 8.7; table 8.3). While the magnitude of protection differs between male and female flies, the protection trend was similar.

Table 8.3: Statistical analysis of the infection survival experimental observations.

Experimental Group	n	Median survival time (MST) in Hrs	% MST Increase	% of surviving flies	ANOVA	T test
Male						
Control	50	4.88		41	p= 0.0013*	
RV	50	>6	>23	55		p≤0.05*
PJ	50	>6	>23	64		p≤0.05*
Female						
Control	50	3.82		38	p= 0.0012*	
RV	50	>5	>31	59		p≤0.05*
PJ	50	>5	>31	78		p≤0.05*

*Significant

8.3.6 PJ promotes sustained physical performance in *D. melanogaster*

The success of healthy living lies in the retention of normal physical activity. *Rasayanas*, like pomegranate, are said to improve energy, strength and stamina (*balya*) of organisms. The physical performance of the flies was measured using the climbing or negative geo-taxis assay. This assay was performed with 20 and 40 days old male and female flies fed on media supplemented with PJ or RV. Even though, significant difference among the groups was not observed in the percentage of flies climbing on 20^{th} day, the PJ group retained the physical activity even on day 40 (aged flies), while the other (control and RV) groups showed significant decline ($P<0.005$) (figure 8.8). Thus, PJ fed flies were found to sustain good physical activity in terms of negative geo-taxis or climbing activity during ageing.

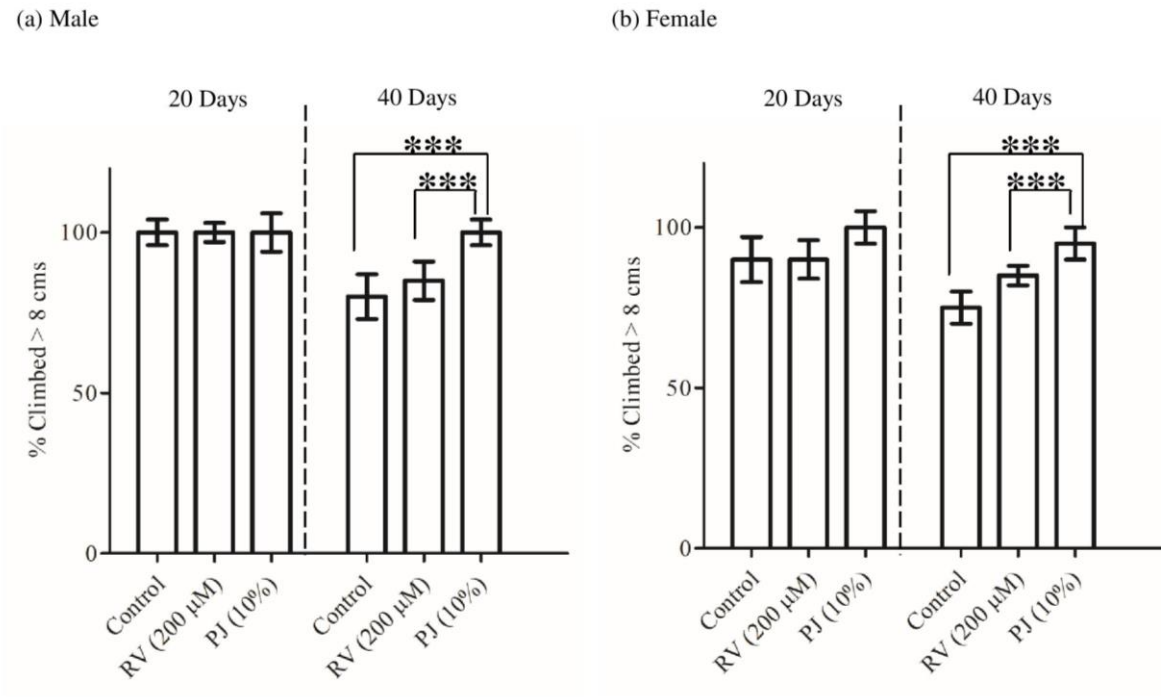


Figure 8.8: Sustained physical performance of *Drosophila* flies fed with PJ. (a) 20 days old male flies did not show any difference in their climbing performance irrespective of feed, but 40 days old PJ fed flies were able to retain their physical performance better than the RV and control group. (b) Female flies at both 20 days and 40 days showed significantly higher climbing performance than the RV and control group. PJ group was able to resist the age related physical activity decline better than the RV and control groups. (***p<0.005).

