FUNCTIONAL BIOAVAILABILITY OF IRON RICH GREENS; UTILISATION AND ACCEPTABILITY

(In vivo study)

A Thesis submitted in partial fulfillment of the requirements for the award of the degree of

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(NUTRITION AND DIETETICS)

By

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CANDIDATE DECLARATION

I hereby certify that the work, which is being presented in the thesis, entitled FUNCTIONAL BIOAVAILABILITY OF IRON RICH GREENS; UTILISATION AND ACCEPTABILITY *(In vivo* study*)* in partial fulfillment of the requirement for the award of degree of Ph.D. (Nutrition and Dietetics) submitted in the School of Agriculture at Lovely Professional University, Phagwara is an authentic record of my work carried out under the supervision of Dr. Prerna Gupta, Assistant Professor, Department Of Food Technology And Nutrition, Dr. Jasvinder Singh Bhatti, Associate Professor, Department of Human Genetics and Molecular Medicine, School of Health Sciences, Central University of Punjab, Bhatinda. The matter presented in this thesis has not been submitted in any other University / Institute for the award of PhD Degree.

(Namrata Sethi)

This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

The PhD Viva-Voce Examination of Namrata Sethi has been held on _____

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Signature of External Examiner

CERTIFICATE

Dated:

This is to certify that the thesis entitled FUNCTIONAL BIOAVAILABILITY OF IRON RICH GREENS; UTILISATION AND ACCEPTABILITY *(In vivo study)* is a bonafide research work carried out by Ms. Namrata Sethi, student of PhD (Nutrition and Dietetics), Lovely Professional University, Phagwara, under my guidance towards the partial fulfillment of the degree in Ph.D. (Nutrition and Dietetics) of Lovely Professional University, Phagwara. This work has not been published or submitted elsewhere for the award of any degree.

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ABSTRACT

Anemia is a severe public health problem prevalent amongst all age groups and affecting varied populations. Despite government policies and initiatives, it has not been curbed. Therefore, the present study aimed to assess the prevalence of anemia in Chandigarh (Rural & Urban) and screen different low-cost green leafy vegetables for iron content using various processing techniques. The study also evaluated the functional bioavailability of the selected green (*in vivo* study) and further developed and assessed a low-cost, functional iron-rich product along with a shelf life assessmentof the developed product.

A survey method was used to study the prevalence of anemia and justify the need for an iron-rich dietary supplement. The survey was conducted on pregnant women from rural and urban areas of Chandigarh. Results reported that more than 50% of pregnant women belonging to the rural population suffered from anemia (50.4%), followed by 44.5% of women affiliated with the urban population. Socio-economic status recorded maximum number of women suffering from moderate anemia belonged to the upper lower class (48.4%). Findings also revealed a direct association between the presence of anemia and socio-economic status.

Five locally available underutilized green leafy vegetables were selected for the presentstudy. Results of the screening of these vegetables for their iron content documented that S1 (Cauliflower greens - *Brassica oleracea var. botrytis*) had the maximum contentof iron 39.25 ± 0.05mg/100g followed by S3 (Amaranth - *Amaranthus viridis* -16.91 ± 0.09), S5 (Radish leaves - *Raphanus raphanistrum subsp. sativus* - 16.75 ± 0.07), S4 (Lettuce - *Lactuca sativa* -1.99 ± 0.01) and S2 (Spinach - *Spinacia oleracea* -1.10 ± 0.03). The standardization of blanching time and temperature was done for dip blanching and microwave blanching by qualitative analysis of peroxidase activity. It was observed that for dip blanching after 3 minutes at 90℃, there was a reduction in peroxidase activity and after 1 minute for microwave blanching. Furthermore, the phytochemical profile of the vegetables was assessed and effect of tray drying was observed.

Cauliflower greens recorded maximum retention of iron 83.33 to 96.65 % and showed high levels of anti oxidant activity (91.65%) and total phenol content (210.60 mg GAE/100g). Amongst the green leafy vegetables, on drying, the ascorbic acid content and beta carotene content of all the samples reduced, but a significant increase was observed in the phenol content and anti oxidant activity. Additionally, screening of the green leafy vegetables was conducted by assessing their anti-nutrient content for fresh as well as dried leaves. Radish leaves had as high as 26mg/100mg of phytic acid, while cauliflower contained quite a good amount of oxalate and alkaloid (97mg/100mg and 72mg/100mg respectively). This was significantly reduced on blanching and drying. The present study revealed that cauliflower greens had a high antioxidant activity and low levels of anti nutritional content, furthermore, the iron content provided by cauliflower amounted to 39.25gm/100mg,making it the most promising amongst all greens.

For testing the bioavailability of iron in cauliflower greens, 36 female Wistar rats, aged2-3 months, weighing 180–250g were utilized. All the experimental animals were divided into 8 groups. Half of the rats were made anemic by the bleeding method. Control and treatment groups I included rats with normal hemoglobin levels and controland treatment group II included anemic rats. Both the treatment groups were fed a supplement of cauliflower greens in varying doses along with the basal diet for aperiodof 60 days. The current study demonstrated a significant difference in body weight of experimental rats after 45 and 60 days of basal diet. An increase in weight of12 and 18%was observed in control group I and in anemic rats control group II when fed with the basal diet. Results revealed that treatment groups I and II of both normal and anemic rats spotted an increase in weight by 15%. However, anemic rats who were fed low, medium and high dose supplement exhibited an increase by 33%, 34% and 38% respectively in their hemoglobin levels as compared to low rise (11-17%) in hemoglobinlevels of normal rats fed with basal diet and supplement. This significant difference owes up to rich iron supplementation. Similar increase was observed in the red blood cell count of the groups following the hemoglobin levels. All the treatment groups reported a significant increase in the red blood cell counts. Furthermore, fecal excretionhas also been placed as an indicator to determine iron absorption in the body. Findingsrevealed that normal groups had lesser absorption (24.64 – 50.37%) than the anemic groups (33.65 – 57.53%). Additionally, high absorption was remarked in treatment groups A (50.37% and57.53%), which were fed high dose supplements. The effectiveness of supplementation is also discerned in the study when testing the locomotor activities of the rats. The anemic treatment groups depicted a higher level of activity than control groups and groups of normal rats fed supplement.

The potency of cauliflower greens, depicted by *in vivo* study, paved the way for product development optimized using response surface methodology. Twenty combinations of cauliflower powder, sugar, and lemon juice were analyzed based on iron content and overall acceptability. Meeting the minimum requirement of iron demands by the body, this beverage provides 8.93mg/100ml of iron. Being rich in lemon juice, the beverage was found to contain a sufficient quantity of Vitamin C (12.40mg/100ml). Sensory scores of the study depicted thatthe beverage was found to be highly acceptable in all parameters with an overall acceptabilityscore of 8.25±0.04. Based on sensory and microbial properties, it was observed that the maximum shelf-life of the beverage was up to 7 days under ambient conditions. The initial microbial and yeast load was 1.68±0.49 Log CFU/ml and 1.85±0.14 Log CFU/ml which increased up to 3.02±0.31Log CFU/ml for total plate count (TPC) and 3.15±0.04 Log CFU/mlfor total yeast and mold count (TYMC) on 7^{th} day. The overall acceptability score decreased from 8.25±0.04 to 6.00±0.03. The average shelf life of the beverage stored under refrigeratedconditions was up to 40 days, with an average overall acceptability score of 7.50±0.03. The TPC at the end of 40 days was 3.10±0.07 Log CFU/ml and the TYMC was found to be 3.19±0.07 Log CFU/ml.

To conclude, this study manifested the need for a low-cost iron-rich supplement to address the growing problem of iron deficiency in women and children. The underutilized cauliflower greens may provide a cheap raw material,that can be furtherprocessed and usedfor product development. The efficacy of cauliflower leaves that increase the hemoglobin levels was estimated from the *in vivo* study, proving that it has high bioavailable iron content.Moreover, product development using cauliflower leaves resulted in developing a beveragewith high overall acceptability and storage lifeof 40 days at refrigerated temperature. Hence,the findings of this study will go a long way in contributing towards the elimination of publichealth problem of anemia in India.

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(Namrata Sethi)

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CHAPTER 1

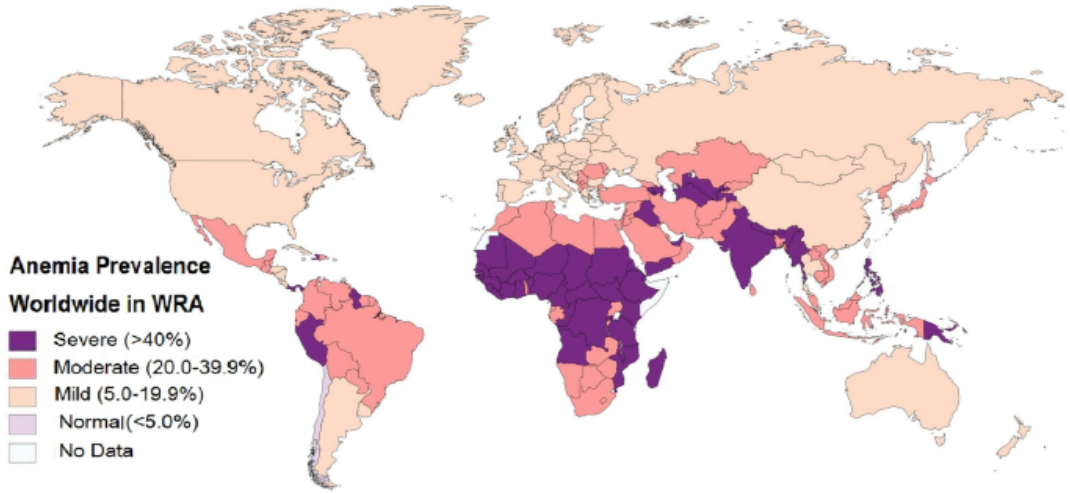
INTRODUCTION

Nutrition is a key determinant of health in today's world. From growth to development, the role of nutritious food for good health has been well established (Bennet et al. 2015, Ohlhorst et al. 2013, Slawson et al. 2013). Diet holds a vital place in determining not just the genetic makeup of an individual but also the aging process (Khandelwal et al. 2013). With the change in lifestyle and eating habits, diseases, especially non-communicable diseases (NCDs), have significantly increased especially in the last decade. WHO (2016) reported that 41 million people die yearly due to NCDs, which account for 71% of the deathsworldwide. NCDs affect all age groups, especially vulnerable populations in countries comprising of low as well as middle-income groups.

