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).
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).
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
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

<table>
<thead>
<tr>
<th>Common name</th>
<th>Scientific name</th>
<th>Aspects studied</th>
<th>Reference¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast</td>
<td><em>Saccharomyces cerevisiae</em></td>
<td>Genetic pathways and interventions</td>
<td>Gershon and Gershon, 2000</td>
</tr>
<tr>
<td>Star ascidian or golden star tunicate</td>
<td><em>Botryllus schlosseri</em></td>
<td>Immunology, stem cell biology, evolutionary biology and regeneration</td>
<td>Rinkevich et al., 2013; Murthy and Ram 2015</td>
</tr>
<tr>
<td>Ciona</td>
<td><em>Ciona intestinalis</em></td>
<td>Ageing and regeneration</td>
<td>Jeffery, 2012</td>
</tr>
<tr>
<td>Hydra</td>
<td><em>Hydra vulgaris, H. oligactis</em></td>
<td>Longevity and immortality</td>
<td>Bellantuono et al., 2015; Tomczyk et al., 2015</td>
</tr>
<tr>
<td>Monogonont rotifers</td>
<td><em>Brachionus manjavacas</em></td>
<td>Life-span and health span</td>
<td>Snell et al., 2014</td>
</tr>
<tr>
<td>Sea urchins</td>
<td><em>Strongylocentrotus franciscanus, S. purpuratus, Lytechinus variegatus</em></td>
<td>Longevity</td>
<td>Bodnar, 2009</td>
</tr>
<tr>
<td>Daphnia</td>
<td><em>Daphnia pulicaria, D. pulex</em></td>
<td>Stress and longevity, dietary restriction</td>
<td>Kim et al., 2014; Schumpert et al., 2014</td>
</tr>
<tr>
<td><em>C. elegans</em></td>
<td><em>Caenorhabditis elegans</em></td>
<td>Ageing mechanisms and interventions</td>
<td>Tissenbaum, 2015</td>
</tr>
<tr>
<td>Fruitfly</td>
<td><em>Drosophila melanogaster</em></td>
<td>Ageing mechanisms and interventions</td>
<td>Brandt and Vilcinskas, 2013; Balasubramanani et al., 2014²</td>
</tr>
<tr>
<td>Zebrafish</td>
<td><em>Danio rerio</em></td>
<td>Tissue regeneration, longevity and interventions</td>
<td>Gilbert et al., 2014</td>
</tr>
<tr>
<td>Turquoise killifish</td>
<td><em>Notho branchiatus furzeri</em></td>
<td>Ageing and disease</td>
<td>Kim et al., 2016</td>
</tr>
<tr>
<td>Mice</td>
<td><em>Mus musculus</em></td>
<td>Ageing mechanisms, age related diseases and interventions</td>
<td>Vanhooren and Libert, 2013</td>
</tr>
<tr>
<td>Naked mole-Rat</td>
<td><em>Heterocephalus glaber</em></td>
<td>Mechanisms of longevity, potential therapies</td>
<td>Gallagher et al., 2011</td>
</tr>
<tr>
<td>Monkey</td>
<td><em>Rhesus macaques</em></td>
<td>Age related pathology and interventions</td>
<td>Roth et al., 2004</td>
</tr>
</tbody>
</table>

¹References shown are only indicative
²Publication 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.](image-url)
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)

![Life cycle of *Drosophila melanogaster*](image)