8.3.7 PJ down regulates both *FOXO* and *TOR* in flies

To determine the possible molecular mechanisms by which PJ extends lifespan, semi-quantitative expression analysis of genes reported to be involved in ageing pathways, *FOXO* and *TOR* was performed. These pathways play a role in lifespan modulation across various living organisms including *Drosophila* and humans (Bjedov and Partridge, 2011; Partridge et al., 2011).

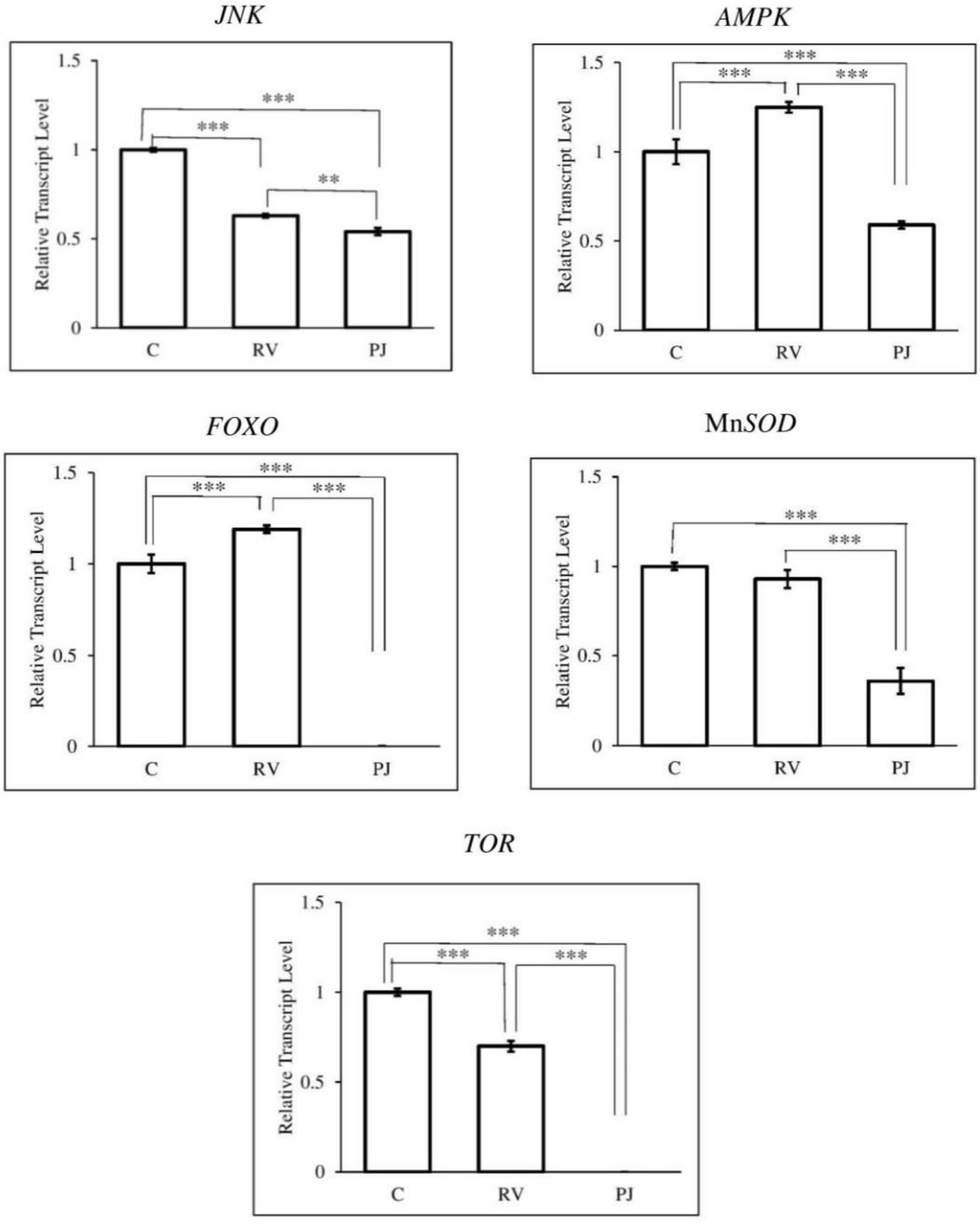


Figure 8.9: Level of expression of selected genes on PJ feeding in *Drosophila melanogaster.* In 20 days old flies fed with PJ, the expression levels of *JNK*, *AMPK*, *FOXO*, Mn*SOD* and *TOR* were found to be significantly ($p<0.0001$) less when compared to the control and RV fed.

Gene expression studies done with 20 days old PJ fed flies indicated a significant decrease ($p<0.0001$) in the transcript level of *JNK*, *AMPK*, *FOXO*, Mn*SOD* and *TOR* (figure 8.9) as compared to that in control. RV feeding on the other hand was found to enhance the expression of *AMPK* and *FOXO* while down regulating *JNK* and *TOR* as expected.