Amongst non-communicable disease, anemia is one disease which has become a concern for both developed and countries under development all over the world. (Jatav et al. 2014). As reported by WHO (2016), 1.62 billion people worldwide suffer from anemia which accounrs for 24.8% of the total population, with India being one of the countries having the maximum number of anemic people (more than 52%) at any given time (National Family Health Survey, 2016). Due to India's geographic and cultural variations, the prevalence of anemia varies from state to state (Nguyen et al. 2018). Causative factors for anemia ranges from low to deficient iron and folic acid intake (Bhatia, 2013). However, low socio economic status, where one can't afford nutritious food, lack of nutrition education also equally contributes to the risk factors of anemia. (Rengma et al. 2015 and Ali et al. 2016). Several socio-demographic factors, including location, gender, ethnicity, or income, also affect the nutritional status of the individuals (Pasricha et al. 2010). Developing countries face a risk of anemia due to nutritional deficiency, blood loss, and malaria (Scully et al.2020).

Figure 1.1:

Conceptual diagram of the prevalence of anemia worldwide



Source: Menshawey et al. (2020)

Anemia is classified into various types such as deficiency due to iron, sickle cell, aplastic and hemolytic anemia. Of all the anemia, iron deficiency anemia is most prevalent and affects a significant number of populations worldwide (CSDH 2008). Most affected anemic individuals are infants, adolescent girls, and pregnant women (Hamlin & Dada 2011). The role of iron and especially green leafy vegetables to improve the status of anemia is indisputable. Green leafy vegetables form an important part of any well-balanced diet. They're high in minerals and vitamins and have a lot of micronutrients in a single serving (Gupta & Parkash 2009). Micronutrients such as calcium and iron are found more in greens than any other food group (Natesh et al. 2017). Despite their high nutritive value, many unexploited foods are available in India, which are not used commonly and belong to the category of underutilized plants (Subhashree et al. 2009, Sheela et al. 2004). Typical Indian diets provide only 25% of the iron needs (ICMR, 2010). Some anti nutritional components found in cereals and other foodstuffs, hamper iron absorption (Gemede & Ratta 2014). To absolve this problem, some basic cooking processes like blanching and drying can be adopted. Still, bioavailability is not solely dependent on the concentration present in food but also its constituents (Amagloh et al. 2012).

Cauliflower leaves are one such underutilized green leafy vegetable which is discarded and not used in the diet. It is the richest source of iron amongst green leafy vegetables providing upto 40mg/100gm (Gopalan et al. 1989). Efficacy and bioavailability of green leafy vegetables have been tested in vivo. Animal models were observed to have increased iron absorption especially in anemic animals. It has been reported by Gheith & Mahmoudy (2018), Srinivas et al. (2013), Joshi et al. (2017) and Baskaran et al. (2019) that due to high iron content of greens, they play an important function in increasing hematopoietic activity and hemoglobin levels. Multiple studies have been conducted on cauliflower greens as an effective product in helping cut down on anemia. Hence, efforts are needed to include such dietary sources that have a high acceptability across all age groups keeping sensory parameters in mind.

Interestingly; functional beverages are one class of food products which find high acceptance amongst all sections of the society. They are most acceptable because of convenience of use, attractiveness due to size, shape and content of the container as well as ease of storage and shelf life. Not just this, by means of a functional beverage, all nutrients, bioactive compounds and probiotics can be delivered at one time (Corbo et al. 2014). A cost effective nutritional supplement of high acceptability available for all age groups and sections of the society is the need of the hour. Although iron supplementation and fortification are commonly adopted strategies for anemia, they still have ill effects like overdose and food oxidation (Lazaro et al. 2017). The present study aimed to evaluate the presence of anemia in Chandigarh population. Further, screening of iron rich green leafy vegetables was done to select and prepare one low cost ready to serve iron rich beverage that can be made available to all sections of the population through government intervention programs.

CHAPTER 2

REVIEW OF LITERATURE

Anemia is a highly prevalent non- communicable disease that has the highest mortality rate especially amongst the vulnerable groups, including pregnant women, mothers who are lactating and adolescent girls. Amongst the several factors, lack of awareness and intake of improper diet are the leading cause of this menace. The review has been divided into various areas based on the data collected and topics which are a part of the study, namely:

- Role of nutrition in onset of diseases
- Prevalence of Anemia and Associated factors
- Use of Green Leafy Vegetables as Functional Foods
- Anti-Nutritional Content of Leaves
- Methods to Improve Bio Availability of Iron
- In Vivo Studies Conducted to Test Bioavailability of Iron Rich Product
- Product Development Using Iron Rich Foods

Role of nutrition in onset of diseases

Nutrition as a science has really evolved over the past few years. Nutritionists are dealing with problems at both ends of the spectrum wherein the world is hovering between under and over nutrition (Zeevi et al. 2015 and Schulze et al. 2018). Overtime the intake of calorie rich but nutrient dense foods has reduced considerably, thereby, increasing obesity and micronutrient deficiencies (Mozaffarian 2016). Some factors responsible for lack of proper nutrition play a role in disease development, including socioeconomic status, ignorance, lack of knowledge, availability of resources, etc. The need of the hour is personalized interventions with special emphasis on low-cost, micronutrient-rich foods to strike a balance between obesity and diseases (Shao et al. 2017).

There is a direct correlation between diet and disease development. Fruits and vegetables constitute a significant part of the diet, are also found to play a role in helping eliminate signs and symptoms of degenerative diseases (Heffron et al. 2017). Skerrett et al. (2010) have observed that consumption of high amounts of trans fats increases the chances of developing diabetes, gall bladder stones, obesity, and cardiovascular diseases as also reported by Wanders et al. (2017). Also, consumption of a high amount of processed foods which is devoid of fiber and rich in refined carbohydrates results in an increase of triglyceride levels. Peluso et al. (2018), conducted a study and reported that a diet providing ample amounts of fibre rich vegetables and fruits can help fight against degenerative diseases, especially cancer due to their rich phytonutrients content. 38 subjects were fed a fibre rich diet including good amounts of fruits and vegetables and one group was given a lesser intake of these fibre rich foods. It was observed that groups on high vegetable consumption showed better antioxidant markers in the body. Similar studies have also been reported by Dhandevi et al. (2015) that reported that despite of having multiple advantages, the intake of fibre rich fruits and vegetables is still inadequate. Miller et al. (2017) conducted a cohort study on 135,335 individuals and observed that a minimum of 3 to 4 servings were successful in helping eliminate the chances of development of cardiovascular diseases.

Ridder et al. (2017) recorded that the general population at large fails to understand dietary recommendations and follow a healthy eating plan, as seen in most industrialized countries. Assessment of food consumption patterns revealed that only one-third of the subjects consumed a diet following healthy eating guidelines. The main finding of the study revealed that socio economic status was the single largest factor responsible for faulty eating habits. There is a rapid increase in the number of cases of NCD's. In a global update by WHO (2016), it was reported that 40% of the adult population of the world is overweight due to improper dietary habits. A balance needs to be maintained between the amount consumed and utilized to ensure homeostasis and keep the body disease-free.

Garg et al. (2015) stated that the biggest challenge in today's world is to improve the health of individuals, when they are either consuming more or having fewer calories. Intervention programs, to a great extent have contributed to eliminate diseases

such as pellagra caused due to deficiency of niacin, scurvy due to vitamin C and rickets due to vitamin. On the other hand, diseases like diabetes, obesity, and other lifestyle-related diseases are spreading like an epidemic. Efforts must be made to understand the role and importance of nutrigenomics, making diets healthier and individual specific.

In another study conducted by Shridhar et al. (2015), it was recorded that the modern ways of eating and modern diets have drastically affected the health of individuals. One such substance is dietary fat, which is known to have an association with metabolic functioning and body composition. A diet rich in fat and poor in fruits and vegetables has a major impact on the health status of an individual. The shift in eating habits over the years has resulted in chronic health problems. Gopalan (2019) also evaluated the changing trends in the Indian scenario and concluded that the link between obesity and chronic diseases further leads to non-communicable diseases. In the past decade, a shift in lifestyle diseases from urban to rural populations has made the burden of diseases rise rapidly. Efforts are needed to preserve the people to ensure maximum potential from the masses for the development of the nation.

Aside from inadequate dietary intake, many other factors have also been associated with degraded nutritional status. It has been well established that lack of sanitation and hygenic facilities such as drinking water, housing conditions and drainage make children and adolescents vulnerable to infections (Mulugeta et al. 2009). Bomela (2009) studied the economic, social, health, and factors related to environment in Central Asian republics affecting a child's nutritional status. The study results revealed that the family's financial status, maternal education status, country they resided in, size of the family, sibship size and availability of drinking water were significant predictors of poor nutritional status. Haboubi & Shaikh (2009) also assessed the nutritional status of 2459 adolescents ranging in age from 10-16 years of Indian origin living in India and UAE to see the effect of different variables such as demographic and socio economic on their nutritional status. The study concluded that the participants belonged to similar ethnicities, but a difference in the nutritional and growth status was observed that could be attributed to the difference in their socio- economic status.