**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***

<table>
<thead>
<tr>
<th>Diet source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prunetin (isoflavone)</td>
<td>Piegholdt et al., 2016</td>
</tr>
<tr>
<td>Naringenin (bioflavonoid)</td>
<td>Chattopadhyay et al., 2016</td>
</tr>
<tr>
<td>Herbal extract-SC100 (<em>Astragalus membranaceus</em> root, <em>Pterocarpus marsupium</em> bark, pine bark oligo-proanthocyanidins, and L-theanine)</td>
<td>Villeponteau et al., 2015</td>
</tr>
<tr>
<td>Proanthocyanidins from <em>Kunlun Chrysanthemum</em> flowers</td>
<td>Jing et al., 2015</td>
</tr>
<tr>
<td>Cinnamon</td>
<td>Schriner et al., 2014</td>
</tr>
<tr>
<td>Nordihydroguaiaretic Acid (a lignin from <em>Larrea tridentate</em>)</td>
<td>Spindler et al., 2015</td>
</tr>
<tr>
<td>Curcumin</td>
<td>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</td>
</tr>
<tr>
<td>Green tea polyphenols</td>
<td>Lopez et al., 2014</td>
</tr>
<tr>
<td>Artemisinin and curcumin</td>
<td>Das et al., 2014</td>
</tr>
<tr>
<td><em>Curcuma longa</em> and <em>Emblica officinalis</em></td>
<td>Rawal et al., 2014</td>
</tr>
<tr>
<td><em>Ludwigia octovalvis</em></td>
<td>Lin et al., 2014</td>
</tr>
<tr>
<td><em>Viscum album coloratum</em> (Korean mistletoe)</td>
<td>Lee et al., 2014</td>
</tr>
<tr>
<td>Cranberry</td>
<td>Sun et al., 2014; Wang et al., 2014</td>
</tr>
<tr>
<td>Pectin</td>
<td>Shaposhnikov et al., 2014</td>
</tr>
<tr>
<td>Lutein (carotenoid)</td>
<td>Zhang et al., 2014</td>
</tr>
<tr>
<td><em>Rhodiola rosea</em></td>
<td>Schriner et al., 2013</td>
</tr>
<tr>
<td>Organically raised bananas, potatoes, raisins and soy beans</td>
<td>Chhabra et al., 2013</td>
</tr>
<tr>
<td><strong>Incarvillea younghusbandii</strong> root extract</td>
<td>Pan et al., 2012 Pan et al., 2008</td>
</tr>
<tr>
<td>-------------------------------------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>Sesamin</td>
<td>Zuo et al., 2013</td>
</tr>
<tr>
<td>Black rice</td>
<td>Zuo et al., 2012</td>
</tr>
<tr>
<td><em>Cynomorium songaricum</em></td>
<td>Liu et al., 2012</td>
</tr>
<tr>
<td>Amalaki Rasayana</td>
<td>Dwivedi et al., 2012</td>
</tr>
<tr>
<td><em>Emblica officinalis</em></td>
<td>Pathak et al., 2011</td>
</tr>
<tr>
<td>Blueberry extract</td>
<td>Peng et al., 2012</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>Bass et al., 2007; Wang et al., 2013</td>
</tr>
<tr>
<td><em>Rosa damascena</em></td>
<td>Schriner et al., 2012</td>
</tr>
<tr>
<td><em>Aloe vera</em> and resveratrol</td>
<td>Chandrashekara et al., 2011</td>
</tr>
<tr>
<td>Orange and lemon juice</td>
<td>Fernández-Bedmar et al., 2011</td>
</tr>
<tr>
<td>Epicatechin</td>
<td>Si et al., 2011</td>
</tr>
<tr>
<td>Glucose and polyphenols</td>
<td>Ortega-Arellano et al., 2011</td>
</tr>
<tr>
<td>Nectarine</td>
<td>Boyd et al., 2011</td>
</tr>
<tr>
<td>Apple polyphenols</td>
<td>Peng et al., 2011</td>
</tr>
<tr>
<td>Genistein</td>
<td>Altun et al., 2011</td>
</tr>
<tr>
<td>Water and ethanol extracts of <em>Stachys lavandulifolia</em></td>
<td>Altun et al., 2010</td>
</tr>
<tr>
<td>Rasayana diet</td>
<td>Priyadarshini et al., 2010</td>
</tr>
<tr>
<td>Black tea theaflavins</td>
<td>Peng et al., 2009</td>
</tr>
<tr>
<td>Cocoa</td>
<td>Bahadorani and Hilliker, 2008</td>
</tr>
<tr>
<td>Caffeine</td>
<td>Nikitin et al., 2008</td>
</tr>
<tr>
<td>Green tea catechins and broccoli</td>
<td>Li et al., 2008</td>
</tr>
<tr>
<td>Rhodiola</td>
<td>Jafari et al., 2007</td>
</tr>
</tbody>
</table>
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)**