8.3.8 PJ feeding completely compensate *FOXO* knockdown but only partially rescues *SOD* knockdown in flies

To confirm the gene expression results, longevity pattern in the *FOXO* and *SOD* knockdown flies were studied. Knockdown of *FOXO* led to decrease lifespan, but when these flies were fed PJ, they completed normal lifespan similar to normal flies (comparable to wild type in figure 8.2) (figure 8.10; table 8.2).

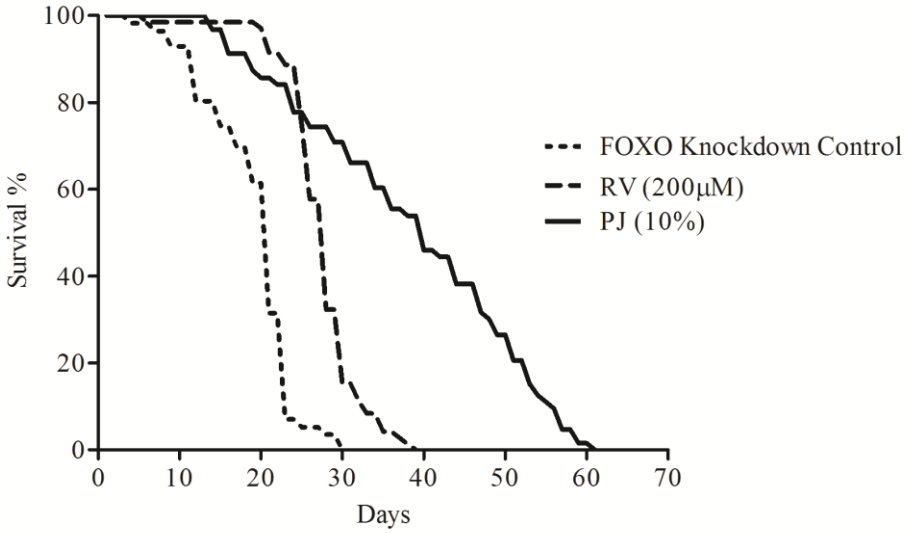


Figure 8.10: Effect of Pomegranate juice supplementation on longevity of *FOXO* knockdown *Drosophila* flies. Log rank test indicated significant ($p<0.0005$) increase in the fly survival by both PJ and RV (table 8.2). But PJ increased the median survival by 78.47 % while it was 25.11 % in the RV group. The maximum life-span in the control flies was observed as 29 days, RV treatment could increase the life-span to 39 days. PJ treatment showed higher life-span of 58 days, which is almost equal to that of the normal flies.