Prevalence of anemia and associated factors

Nguyen et al. (2018) scrutinized the trends in presence of anemia among the vulnerable groups in India. According to NFHS 3 and NFHS 4 reports, the data was assembled the to depict change in the condition. Sampling design at two-stages was done which was further divided to have rural and urban samples. The sample encompassed 2,45,346 children below 6 years, 37,165 pregnant women and 7,60,460 non-pregnant women in the reproductive age. They concluded that hemoglobin and anemia levels improved significantly in children (4.5 g/L) whereas in pregnant women the level was improved by 3.2 g/L. This improvement can be owed to health and nutrition interventions in children. At the same time, education and money seemed to have improved the hemoglobin levels amongst pregnant women. Therefore, it can be concluded that it is essential to generate awareness through appropriate educational techniques, especially for women, as they bear and rear children. A better socio - economic status, improved water, hygiene, and sanitation practices can further accelerate good health.

Insufficient intake of diet is also a major factor contributing to onset of anemia. Bandyopadhyay et al. (2017) explored that anemia is a standout amongst the most well-known and obstinate dietary issues universally influencing nations with significant outcomes on an individuals' well-being just as social and economic development. Youngsters especially adolescents were reported to be at a high danger of iron insufficiency and anemia because of quickened increment in the need for iron, combined with poor dietary admission, high rate of contamination, and worm pervasion. The study here intended to survey the effectiveness of health training mediation on benchmark learning of anemia avoidance among school-going teenagers. In this quasi-experimental intercession study, before and after comparison, groups were taken and led in one secondary school of West Bengal. The study was conducted among 181 students of standard VIII and IX by regulating a pre-structured questionnaire dependent on the accompanying five spaces – the importance of wearing slippers, health benefits of eating a balanced diet, personal hygiene, details about anemia and its management. Out of the 181 understudies, 84 (46.4%) were from Class VIII, and the rest in Class IX where about 86 (47.5%) were boys and 95 (52.5%) were girls; with

70.2% in age group of 14– 16 years'. Knowledge concerning etiology and harmful effects of anemia, vegetable and animal wellspring of iron, vitamin rich natural products, and foods avoiding iron assimilation impressively improved in post-test. It was further evaluated that formation on the utilization of shoe was essentially superior to preliminary stage ($P < 0.001$). Concerning the learning on handwashing, paired sample t test demonstrated a huge mean difference among the respondents after the mediation ($P < 0.001$).

In another study Rishi et al. (2016) students from South India studying in a university were analyzed. This study was conducted in Chennai, Tamil Nadu, India between January 2016 to July 2016. The sample size comprised of 568 students falling in the age group of 18 to 45 years who were studying Health Science subjects took part in the study, with 113 males and 455 females. A higher prevalence was found amongst female students as compared to male students (97 percent vs. 68 percent, p=0.0001). Anemia was present in 43 percent however, only a negligible percentage of students had severe anemia. Similar results of high prevalence amongst adolescents were reported by Durrani (2019), Webb et al. (2018), Goyal et al. (2018) and Patil et al. (2018). Intriguingly, not only in adolescents, but a high prevalence of anemia was also observed amongst school going children as well.

Garg & Bhalla (2016) assessed schools of rural area (Jand sahib) of Faridkot District, India, to study the presence of anemia amongst students attending those schools. In a cross- sectional survey from January 2010 to December 2010 in households was conducted on 463 students in rural schools. Children selected were aged between 5-16 years and through systematic random sampling method they were selected. After analysis and interpretation of data, it was found that amongst males, 91.69% were anemic and on the other hand, amongst females, 94.89% were anemic. Overall incidence of anemia among males and females was recorded to be 85.74%. Therefore, findings revealed that the incidence of anemia tends to be very high in school going children. Mengistu et al. (2019), Khan et al. (2017) and Kumari et al. (2017) reported similar findings.

In another cross – sectional study by Basu et al. (2016) residing in the district Amroha, Uttar Pradesh, anemic status along with the related socio demographic factors

of 604 adolescent girls was assessed. 69.2% girls were found to be anemic. While the prevalence of anemia based on the categories of mild, moderate and severe was 39.7%, 28.3% and 1.2% respectively. A significant association was found between low socio-economic status, large family size and poor education of the parents and anemic status of the girls.

From the year 2003 to 2012, Joo et al. (2016) extensively examined the data of five cycles of NHANES, with the objective to find out the current rate of anemia in the US population based on the different categories. The NHANES cycle conducts surveys every two years, which included males and females in ratio almost equal to 1:1. Around 776 pregnant women were also a part of the study separately. The tabulation of data was done by following the definitions of anemia on the basis of hemoglobin (serum Hb) as per World Health Organization. The results of the research observed that out of nearly 41,000 people, 5.6% of the US population were anemic and 1.5% were moderate-severe anemic during the decade 2003-2012. Anemia was found to range from 3.5% and for moderate - severe 0.5% respectively, and in females was 7.6% and 2.5% respectively. Anemia in males increased consistently, whereas, in women, the increase was primarily in two phases- 40-49years and 80-85 years. While Hispanics showed higher rates of the ailment as compared to the whites. The overall prevalence of anemia and moderate-severe anemia has doubled from 4% to 7.1%, and from 1% to 1.9% respectively in the years 2003–2004 and 2011–2012. Therefore, it can be concluded that anemia is not just a concern for public health in third world countries but also in first world countries. Nutritional and lifestyle interventions can aid in such grave situations. Hence, generating awareness about iron rich foods, its consumption and ways to increase its bioavailability along with reduction in smoking and alcohol consumption may lead to a better lifestyle and holistic health.

Mangla & Singla (2016) investigated anemia prevalence in total of 853 pregnant women (with pregnancy of more than 37 weeks) in rural India and this investigation was carried out using Hemoglobin estimation. According to ICMR standards, anemia was found in 98 percent of pregnant women in this rural Indian location. The results categorized as mild, moerate, severe and very severe anemia reported 41.76 percent, 37.05 percent, 15.88 percent and 3.29 percent respectively. The mean hemoglobin was

also determined to be 8.845, concluding that higher prevalence of anemia is observed amongst rural areas. Gupta et al. (2013) conducted a study in which anemia was investigated in 1221 stable young males and females from rural Punjab. During health check-up camps in villages around Bathinda in rural Punjab. The prevalence of anemia was assessed in males belonging to the age group of 5-20 years and females (5-30 years), taking into account their BMI, activity profile, and socioeconomic status. Hemoglobin test was conducted by Cyan methemoglobin method while the results were analyzed on a percentage scale. The findings reported that females (5-30 years) had anemia at a rate of 89.5 percent, with 49.8 percent, 38.2 percent having moderate, and 1.5 percent having mild, moderate and severe anemia repectively, while males (5-20 years) had anemia at a rate of 89.9 percent, with 51.2 percent, 38 percent and 0.7 percent under the same categories. Males and females who had lesser weight (low BMI) and lived a sedentary lifestyle and belonged to low income groups showed higher prevalence. Furthermore, they recommended a well -planned study consisted of anthropometry and dietary intake parameters. Moreover, they concluded that such data can give a better insight and help devise interventions for all age groups, irrespective of gender. Similar results were observed by Little et al. (2018) who reported 57.2% women suffering from anemia in a rural setting South India and Shedole et al. (2017) reported that more pregnant women were recorded as being anemic as compared to men or children.

The most vulnerable group affected by anemia include preschool children and pregnant and lactating women. To assess this situation Coopman (2015) conducted a systematic review. Latest available data of hemoglobin of children below 6 years and females aged 15-49 years since the year 2000 was checked. According to results among children, anemia in Haiti, Bolivia and Guatemala was 60.6%, 61.3% and 47.7% respectively, showing severe anemic health problem. While in case of women of child bearing age, highest prevalence was observed in Haiti, that is, 45.5% followed by Panama with 40%. Lowest prevalence of anemia in children as well as women was reported in Chile (4% and 5.1% respectively). The research concluded that that the vulnerable population will continue to be at high risk until interventions with respect to

iron are not done. Hygiene, sanitation, awareness, health care systems are considered to be one of the important aspects of playing a role in reducing the prevalence rates.

Kaur et al. (2015) surveyed presence of anemia amongst 250 girls who were adolscents and belonging to rural background of District Karnal, Haryana. 88% girls were found to be suffering from various grades of anemia. According to the report, the majority of girls are vegetarians who eat two meals a day. Almost 75% of the girls skipped meals on a regular basis. The study concluded that the girls' anemic status is caused by low dietary intake.

WHO (2016) reported that anemia is a major health problem affecting large numbers with high prevalence throughout the world. In a report prepared by WHO (2016) assessing the prevalence of anemia over a temporal period from 1995- 2011, it was observed that the hemoglobin concentration in children was 111 g/L, 126g/L in non-pregnant, and 114g/L in pregnant women. Here hemoglobin level is the indicator for anemia. Various nutrition and prophylaxis programs can help in generating awareness about the severity of the problem at the grass root level, therefore, have been recommended.

Kaur et al. (2014) investigated the prevalence, styles of anemia and hematopoietic levels of old age belonging to 60 years and above attending a hospital. Around 2540 subjects were assessed for signs and symptoms of anemia and were further 100 anemic subjects were then non-consecutively selected for screening. It was estimated that prevalence percentages of anemia were around 71%. Moreover, it was also noted that the maximum percentage of anemia was reported in individuals belonging to 60-69 years.