<table>
<thead>
<tr>
<th>Group</th>
<th>Cut-off value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Children aged 0.5-5 years</td>
<td>&lt;11.0 g/dl</td>
</tr>
<tr>
<td>Children aged 5-11 years</td>
<td>&lt;11.5 g/dl</td>
</tr>
<tr>
<td>Children aged 12-13 years</td>
<td>&lt;12.0 g/dl</td>
</tr>
<tr>
<td>Men</td>
<td>&lt;13.0 g/dl</td>
</tr>
<tr>
<td>Non-pregnant women</td>
<td>&lt;12.0 g/dl</td>
</tr>
<tr>
<td>Pregnant women</td>
<td>&lt;11.0 g/dl</td>
</tr>
</tbody>
</table>
Chapter 3

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).

---

**Figure 3.6: Stages in the development of iron deficiency**

- **Iron Deficiency Anemia (IDA)**: Blood haemoglobin concentration falls below the lower limit of normal.
- **Latent**: Iron stores are exhausted, but the blood haemoglobin level remains normal.
- **Prelatent**: Reduction in iron stores without reduced serum iron levels.
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

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

*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 (37°C) and with pH2. The second step simulates intestinal phase, where digestion at physiological temperature (37°C) 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 understanding (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 multicellular 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 \textit{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 \textit{S. cerevisiae}**

The steps involved in iron metabolism in \textit{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, \textit{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
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.

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

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


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 disulphonic 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.
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)

<table>
<thead>
<tr>
<th>Form of Iron</th>
<th>Fe normal medium</th>
<th>Fe deficient medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe$^{2+}$</td>
<td>~100 µM</td>
<td>Present</td>
</tr>
<tr>
<td>Vacuolar Fe$^{3+}$</td>
<td>~300 µM</td>
<td>Absent</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>Fe$^{2+}$ ions and substantial amounts of Fe$^{3+}$ nanoparticles</td>
<td>Fe-S clusters and Fe$^{3+}$</td>
</tr>
<tr>
<td>Fe content</td>
<td>400 – 450 µM (even at 250-fold excess Fe in media)</td>
<td>~150 µM</td>
</tr>
</tbody>
</table>
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](image)

3.5.1 Taxonomy

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Plantae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phylum</td>
<td>Angiosperms</td>
</tr>
<tr>
<td>Class</td>
<td>Eudicots</td>
</tr>
<tr>
<td>Sub-class</td>
<td>Rosids</td>
</tr>
<tr>
<td>Order</td>
<td>Myrtales</td>
</tr>
<tr>
<td>Family</td>
<td>Punicaceae</td>
</tr>
<tr>
<td>Genus</td>
<td>Punica</td>
</tr>
<tr>
<td>Species</td>
<td>granatum</td>
</tr>
<tr>
<td>Botanical Name</td>
<td>Punica granatum L.</td>
</tr>
</tbody>
</table>


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)

![Diagram of Tannins present in PJ](image)

**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 antioxidants 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)

![Chemical structures of flavonoids](image)

**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).

![Chemical structures of phenolic acids](image)

**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](image1)

![Serotonin](image2)

![Melatonin](image3)

**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)
Figure 3.14: Organic acids present in PJ
3.5.3.4 Sugars

Sugar content of juices are expressed in °B (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 °Brix (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).

![Glucose and Sucrose structures]

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).
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

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


Hidalgo IJ, Raub TJ, Borchardt RT. 1989. Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. Gastroenterology 96(3):736-49.


Rom O, Aviram M. 2016. Paraoxonase2 (PON2) and oxidative stress involvement in pomegranate juice protection against cigarette smoke-induced macrophage cholesterol accumulation. Chemical Biological Interactions 6. pii:S0009-2797(16)30179-X.


Wu TF, Hsu LT, Tsang BX, Huang LC, Shih WY, Chen LY. 2016. Clarification of the molecular pathway of Taiwan local pomegranate fruit juice underlying the inhibition of urinary bladder urothelial carcinoma cell by proteomics strategy. BMC Complementary and Alternative Medicine 16:96.