Knockdown of *SOD* was found to be lethal to flies, which reduced the lifespan by about 85% when compared to the *SOD* normal flies. PJ fed *SOD* knockdown flies showed 42% increase in the mean survival compared to the *SOD* knockdown control ($p<0.005$) and also it significantly increased the life-span from 23 to 34 days ($p<0.05$). However, they could not complete their full life-span of approximately 60 days like the normal flies (figure 8.11; table 8.2).

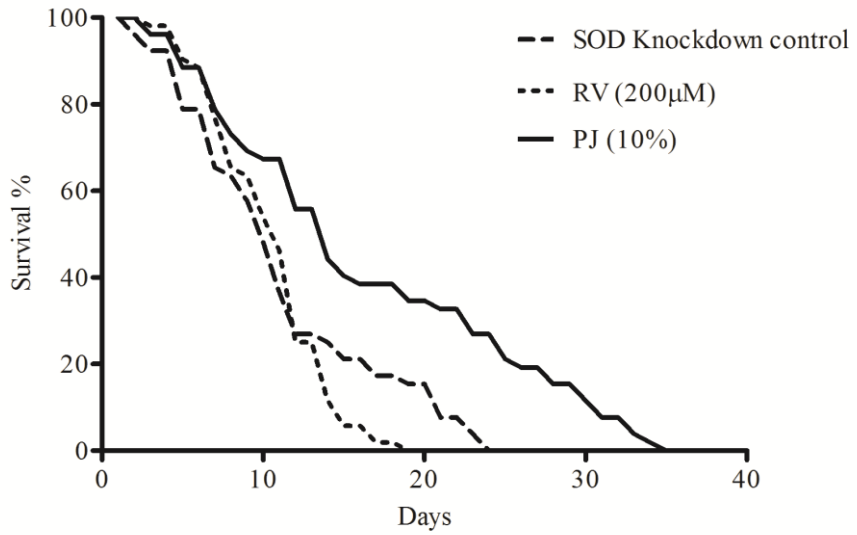


Figure 8.11. Effect of Pomegranate juice supplementation on longevity of Mn*SOD* knockdown *Drosophila*. Log rank test indicated significant ($p<0.005$) increase in the fly survival by both PJ and RV (table 2). But PJ increased the median survival by 41.93 % while it was 10.75 % in the RV group. The maximum life-span in the control flies was observed as 23 days, while in RV supplemented group maximum life-span was observed to be only 18 days. PJ treatment showed highest life-span of 34 days.

These observations indicate that the lifespan enhancing activity of PJ may be through the down regulation of the TOR pathway. Also, it appears PJ is capable of rescuing or compensating the loss of function of *FOXO*, since knockdown of *FOXO* gene did not affect PJ's activity. At least partial expression of *SOD* in the host is required for PJ's health protecting effect, since PJ could only partially rescue the *SOD* knockdown flies.

8.4 Discussion

Developing scientifically validated, culturally acceptable, appropriate guidelines for healthy eating is recognized as one of the thrust areas for reducing age-related functional decline (World Health Organisation, 2002). An Australian longitudinal analysis to guide health promotion concludes that health, life style, and gender influences healthy ageing (Kendig et al., 2014). Regular consumption of fruits is said to reduce the risk of age related functional decline (Jong et al., 2014). The biological effects of herbs are primarily linked to phytochemicals present in them (Si and Liu, 2014). In Ayurveda, *Rasayana*s are said to improve immunity, impart vitality, are good aphrodisiacs and are considered to delay the ageing process (Sharma, 2004; Balasubramani et al., 2011). Pomegranate has been mentioned in Ayurveda as a Rasayana with the above properties. Work summarized in this chapter has shown that PJ fed flies live and reproduce longer, are better protected against stress and infection and are active even when aged.