Diet is known to be a major causative factor in development of anemia. (Nair et al. 2018, Beck et al.2014 and Singh et al.2016). Due to its high prevalence, many studies have assessed the relation between diet and anemia. As a result, Walczyk et al. (2008) observed the relationship between status of iron in young women eating a rice-based meal and level of absorption of iron. Here forty women aged 18-35 years were examined for their status of iron absorption based on consumption of ascorbic acid under the control group and treatment group, where the iron in control group was

replete. Association between tea consumption and absorption of iron was recorded. It was found that the mean fractional iron absorption was 2.5 times more in the iron-deficiency anemia (IDA) group in comparison with the control group. It was further revealed in the study that consumption of tea harmed iron absorption. The findings of the study also reported that ascorbic acid (AA) helped in boosting iron absorption. The iron absorption from rice meal was 17.5% in the anemic samples and only 7% were recorded in the control subjects. The findings of the study were in accordance with such studies conducted elsewhere (Kotecha 2011). The iron absorption was observed to be affected by the previously existent iron status in the two groups under study. Therefore, the results from this study reinforce the earlier findings (Fan et al. 2016) that consumption of tea inhibits the absorption of iron in the body to a considerable extent.

The main finding of various studies showed more or less an inverse relationship between anemia and socio- economic status of the family (Kapoor & Aneja 1992, Kumar et al. 2008, Sharma et al. 2005, Chaudhary & Dhage 2008, Deshpande et al. 2013 and Twara et al. 2015). Other factors that showed an association with anemic status include parents' educational and occupational status, family size and structure, poor dietary habits, type of diet, menarche status, and other lifestyle and nutritional aspects. (Rajaratnam et al. 2000, Kaur et al. 2006, Kordas et al. 2008, Gupta & Prakash 2009, Kaur & Kochar 2009, Siddharam & Venkitish 2011, Deshpande et al. 2013, Twara et al. 2015 and Basu et al. 2016). Thus, not just dietary intake, many other factors contribute to the high prevalence rate of anemia. Efforts are needed to suppress all such factors as per the need of the population.

Use of green leafy vegetables as functional foods

Green Leafy vegetables play a key role in formulating a balanced diet. They are a not only a good source of minerals and vitamins that provide many micronutrients in a single bowl and also provide good amounts of carotenoids, flavonoids and other non-flavonoid phenolic compounds which fall under the category of bioactive compounds. (Gupta & Parkash 2009). So, it can be said that GLV's are an important source of all nurients and should be included in the diet to help minimize the burden of non-communicable diseases especially anemia.

Pollock (2016) did a meta-analysis to check the incidence of cardiovascular disease based on the amount of GLV's and vegetables belonging to the cruciferous family consumed in the diet. The connection between GLV's consumption and CVD was investigated. In the end, 8 studies were included based on all the criterias specified and the researchers looked into the association between the presence of cardiovascular disease and the consumption of GLV's. A greater intake of GLV was found to minimize the risk of several forms of CVD in the sample. The study suggested that people consume GLV regularly and that government agencies hold public awareness campaigns to inform people about the benefits of GLV consumption.

In the South-East Zone of Nigeria, a research was conducted to assess the composition of Indian based GLV's by Otitoju et al. in 2014. The nutrient composition of *Psychotria sp., Cindoscolus aconitifolics*, and *Telfairia Occidentalis* showed a 62.30 percent and 12.87 percent moisture content in raw and dried samples, *C.aconitifolius* had 82.16 percent and 12.87 percent, and *T.occidentalis* had 86.28 percent and 9.82 percent moisture content in raw and dried samples, respectively. A range of 11.75-27.32% was observed in the crude protein content in *Psychrotia sp*., *C.aconitifolius*, reported values of 4.83-24.13 percent and 5.26-35.06 percent in *T.occidentalis*. These GLVs have been found to be high in pro-vitamin A, B2, C, and E and minerals are also abundant in these green leafy vegetables. As a result, *Psychorita sp.* and *Cnidoscolus aconitifolius* were discovered to be excellent macro and micronutrient sources.

The term "functional" can be applied to food and used for a broader spectrum like providing additional physiological benefits beyond that of meeting basic nutritional needs (Raut et al. 2014). Impact of awareness program for women on knowledge and consumption of functional food was conducted by Faber et al. (2007) who investigated the percent contribution of GLV's with respect to micronutrient intake amongst small children of a rural setting in Africa belonging to the age group of 2-5 years. The cross sectional survey reported that GLV's made a major contribution to the dietary intake of important micronutrients amongst the children.

Kumar et al. (2013) conducted a review on edible leafy vegetables in South India to assess their pharmacological benefits. They observed that all the commonly

consumed greens had beneficial phytonutrients and polyphenolic compounds (Ramulu & Rao 2003) that proved beneficial in cancer, diabetes, hepatotoxicity nephrotoxicity and many microbial attacks.

In another study by Yadav & Dixit (2003), nine commonly consumed green leafy vegetables were taken. Genotypes of bathua, amaranth, and spinach were taken. The edible portion of the plant was harvested about 60 days after sowing when it was at marketable level, and fresh leaf samples were cleaned under both tap water and double-distilled water. The samples were then washed, dried with filter paper, and analyzed for nutrients like ascorbic acid and non-nutrients. These leafy vegetables, including amaranth, chenopod, spinach, were found to have high antioxidant activity and their uses extend to formation in different nutraceutical and pharmacological products.

Anti-nutritional content of leaves

Anti-nutritional factors are key in deciding the use of plant products for human consumption and as part of the daily diet to help alleviate micronutrient deficiencies. Anti-nutrients inhibit the uptake of nutrients by plants, but some also have beneficial uses (Gemede et al. 2015). The commonly found anti-nutrients in green leafy vegetables include tannins, phytates, oxalates, nitrates, saponins, alkaloids, and lectins, to name a few (Gemede & Ratta 2014, Akubugwo et al. 2007 and Natesh et al. 2017).

Natesh et al. (2017) conducted a review and reported that the genre of vegetables was different and classified according to the levels and classes of nutrients as well as anti nutrients present in them. Anti-nutritional factors might harmthe health of humans and their consumption must be limited. Various methods used during processing can help reduce the quantity of these factors in the diet. Blanching and heat treatment during cooking help to reduce the amount of phytic and oxalic acid (Oboh & Akindahunsi 2004). Drying as a processing treatment affects the anti nutrient levels of GLV's. (Yadav et al. 2003 and Ramulu & Rao 2003).

Saad et al. (2017) studied the potential to act as a herbicide and chemical composition of cauliflower, cabbage and turnip (*Brassica oleracea var. botrytis and*

Brassica oleracea var. gongylodes) According to the previous reports, content of pehnols, alkaloids, flavonoids, flavones and proanthocyanidins are higher in the extract using aqueous medium. (Bahorun et al. 2004, Scalzo et al. 2008, Vallejo et al. 2003 and Wu & Prior 2005). Because of the alkaloid content, brassica family vegetables have a higher antioxidant potential and must be made an important part of the daily diet.

In 2017, Gowri & Manimegalai assessed the phytochemical and XRDanalysis of cauliflower leaf. It was reported that this property of cauliflower leaves makes it an important anti-bacterial anti-carcinogenic agent.

Chauhan et al. (2016) also studied the beneficial and antioxidant properties of *Brassica oleracea* varieties. They found that red cabbage contains many bioactive substances and has anti-inflammatory, analgesic, anti-bacterial effects, and antidiabetic effects. In another study conducted by Ranjitha & Sudha (2015), it was reported that alkaloids belong to different classes and depending upon their consumption, their health benefits are manifested. Similar results were reported by Talreja & Moon (2015), where the brassica family was assessed for their phytochemical profiling and presence of anti-cancer compounds. Glucosinolates found in these plants were isolated and are known to act and provide protection against cancer, bacteri and fungi.

In another research phyto chemical content of GLV's was studied. Otitoju et al. (2014) conducted a study to find out the qualitative as well as quantitativecontent in vegetables in three different states i.e., raw cooked and shade dried leaves where six greens were selected randomly like *Fices Thomingii, MucunaPruriens, Ficus Capensis, Pterocarpus Santalenoids, Vitex Doniana, and Myrianthus Arboreum.* Qualitative phytochemical analysis revealed that all the green leafy vegetables had anthocyanin, flavonoids, carotenoids, oxalate, saponin, and tannins. Inthe case of alkaloids, *M.Pruriens*lacked in it, and only *F. Capensis*was having Glycoside. Quantitative phytochemical analysis revealed that the highest content of alkaloids and flavonoids was found in *M.Pruriens* (3.76gm / 100gm) and *F.Capensis*(4.29gm/100gm), respectively, whereas it also had the highest saponin and tannin content (5.86gm/100gm and 3.61gm/100gm respectively). The meanphytochemical composition also revealed that these two vegetables have the highest range. The study concluded that all the

selected vegetables are rich in phytochemicals. Hence they should be utilized for various therapeutic purposes, either in raw or cooked form.

Gemede & Ratta (2014) reviewed the anti-nutrient content in plant foods, intending to investigate their health benefits as well as their adverse effects. They reported that anti-nutrients are those chemicals that hinder the utilization of essential nutrients present in food. But sometimes these are beneficial also if consumed in a suitable amount. Anti-nutritional factors like tannins, lectins, phytic acid, saponins have beneficial health effects if consumed in small amounts. Researchers have shown them to help lower blood sugar levels, improve insulin response, decrease cholesterol and triglyceride levels, as well as have cancer-fighting ability proving that consumption of anti-nutritional factors is not always harmful. A balance needs to be maintained to avail the health benefits of these compounds and maintain the nutritional value.