8.4.1 PJ extends longevity of flies

As per Ayurveda, of the *Rasayana* action of pomegranate provides satiation (*trupti*); i.e., the organism could be satisfied with lesser intake of food when supplemented with PJ. While there was no significant difference in the feed intake by RV and PJ fed groups, the latter out-performed the former in all lifespan and health span parameters tested. The current study corroborates earlier reports that resveratrol enhances lifespan in *Drosophila* (Bass et al., 2007; Kaeberlein, 2010). In this study, we have observed that RV increased median lifespan of *Drosophila* by 11%. RV substitution imparted comparatively lesser protective ability against H_2O_2, paraquat and infection stress to the flies when compared to PJ fed group. However, RV did not change the fecundity

of the flies as compared to the control, while PJ had enhanced and extended the fecundity of flies. Estimation of the feed intake showed a slightly reduced feed intake quantity (figure 8.3) for both PJ and RV supplemented groups when compared to the control. Earlier reports also indicate the possible dietary restriction by resveratrol (Bass et al., 2007). This observation was more marked in the male flies.

According to the antagonistic pleiotrophy theory, higher levels of reproduction are negatively correlated with survival. This concept of 'trade-off' between longevity and reproduction or "cost of reproduction" has been widely accepted and demonstrated in a number of experimental studies (Iliadi et al., 2012). The current experimental setup indicated that PJ is capable of simultaneously enhancing fecundity as well as survival. Ayurveda texts also indicate that, an efficiently practiced Rasayana is capable of improving overall wellbeing and quality of life of individual.

8.4.2 Potential molecular targets of PJ in flies

Earlier studies have shown that resveratrol enhances lifespan from yeast to mammalian models by down regulating *TOR* and activating *AMPK* (Kaeberlein, 2010; Johnson et al., 2013). Down regulation of *JNK* is also considered as pathway to controlling several pathological conditions (Cui et al., 2007). Roy et al., (2011) have shown that resveratrol inhibits the growth of orthotopic pancreatic tumors through activation of *FOXO* transcription factors. *FOXO* expression has an auto-feedback regulation and controls many other downstream targets including *SOD* (Xiong et al., 2011; Salih et al., 2008). Our observations with RV corroborate these findings. Interestingly, PJ substitution has shown significant down-regulation of both *FOXO* and *TOR* dependent pathways. An increased Mn*SOD* expression is strongly correlated to increase in lifespan (Curtis et al., 2007), but our observations with PJ shows

decrease in Mn*SOD* expression levels as well. POMx (a polyphenol rich commercial pomegranate fruit extract has been shown to inhibit inflammation by blocking *JNK* and other inflammatory pathway genes in human KU812 cells. In summary, lifespan enhancement by PJ in *Drosophila* might be through down regulation of *TOR* and possibly by compensating *FOXO* pathway.

8.4.3 Feeding behaviour

Feeding behaviour and nutritional constituents of a culture medium are important factors in the lifespan determination of *D. melanogaster* (Grandison et al., 2010). Bass et al., (2007) has reported that RV enhances life-span by dietary restriction in *Drosophila* and *C. elegans*. PJ too showed a slight reduction in the feed intake, but PJ fed flies outperformed the RV fed flies in longevity, fecundity, stress resistance, immunity and physical performance. The probable reason behind reduced food intake in the PJ group may be due to the '*Triptikra*' (satiating) property of pomegranate (Dash, 1994; Chunekar, 2004).

8.4.4 PJ imparts stress resistance to flies

Elevated resistance to various environmental stresses, including oxidative stress is one of the phenotypes of long lived mutant flies (Peng et al., 2011). The experimental observations indicate that, PJ feeding could play a role in ameliorating the free radical induced damage, which is a major reason for ageing (Faria and Calhau, 2011). Curcumin (Lee et al., 2010), apple polyphenols (Peng et al., 2011) and cranberry extract (Wang et al., 2014) are some of the herbs and fruits that have been reported to impart protection against H_2O_2 and paraquat induced free radicals in *Drosophila* and extend lifespan. The polyphenol and vitamin C content in pomegranate juice may be responsible for imparting the resistance against free radical induced stress (Wanget

al., 2010). A significant correlation has been observed with polyphenol content of pomegranate preparations to its anti-oxidant activity. Higher polyphenol content has been shown to enhance the antioxidant activity of pomegranate (Madrigal-Carballo et al., 2009).

8.4.5 PJ improves immunity in flies

Drosophila, although devoid of an adaptive immune system, harbors an innate immune response with striking similarities with mammalian defense mechanisms. The *Drosophila* innate immune response uses pattern recognition receptors to activate phagocytosis by plasmatocytes, proteolytic clotting cascades in the hemolymph and production of specific antimicrobial peptides (AMPs) in the fly fat body (Alarco et al., 2004). Generally, the expression of specific anti-fungal peptides is controlled by a signaling pathway orchestrated by the Toll-like receptor (TLR) (Chamilos et al., 2006). Protection against fungal infection in flies however may or may not involve TLR's (Alarco et al., 2004; Chamilos et al., 2006). The exact mechanism by which PJ exerted protection to the flies is yet to be conclusively elucidated. Some of the polyphenol rich herbal preparations like green tea extract and resveratrol have been shown to have different effect on the various classes of TLRs (Byun et al., 2012; Capiralla et al., 2012).

Protection against free radical induced stress, improved infection survival and climbing performance assays show that pomegranate supplementation helps in reducing age related functional decline in the flies. Sex specific differences in the lifespan are common response in *Drosophila*. Sex specific differences in the lifespan modulation has also been independently observed with dietary restriction (Magwere et al., 2004) and also by feeding an herbal extract (SC100) (Villeponteau et al., 2015).

This study justifies the Ayurvedic claims that pomegranate is a *Rasayana* by being a multi-functional health promoter. *Drosophila* has been a strong candidate as a model in contemporary ageing research. *D. melanogaster* has also been used as a model by researchers to study *Rasayanas*. An insect specific Rasayana was developed that was found to increase lifespan by 50% in *Drosophila* model (Priyadarshini et al., 2010). Dwivedi et al., (2012) reported that Amalaki *Rasayana*, an herbal formulation and Rasa-Sindoor (an organo-metallic derivative of mercury) was capable of increasing lifespan and fecundity in fruit flies.

Ageing population and the associated physical, physiological and neurological deficiencies are being recognised as the major challenges for healthcare in the 21st century while people are living longer; they are ageing prematurely (WHO & US National Institute of Ageing, 2011). Ageing is fundamentally more complex than many other problems that biologists study and would require a holistic approach like what Ayurveda offers and a systems biology approach for a better understanding. Scientific exploration of *Rasayana*, a specific branch of Ayurveda that deals with interventions for ageing (*jarachikitsa*) can provide better insights into the ageing process and also provide affordable ways to delay ageing and maintain good health through nutrition.

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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

Rasayana Karma* of *Dadima	**One of the Probable functional correlations**	**Function / Marker selected**	**Model used**
Agnivyapara (*Jataragni*)	Improves digestion & uptake of nutrients	Bioavailability of iron	Cell free & cell based (Caco-2 and HepG2) *in vitro* assays
Dhatuphoshana	Nourishes tissues	Ferritin	Caco2 and HepG2 cells
		Heme	Yeast
Panduhara (overall)	Anti-anemic	Iron status	Anemic Yeast (*Saccharomyces cerevisiae*)
Ayuvardhana	Life-span extension	Longevity	*Drosophila melanogaster* lifespan
Vayasthapana	Delay ageing	Delay in age related functional decline	*D. melanogaster* climbing assay
Vyadhikshamatva	Immunity	Resistance against infection	*Candida albicans* infection in *D. melanogaster*
	Stress resistance	Resistance against free radical stress	Paraquot and H_2O_2 induced stress in *D. melanogaster*
Vrshya	Fecundity	Reproductive ability	Off spring production in *D. melanogaster*

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 (Mn*SOD*) 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 Mn*SOD* 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 Mn*SOD*. 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 urolithin 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 (Kiefte-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.