Onyeka & Nwambekwe (2007) examined phytochemical content of some GLV's which are consumed in the South East region of Nigeria in raw form and also assessed the effect of cooking on them. The study was performed on eight green leafy vegetables, which include: -Ugu, Nchanwu, Okazi, Oha, Nturukpa, Ahihara, and Onugbo. The study compared phytochemical content in the raw and cooked state by using statistical tools like mean and ANOVA, and results indicated that Oha was rich in anthocyanins, flavonoids, and carotenoids. In contrast, Onugbo was found to be a rich source of steroids and tannins. Ahihora had the highest content of alkaloids. It also revealed that flavonoids, alkaloids, and anthocyanin content of selected green leafy vegetables were significantly reduced after cooking, whereas tannins and steroids content was not affected at all. The study suggested the consumption of a mixture of selected green leafy vegetables to meet the need of phytochemicals in body.

Methods to improve bioavailability of iron

The availability of iron from its two forms, heme and non heme i.e. vegetarian food sources is comparatively lesser than heme iron. (Young et al. 2018, Yang et al. 2006, Hurrel & Egli 2010 and Dainty et al. 2014). Typical Indian diets provide only 25% of the iron needs (ICMR 2010). Efforts should be made to increase the form of bioavailable iron from daily meals. It was investigated through secondary data that

cereal-legumes-based gruels added to the infant's and young children's diet have low energy and low bioavailability of iron. Home-basedcomplimentary food leads to deficiency of iron in the body and further anemia. Various researchers recommended many ways to enhance the amount of available and ioniasable iron in plant-based complementary food, like phytic acid enzymatic degradation, increased consumption of ascorbic acid, etc.

Hoppe et al. (2015) observed the role of probiotics strain *Lactobacillus Plantarum* 299v on iron absorption. Two trials were conducted in healthy women. The first trial involved the assessment of a drink based on fruits which had 109 colony-forming units (CFU) Lp299v in comparison to a drink that did not contain the probiotics strain and served as control. The second trial had higher colony-forming units (1010). Ferrous lactate was the form of iron available in the drink in the amount of 5mg. The results showed that the drink containing 109 CFU showed a mean absorption of (28·6%) which was found to be significantly higher as compared to the control drink, which showed absorption of (18·5%), n 10, P<0·028). The results of trial two did not show much significant difference between the iron absorption of both drinks. The study concluded that probiotics can be used to uplift iron absorption and improve iron bioavailability.

The role of sugar in iron absorption was studied by Christides& Sharp (2013). Due to the increase in the consumption of sugar, its effect was studied on the oxidation of iron. It was observed that consumption of fructose helped elevate the formation of ferritin which is induced by iron. Even the consumption of derivative of sugar (HFCS) had similar effects on the iron which was induced by ferritin. It was thereby concluded that fructose helps improve the bioavailability of iron and has an important patho-physiological consequences.

Microorganisms also help in overall reduction of anti nutrients found in food, as well as increase the content of available iron. Peral millet sprouts when feremented with different strains of *Saccharomyces* and *Lacto-bacillus* at 30 °C for 72 h, showed a reduction of 88.3% in the total phytic acid content of the millets. (Kaur et al. 2011).

Yang & Tsou (2006) studied the relationship of cooking on content of iron in vegetables and fruits and reported that boiling help improves the dialyzable iron content better than blanching, drying, or other processing methods. Gupta et al (2015) also recorded the effect of reducing the phytic acid content on iron absorption. They also stated that milling and soaking grains before cooking helps reduce the phytic acid content. Similar studies were reported by (Lestienne et al. 2005, Perlas & Gibson 2002, Coulibaly et al. 2011, Greiner & Konietzny 2006).

Bencomo et al. (2003) evaluated the effectiveness when foods rich in ascorbic acid was used to improve the absorption of iron in certain Mexican diets. This study involved measuring the stable isotopes of iron. The locale of the study was a rural community clinic in Mexico where fifteen non-pregnant, non-lactating, and iron-deficient women were examined. The results indicated that adding 25mg of ascorbic acid to meals twice in a day i.e. both breakfast and lunch for 2 weeks, had shown a significant increase in iron absorption and may improve the iron status of the respondents. Imtiaz et al. (2003), Nair & Iyengar (2009) and Nair et al. (2013), recorded similar observations where ascorbic acid was used to enhance the bioavailability of iron.

Thus, various strategies can be employed to help improve iron absorption and bioavailability. Efforts are needed for better implementation of various processing methods and different techniques in our daily cooking to help combat iron deficiency anemia.

***In vivo* studies conducted to test bioavailability of iron-rich products**

In Vivo studies using animals as test sublects have been used extensively to assess the metabolic responses of food stuffs and their toxicity. Several research studies have supported animal experiments to test the bioavailability of iron and its various formulations.

In anemic rats treated with phenylhydrazine, researchers Geith & Mahmoudy (2018) tested the efficacy of leaves and stalk of *Beta vulgaris on* the hematopoietic ability of male albino rats. The extract was made by macerating the leaves in hydro-ethanol for 48 hours. Two doses of 20mg/kg phenyl hydrazine were administered

intraperitoneally to induce anemia. The animals were divided into five classes, with one receiving standard treatment which included ferrous ascorbate and folic acid in the amounts 13.5 mg/kg, the second class was provided an extract of *B. Vulgaris* in the amount raning from 100 – 200mg/kg and the third class was left untreated and lebeled as control for normal and anemic rats. Blood tests revealed a substantial rise in hematopoietic levels, which can be used for pharmaceutical research.

Joshi et al. (2017) also examined anti-anemic activity in phenylhydrazine induced anemic rats by using hydro-alcoholic extract of leaves of *Lycium barbarumin*. Anemia was induced by giving phenyl hydrazine intraperitoneally for up to two days. Five groups comprising of six animals were divided namely: a regular control group, an anemic control group, a third group given Vit. B_{12} as a standard reference control, and a fourth group was given 100mg/kg of leaves extracted using water and alcohol. The test control-I was given a 200mg/kg hydro-alcoholic extract from the leaves of *Lycium barbarum,* and the test control-II was given a 200mg/kg hydro-alcoholic extract from the leaves of *Lycium barbarum*. This regular oral route of administering test drugs was observed for 28 days. Blood was drawn through a tail puncture on the 29th day and checked for Hb, RBC, and Hematocrit expressed as percentage. A significant increase was observed in the water alcohol extract as well as Vit B_{12} on the hemoglobin, RBC's and hematocrit level indicating that these leaves do exhibit anti-anemic behavior, according to the report.

In a similar study, the role of a water-ethanol extract of *Allium tricoccum* fruit was investigated on fighting anemia by Gupta et al. (2013) using phenyl hydrazine-induced anemic rats. Animals were divided into five classes, each comprising about six animals. The first group acted as a regular control group, the second as anemic control, the third as a standard reference control when given Vitamin B_{12} complex, and the fourth as test control-I received 100mg/kg of extract of *Allium tricoccum* fruit, while the the 5th group, designated as test control-II, received around 200mg/kg of the leaf extract. The hydro-alcoholic fruit extract of *Allium tricoccum* has anti-anemic activity against phenyl hydrazine-induced anemia in rats, according to the findings. The high iron content of the *Allium tricoccum* fruit can contribute to the plant's anti-anemic effect.

Baskaran et al. (2019) also evaluated the anti-anemic activity in phenyl hydrazine induced rats but this time using ethanol leaf extract of *Kedrostis foetidissima.* For 2 days' phenyl hydrazine (60mg/kg) was administered intra peritoneal in rats to induce anemia. Here instead of 5 groups like in previous study, four groups were made containing 6 animals each. Normal control batch served as group I, while group II served as anemic control ones, group III administered with Vitamin B_{12} was reference control and final group that is group IV animals were given about 200mg/kg of ethanolic leaf extract of the plant. All the test drugs were administered through oral route for 28 days daily. On 29^{th} day, through tail puncture and under effect of anesthesia blood was withdrawn which was then tested for the estimation of RBC, Hb and percentage Hematocrit. *Kedrostis foetidissima* and Vitamin B_{12} showed a rise in the RBC, Hb and Hematocrit levels when administered, concluding that it exhibits anti-anemic activity. Modupe et al. (2018) conducted another study and reported the effect of *theobroma cacao* in iron deficient anemic rats. Rats were fed a diet lacking in iron to test the difference in efficacy of *theobroma cacao* and *mangifera indica*. It was reported that *theobroma cacao* at a lower dose of 25mg/kg had better anti anemic properties as compared to mangifera indica at the same dosage level. Thus, its use at this low dose can be considered beneficial for the treatment of anemia.

Srinivas et al (2013) examined the anti-anemic effect of *schrebera swietenioides roxb.* in rat models. The therapeutic value of plants in the treatment of anemia was explored in this report. All of the solvents used were UV-resistant. Both male and female Albino rats (bodyweight 250g) were utilized in this study, and they were placed in a specific setting. They were held in polypropylene cages in groups of three at ambient temperature (25°C), relative humidity (55%), and 12 hour/12 hr light-dark cycles. Leaf extract, stem bark extract, and root bark all had their total phenolic content measured. For three days, anemia was induced using phenyl hydrazine (PHZ) at a dose level of 40 mg/kg expressed as body weight. SRE doses of 200 and 350 mg/kg expressed as body weight were fed to anemic rats. On days 4 and 14, the rats were tested for hematological parameters including, red blood cell, packed cell volume and heamoglobin. The animals were separated into four classes. During the entire time, the first group received only normal saline in a day. The second PHZ control group was

given PHZ 40mg/kg once daily for a period of three days. For three days, Test group 1 obtained PHZ 40 mg/kg body weight and SRE 200 mg/kg day orally. The fourth party, the plant extract control, receives SRE orally 350 mg/kg/day for the entire time of study. According to the findings, the existence of carbohydrates, saponins, polyphenols, tannins, and flavonoids was observed in the phytochemical sreening. SRE was found to be high in total phenols using the Folin–Ciocalteu process, with a value of 266 mg GAE/g dry extract. Anemia was successfully induced in Groups II, III, IV, and V, as shown by a 51 percent reduction in RBC count, 53.85 percent reduction in Hb material, and 54.9 percent reduction in PCV. According to hematological parameters, at a dosage of 350 mg/kg, SRE significantly (p0.05) improved Hb, RBC count, and PCV on day 14.