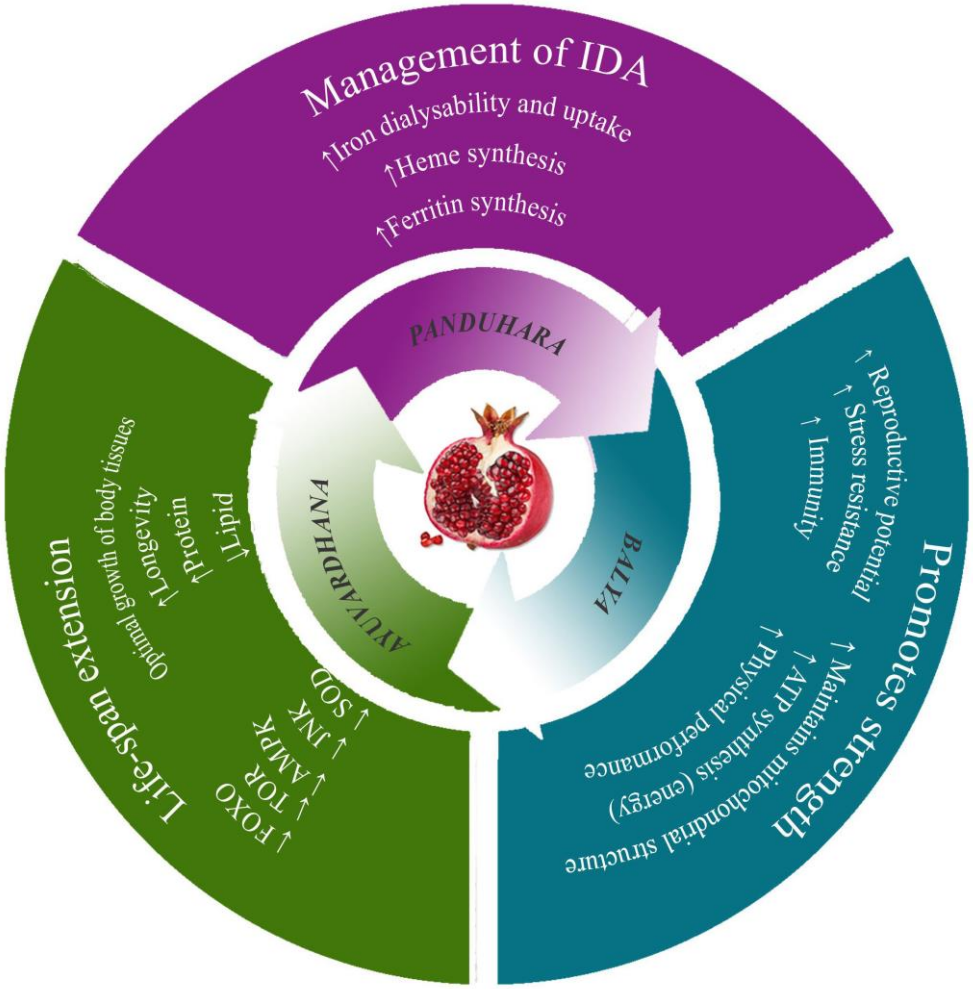

Figure 9.1: Effect of Pomegranate juice on IDA and wellness

Models have been used in this study for understanding certain aspects of *Rasayana karmas* like *agnivyapara* (digestion and metabolism), *dhatuphoshana* (improve quality of cells and tissues by providing nutrition), *panduhara* (anti-anemic), *vrshya* (reproductive potential), *ayuvardhana* (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.

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ANNEXURE I

Table showing RNA transcript accession numbers of the *Drosophila* genes used for semi-quantitative gene expression studies.