Thus, the use of rats specifically for testing the efficacy of iron-rich plant products has long been proved to be successful. The present study includes the use of such a model to determine efficacy and bioavailability of iron from an underutilized green leafy vegetable.

Product development using iron rich foods

Many researchers have conducted studies and developed products rich in iron. Banka et al. (2017) conducted a study to develop iron rich products using underutilized greens. The acceptability of the products was limited to 5% of the dried powder incorporated. Singh et al. (2019) obtained similar results when they supplemented some commonly consumed snacks with cauliflower leaves powder.

Srebernich et al. (2015) developed iron rich meat loaf with pork liver for school children. The aim was to develop meat loaves to provide 15-20% of the daily requirements of children for iron. Two formulations were prepared containing 9.98% and 13.31% pork liver, respectively. Biochemical, physicochemical, sensory, and microbiological analysis of the product was carried out. An increase was found in the iron and zinc content with a reduction in the fat content. Formulation B had higher iron levels (152.73%), lesser fat content (reduction of 31.5%), and better sensory acceptability.

Alaunyte et al. (2014) studied the effect of iron status in female runners as a result of dietary intervention using a teff bread enriched with iron. The study was carried out over a period of 6 weeks, wherein the participants were asked to substitute their bread with iteff bread enriched with iron. A significant increase was seen in the iron intake of the subjects pre-andpost-intervention (from 10.7 to 18.5mg/day). An increase was also observed in the iron content of the tissues. Thus, dietary interventions with iron-rich products have a marked effect on the biochemical parameters associated with iron.

Joshi & Mathur (2015) also assessed the acceptability of food products by value addition using cauliflower greens and other dried leaf mixtures to help increase the micronutrient content of commonly consumed food products. Powdered leaves of beetroot, cauliflower, carrot, and turnip were incorporated in twenty recipes. The leaf mixture contained appreciable amounts of micronutrients like iron (77.10%), protein (25.77%), and calcium (3.77%). It improved the nutritional content of the foodstuffs significantly in comparison with the sample not fed the cauliflower leaf mixture. In terms of sensory evaluation, products incorporated with a 10% proportion of leaf mixture were highly acceptable. Similar studies were conducted by Pankar & Bornare (2018), Srikanth et al. (2018), Wani & Sood (2014) and Bhuvaneswari K.M& Ramya (2014) to assess the addition of cauliflower leaves powder as a supplement in daily commonly consumed recipes. All food products showed acceptability of up to 10% of the leaf powder.

In another study by Wani et al. (2011), supplementation was done using noodles made with cauliflower green in dried form. Cauliflower leaves was mixed with wheat flour after malting in different proportions. Sensory and physico chemical analysis of the noodles was done after a period of 90 days. The supplemented product had a higher content of protein, iron, ash and fiber, as well as vitamin A. Dried cauliflower leaves up to 10% was considered acceptable after organoleptic evaluation.

Joshi & Mathur (2010) conducted a study to assess the bioavailability of iron form these underutilized green leafy vegetables, they observed that all the leaves had a higher absorption level and bioavailability of iron as compared to normal vegetarian diets. The study showed that carrot leaves powder showed the maximum absorption of

iron at 27.27% and the lowest was observed in the turnip greens at 14.47%. These values for iron bioavailability are significantly higher as compared to iron absorption from our daily diets.

Multiple studies have been reported to assess the use of cauliflower greens as an effective product in helping cut down on anemia as a public health problem. Kumar & Bhawani (2004) tested the efficacy of cauliflower greens on hemoglobin levels. The group fed the greens reported an increase in the hemoglobin levels which was significant post the intervention. ($t=9.4$, $P<0.05$).

Thus, the use of underutilized and commonly consumed green leafy vegetables has been well documented. Their efficacy in helping increase hemoglobin levels and help combat anemia has gained tremendous importance. The prevalence of anemia has been well established with the review collected, and the role of diet for good health and prevention of anemia is indubitable. The role of green leafy vegetables as functional foods, despite their anti-nutrient content, plays an essential part in dealing with micronutrient deficiencies. The need of the hour is to develop a food product that can be consumed daily as part of the diet of all age groups and all sections of society to help solve this severe public health problem. The present study aims to develop such a product using low-cost underutilized ingredients and provide a solution aiming to cater to all sections and groups of the society at large.

HYPOTHESIS

H_1 - It is expected that there will be significant association between socio economic status and anemia in pregnant women

H_2 – It is expected that green leafy vegetables are a rich source of iron and phytochemicals and can be processed and utilized in the daily diet

H_3– It is expected that there will be significant difference amongst the hematological profile between treatment groups when tested *in-vivo*foriron rich vegetables like cauliflower greens

H_4 – It is expected that product optimization and development using cauliflower greens can be done with high sensory acceptability and shelf life

AIMS AND OBJECTIVES

1. To study the prevalence of anemia in Chandigarh amongst one vulnerable group (Rural and Urban)

2. To screen different low-cost green leafy vegetables for iron content using various processing techniques

3. To assess the functional bioavailability of the selected green (in-vivo study)

4. To develop and evaluate a low-cost, functional iron-rich product, shelf–life assessment and storage study of the developed product

CHAPTER -3

MATERIALS AND METHODOLOGY

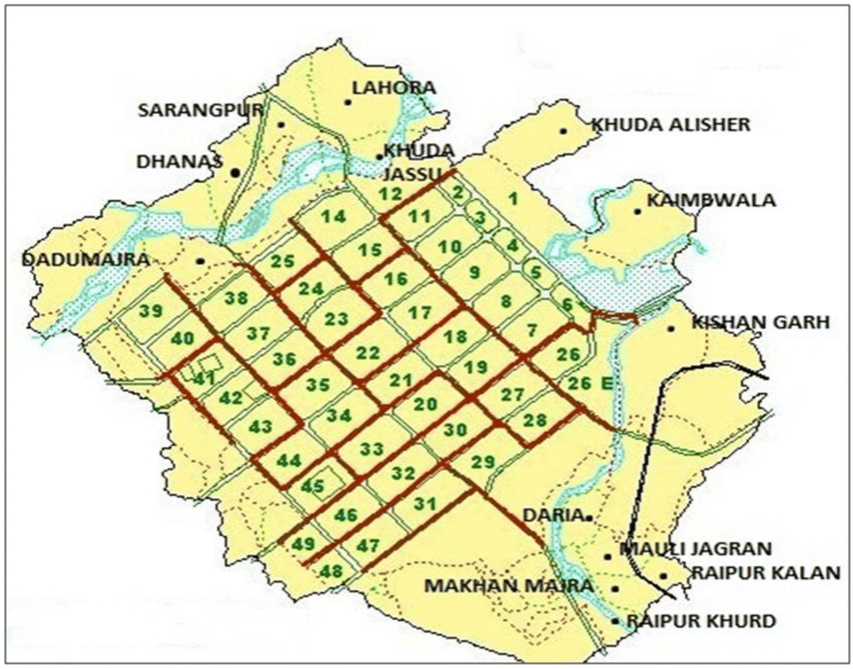
The present study aimed to survey and report the rate of prevalence of anemia amongst one of the vulnerable populations in Chandigarh's rural and urban areas and develop an iron- rich product after the screening of some GLV's. The functional bioavailability of the iron content was analyzed *in vivo.* As per the objectives of the study, the methodology was divided into four heads.

3.1 Survey on prevalence of anemia

The study was conducted using a cross-sectional survey amongst pregnant women to assess the presence of anemia. The locale of the study, Chandigarh, is divided into 56 city sectors, 23 villages and 15 slums as reported by the census of India (Census 2011) and is a Union Territory located in the Northern part of India. (Khaiwal et al. 2019).

Figure 3.1:

Map showing villages falling under rural areas of Chandigarh



Source: Adapted from Khaiwal et al. (2019)

The city has two community health centers in Sector 45 and Sector 22 which provide health facilities and cater to all sections of the society. The survey was conducted in Civil Hospital Sector 22 as it was more representative of the sample. The gynecology department of this hospital was well established and was also awarded the Kaya Kalp award for the year 2018-2019 for maximum number of deliveries (National Health Mission Chandigarh Administration, 2019). Selection of the subjects was done on the following criterions:

Inclusion Criteria:

- Pregnant women belonging to both rural and urban areas of Chandigarh visiting the civil hospital
- Women who were 15 - 49 years of age and in their first trimester of pregnancy

Exclusion Criteria:

- Respondents who did not have interest to participate in the study
- Respondents who were not residents of areas in or around Chandigarh

Sampling method

The method of sampling used for data collection was stratified random sampling. It is a useful method when a particular population is under focus, and the need is to reach a target sample quickly. This method helps in increasing the probability of selection of data items and give better results or estimates.

Setting

242 Pregnant women visiting the Civil Hospital were selected for the study keeping in mind the inclusion criteria and exclusion criteria as defined above. The tool used was a structured interview schedule using a self- designed questionnaire on demographic information, socio economic status, hemoglobin level, clinical signs of anemia. The questions in the questionnaire were simple and unambiguous. The questions were explained to some of the respondents in local language for their convenience and understanding.

Figure 3.2:

Sampling Design showing the division of health centers and further categorization in areas of Chandigarh as per NRHM

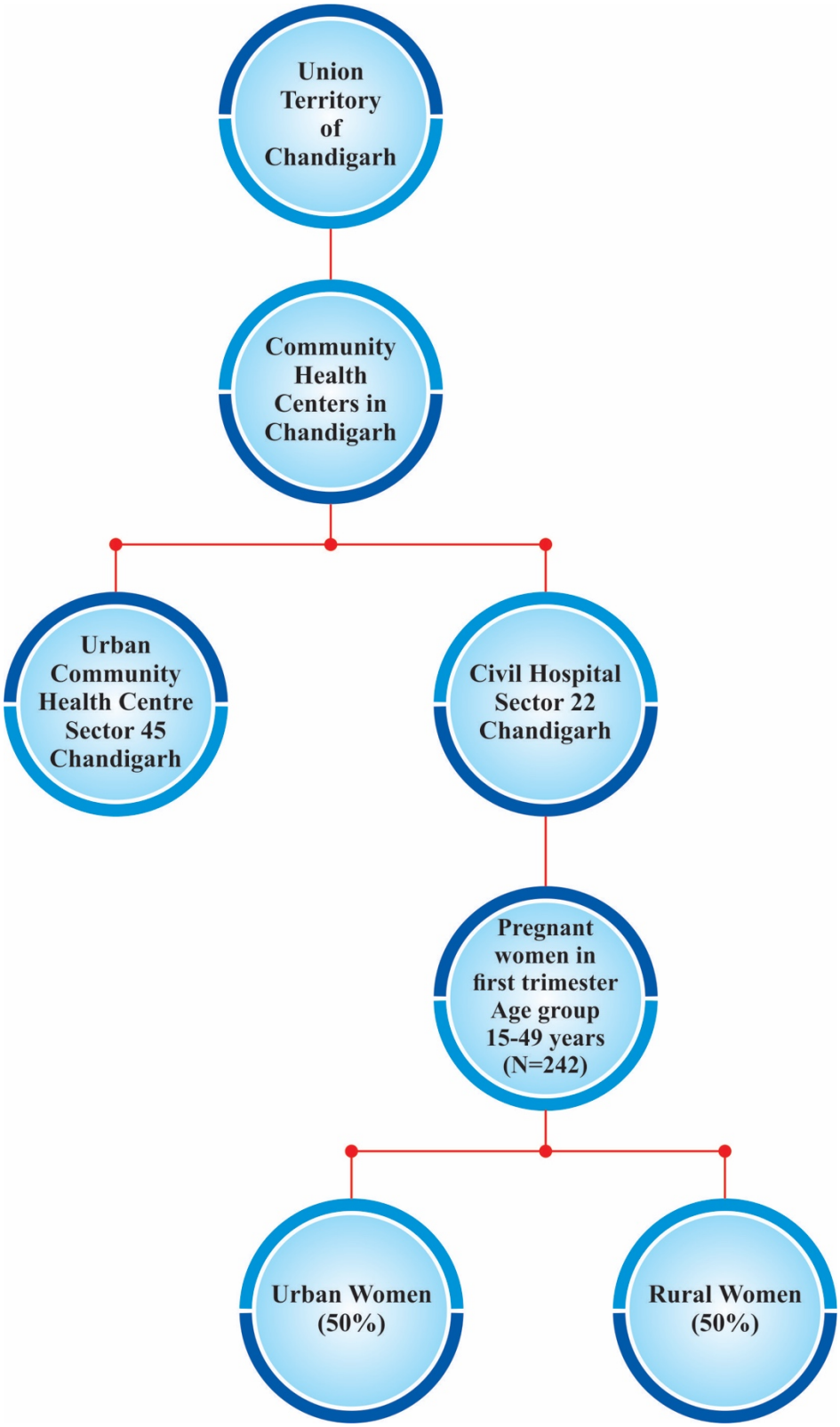


Photo 1:

Data collection using structured interview schedule. Locale of investigation – Civil Hospital, Sec-22, Chandigarh

3.2 Screening of green leafy vegetables

5 varieties of green leafy vegetables were screened for their physico chemical and phytochemicalcontent using various processing techniques and further tested for their anti - nutritional content

Selection of green leafy vegetables

Five locally, seasonally available and underexploited GLV's were collected from the local market of Chandigarh, Sector 26 and further tested and processed. The vegetables were selected based upon seasonal availability and consumption. The leaves randomly selected for the study wereCauliflower leaves - *Brassica oleracea var. botrytis*, Spinach - *Spinacia oleracea* , Amaranth - *Amaranthus viridis,*Lettuce - *Lactuca sativa,* Radish leaves - *Raphanus raphanistrum subsp. Sativus,.*

The stalks were removed and the leaves were cleaned under running water to remove any foreign particles. They were spread and air dried and further used for assessment and screening.

Photo 2:

Various Green leafy vegetables used in the study

Cauliflower Leaves

Spinach Leaves

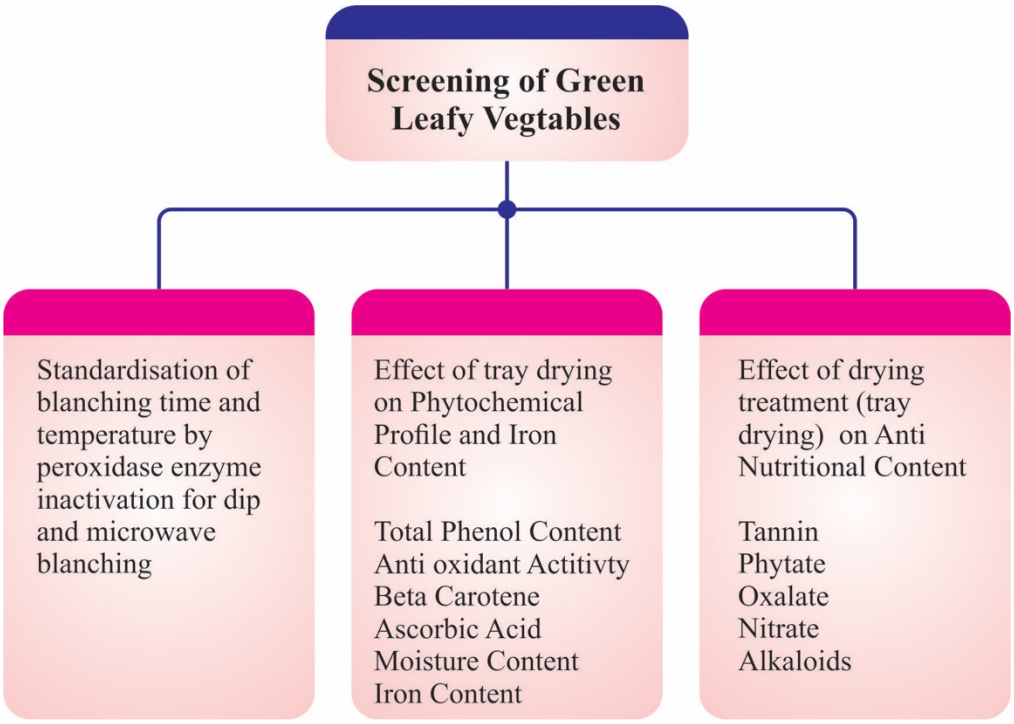
Amaranth Leaves

Lettuce Leaves

Radish leaves

Figure 3.3:

Screening process used for green leafy vegetable



Sample Preparation

Freshly collected leaves of underutilized vegetables as collected from the local markets of Chandigarh were separated, weighed and washed. To dry by direct contact with air and to extract the excess water, the leaves were spread out on a tray after washing.

Dip Blanching

The leaves were washed properly in tap water to remove external impurities. 100gm of leaves were weighed accurately and were dipped in water at the different time and temperature combinations. Using a water bath, at a constant temperature the blanching process was carried out and further finished by dipping the blanched leaves in ice cold water immediately. (Severini et al. 2016).

Microwave Blanching

The leaves were washed properly in tap water to remove external impurities. 100gm of leaves were weighed accurately. The vegetables were covered with 100ml water and

blanched in a microwave oven (Samsung MW73AD-B/XTL) at high power (2450 MHz -900Watts) for a period of 1, 3 and 5 minutes. Once blanched, the leaves were immediately submerged in cold water and allowed to cool for the same amount of time as they were heated. (Severini et al. 2016).

Tray Drying

To extract the impurities, the leaves were washed in water. The leaves were drained of excess water. 200g of leaves were precisely measured. The oven was preheated to 60°C (Universal Hot Air Oven, Temp Star 605x605x605), and the single layer filled trays were held until the leaves were fully dry. (Satwase et al. 2013).

Estimation:

Peroxidase Activity

100 g of leaves were tied up loosely and dipped in boiling water and blanched as per protocol. Sample was ground using mortar and pestle with water. After this, the sample was strained using a filter paper in a test tube. About 5 ml of content was taken and to it 1 ml of 1% guaicol solution (in 95% alcohol) and 1 ml 0.5% of H_2O_2 added. After allowing the test tube to stand for 5 min, the development of brown color in the content indicated the presence of peroxidase, the most heat resistant enzyme. (Manzoor et al.2019).

Moisture Content

A petri dish with lid was weighed.10 gm sample was weighed into the petridish and spread evenly for uniform drying. Oven was set at 100 to 105°C and the petridish with sample was placed inside the oven with lid open for 4 hours.Petri dish was transferred to a dessicator for cooling.Then the weight of the petri dish with sample was noted.the readings were recorded till weight remained constant consecutively. (AOAC,2005).