S. No	Gene Name	Accession no. of mRNA transcript variants
1	*AMPK*	NM_057965; NM_206604 and NM_166880
2	*JNK*	AF_061255
3	*FOXO*	NM_001275628; NM_206482; NM_206483 and NM_142073
4	Mn*SOD*	NM_057577
5	*TOR*	NM_001273974 and NM_057719

GLOSSARY OF AYURVEDA TERMS USED IN THE THESIS

Agni deepana / Agnivyapara	:	Improve digestive process
Analam	:	Digestive and metabolic processes
Agni	:	Energy involved in metabolic processes
Agnidipaka	:	Appetizer
Agnivardhaka	:	Improves digestion and metabolism
Ahara	:	Food
Ahara Parinamakara Bhavas	:	Process by which tissues are formed from food
Ama	:	Undigested or semi-digested food (toxin)
Amla	:	Sour
Apya	:	Water
Asthi	:	Bone tissue
Ayana	:	Channels of circulation
Ayuvardhana	:	Lifespan enhancement
Balam	:	Physical strength
Balya	:	Strength
Bhutagni	:	Energy which metabolizes each of the five elements
Brmhaniya	:	Increases body weight / bulk promotion
Dadima	:	Pomegranate
Dasa vidha pariksha	:	Ten factors examined to determine health of an individual
Dehagni	:	Energy responsible for life
Desham	:	Residing location
Dhamani	:	Blood vessels
Dhathupaka	:	Relationship between nutrition and tissues
Dhatu	:	Tissue
Dhatuposhana	:	Nourish tissues
Dhatuvrddhikara	:	Improve tissue structure and function
Dhatvagni	:	Metabolic force present in tissues

Dipana	:	Improve digestion
Dosha	:	Humors
Drvyaguna	:	Ayurveda pharmacology / functional property
Dushyam	:	Body tissues
Hrdaya	:	Heart
Indriyas	:	Sense organs
Jara chikitsa	:	Ayurvedic geriatrics
Jatharagni	:	Energy responsible for digestion and absorption
Jeevana	:	Life
Jivaniya	:	Life giving
Kalam	:	Seasons/ time
Kapha	:	Biological humor responsible for structure formation
Madhura	:	Sweet
Majja	:	Bone marrow
Mala	:	Essential by-products during tissue formation
Mamsa	:	Muscle
Mamsapushti	:	Nourishment of muscle tissue
Mandagni	:	Reduced digestive power
Meda	:	Fat
Nabhasa	:	Space
Oja	:	Life force
Pachakapitta	:	The humor that drives metabolic process
Pachana	:	Improve factors that digest food
Pandu	:	Anemia
Panduhara	:	Anti-anemic
Parthiva	:	Earth
Pathya	:	Food supplement

Pitta	:	Biological humor responsible for transformation
Poshana	:	Nutritive property
Prakriti	:	Genetic and phenetic constitution
Prana	:	Life energy
Rakta dhatu	:	Blood
Rasa dhatu	:	Plasma
Rasa	:	'Chyme' – essence of nutrition
Rasayana Karma	:	Rasayana actions
Rasayana tantra	:	Rasayana therapy
Rasayana	:	A branch of Ayurveda meant for rejuvenation
Rocana	:	Stimulating taste
Sama mala	:	Homeostasis in waste generation and elimination
Samana	:	Palliative treatment
Saptadhatu	:	Seven tissues
Satmyam	:	Habituation
Satvam	:	Mental strength or temperament
Shaarira	:	Anatomy and physiology
Snehana	:	Oleation therapy
Sodhana	:	Purification therapy
Srotaprasadana	:	Purify channels
Srotas	:	Micro- and macro- circulatory channels in body
Sroto shodhaka	:	Enhancement of micro-circulation
Srotorodha	:	Blockage of channels
Srotosodhana	:	Purification of channels
Sukra	:	Reproductive tissues
Svasthya	:	Wellness
Teja	:	Fire
Vamana	:	Emesis

Varna Prasadana	:	Complexion
Vata	:	Biological humor responsible for movement
Vaya	:	Age
Vayasthapana	:	Delaying ageing process
Vayavya	:	Vayu / air
Virechana	:	Purgation
Virya	:	Potency
Vipaka	:	Taste after digestion
Vrshya	:	Aphrodisiac
Vyadhikshamatva	:	Resistance to disease / immunity