Calculation:

$$\text{Moisture \% (g)} = \frac{\text{Sample initial Wt (W1)} - \text{Final Wt (W2)} \times 100}{\text{W1}}$$

Iron Estimation

A sample of 10gms was taken and burned on a hot plate. After crushing the leaves, 5gms were placed in duplicate crucibles and after ashing in a muffle furnace at a temperature of 550°C for 4 hours, crucibles were cooled to room temperature and the ash was dissolved in 25 mL of dilute hydrochloric acid. The remaining distilled water was then added to the mixture in a 250ml volumetric flask. The material was filtered and iron levels were determined using the filtrate. 1 mL of hydroxylamine hydrochloride solution was added to the prepared extract in a 25mL volumetric flask. 5 mL buffer + 1 mL ortho- phenanthroline solution. The contents were made upto volume by using distilled water. A spectrophotometer was used to calculate the absorbance and strength of the color produced at 510 nm.2 ml of conc. A dilution of HCL upto 100ml was done and 10 ml of this solution was used as blank for each sample. Six separate 100 ml volumetric flasks were filled with different concentrations of regular iron solution, 2 ml of conc. HCL, and volume was made up. With the reading of the standard solution, a calibration curve was drawn. The iron content in the unknown sample was determined by multiplying the standard curve by the dilution factor (AOAC, 2005).

Ascorbic Acid

5gm of fresh sample was weighed and 4% oxalic acid was used to extract it. Volume was made upto 100ml and after centrifuging, 5 ml of the supernatant was pipeted and to this 10ml oxalic acid (4%) was added. This was titrated against the dye 2,6-dichlorophenolindophenol. Pink colour was denoted as the end point and the standard used was ascorbic acid.

$$\text{Amount of ascorbic acid mg/100gsample} = \frac{0.5\text{mg}}{\text{V1}} \; x \frac{\text{V2}}{5} x \frac{100}{\text{SW}} \; x \; 100$$

V1 - titer value of the standard solution

V2- volume of sample extract

SW - weight of the sample. (Sadasivam&Balasubramaniam, 1987).

Beta Carotene

10ml of fresh sample was taken, macerated and added to a flask containing 50ml of 95% ethanol. This was kept in a water bath at a temperature of 70-80°C for 20 minutes. The supernantant was allowed to cool. 15ml distilled water was added to the ethanol to bring the concentration to 85% and it was cooled for 15 minutes. After transferring the mixture into a separating funnel, 25ml petroleum ether was taken and ethanol was added to it. The contents were mixed and the funnel was allowed to stand till two layers of separate liquid were obtained. In a beaker, the bottom layer was collected and the top layer in a 250ml conical flask. In a funnel, the bottom layer was re extracted using 10ml petroleum ether till it became light yellow. All the petroleum ether was taken in a flask and kept in a separating funnel to reextract with 50ml of 80% ethanol. After measuring the final extract, it was kept for further analysis. Spectrophotometer was used to measure the absorbance of the extract at 436nm. The formula used for calculation was C=A/EL

C= concentration of carotene

A= absorbance

E=extinction coefficient

L= thickness of cuvettes (path length) =1cm

E of β-carotene =1.25x104 µg/l (AOAC, 2005)

Total Phenol Content

75ml (95% v/v) ethanol was used to extract 10gm of fresh sample at 40°C for 10 min thrice. Known volumes of ditilled water (5ml) were used to dissolve the residue and aliquots of 0.2-2ml were pipetted in test tubes to raise volume upto 3 ml using water. After adding 0.5ml of Folin- Ciocalteu reagent, 2ml of 20% Na_2CO_3 was added to the solution and proper mixing was done. After placing in boiling water for 1 min, the test tues were cooled and absorbance was recorded at 650nm. Gallic acid was used to prepare a standard curve and values were noted as mg G.A.E/100g. (Sadasivam&Manickam, 2004).

Anti oxidant Activity

10gm sample was taken and extracted using ethanol and then dried. Extract was made soluble in ethanol and aliquots were prepared to 1.0mg/ml final concentration. Further dilution was done to 5, 10, 25, 50, 125 and 250µg/mL using ethanol. 0.3 mM DPPH in volume of 1ml was mixed with ethanol solution and further 2.5ml of all the samples of different concentrations. The mixture was left to stand and absorbance was read at 518nm after 30 minutes. Linear regression was used to calculate EC50 values. Antioxidant activity (AA) was expressed as percentage with the formula:

AA% = 100 – (Abssample -Abs blank) X 100/ Abscontrol

Blank solution – Ethanol (1ml) and Plant extract (2.5ml)

Negative control – DPPH (1ml) and Ethanol (2.5ml)

Positive control – Standard solutions (AOAC, 2005)

Anti Nutritional factors analysis:

Phytic Acid

0.5M Nitric acid was taken in a volume of 20ml and after 3-4hrs with continuous shaking, 0.5gm of sample was extracted with it. Distilled water was mixed with 0.2-1.0 mL of regular solution of sodium phytate and final volume was made to 1.4 mL.1.0 mL. Ammonium ferric sulphate solution which contained 50 g iron was added to this. In a boiling water pan, the contents were mixed, covered with a stopper for 20 minutes. After cooling the contents of the test tube, 5 mL amyl alcohol with 0.1 mL ammonium thiocyanate solution were added and shook vigorously. They were then centrifuged at a low temperature for a limited period. Using a spectrophotometer and a blank of amyl alcohol, at 465nm, the colour was determined in the amyl layer. The standard curve was used to measure the volume of phytic acid. (AOAC, 2005).

Nitrates

The sample was chopped and grounded till it was homogeneous in a mortar. In a 250ml beaker, 70ml distilled water was taken and 10gm of the sample was added to it. The beaker was filled with 2.5 mL of 4 percent NaOH and heated to 80°C for 25 minutes

while shaking continuously. In a 100ml volumetric flask, the solution was added and brought to volume with distilled water. Solution of volume 4 ml was taken in a test tube and cooled. 5 percent Ag_2SO_4 solution, 7ml 98 percent H_2SO_4, and 0.1ml 5 percent phenol solution were applied to this. The solution was left to stand for 20 minutes, trembling every now and then. In a 50ml separating funnel, toluene was applied to remove the resulting mixture, which was shaken for 5-10 minutes. After discarding the aqueous layer and washing the organic phase twice using distilled water (10ml), shaking every 2 minutes and discarding each time, 10ml of 10% Na_2CO_3 was added and shaken for 1 minute. At 407nm, the absorbance was measured. Nitrate was calculated using the formula since 4ml of the 100ml filtrate was used for analysis (AOAC, 2005).

$$\text{Nitrate} = \frac{\text{C x 100}}{\text{Ws x 4}}$$

C = Concentration of nitrate from calibration graph (µg cm3)

Ws = Weight of slurry (g).

Tannins

To 1 gm of sample in a flask, 40ml of 10 percent methanol was added. After refluxing for 1-2 hours using water bath, the volume was made up to 50ml with methanol and filtered. 0-10ml aliquots of normal tannic solution and 1 ml of sample were pipetted into a 100ml volumetric flask having 7.5 ml distilled water. 5 mL Folin-Denis reagent plus 10 mL saturated sodium carbonate solution was added to this solution and diluted with water to make up volume. After mixing the solution, the absorbance was measured at 760nm after 30 minutes. The standard curve was used to measure the sum of tannins. (AOAC, 2005).

Oxalates

The leaves were dried and powdered. In a 50ml volumetric flask, 0.5g of powdered leaves were added along with 50ml 0.25 N HCL was added. The mixture was boiled for 15 minutes and then brought to room temperature. 0.25N HCl was used to make up the volume and then extract was used for further determination. Aliquots of mixture containing 2ml standard oxalic acid solutions ranging from 0.100 -1.00 mg/ml in 1N H_2SO_4 were made. 2 ml of1 N sulfuric acid was used for the preparation of the blank.

After adding 2 ml indole which was prepared fresh, the test tubes were placed in a water bath at 80-90◦C. After cooling, absorbance was read at 525nm using a spectrophotometer. (Naik et al. 2015).

Alkaloids

The leaves were dried and powdered. In a 250ml beaker, 200ml acetic acid (10%) was taken and 2.5gm sample was added to it and kept for 4 hours. After concentrating the extract to $1/4^{th}$ of the original volume on a water bath, 15 drops of concentrated NH_4OH were added dropwise to completel filter out the precipitate. After allowing the mixture to stand for 3 hours, the supernatant was discarded. After washing the precipitates with 20ml of 0.1M , NH_4OH, they were filtered and dried in an oven. The weight was taken on an electronic weighing balance. Alkaloids were expressed by the formula

$$\% \text{ Alkaloid } = \frac{\text{Weight of alkaloid } \text{ X } 100}{\text{Weight of sample}}$$

(Chukwuma et al.2016)

3.3 Functional Bioavailability of the selected green (*in-vivo study*)

An *in vivo study* conducted on Wistar rats to assess the functional bioavailability of the selected green and its effects on blood parameters such as hemoglobin and red blood cell.

Species, strain and number of animals

Species	Wistar rats Female
Number	36
Weight	180-250gm
Humidity	30-70%
Light/Dark cycle	12 hourly
Temperature	22°C

The animals were held in polypropylene cages under sanitary and normal environmental conditions. They were given one week to acclimate in the animal house before the study began. Both animals were given a regular rat pellet diet and free access to water. The study protocols followed were as approved by Institutional Animal Ethics Committee, Panjab University, Chandigarh. All the animal testing was done under the approval of Institutional Animal Ethical Committee (IAEC) with reference number PU/45/99/CPCSEA/IAEC/2018/230. CPCSEA guidelines were duly followed.

Division and grouping

The rats were divided into 8 groups. Hemoglobin was analyzed initially to assess the anemia status of the rats. Anemia was induced by using the process of bleeding in half the groups/rats. The process involves bleeding the rats by 10% to 20% of blood volume. Blood volume of each rat is a function of its weight. The formula is

Blood volume (ml) = 0.06X body weight (g) +0.77 (Lee & Blaufox 1985). The rats were bled after inducing light anesthesia first. The hemoglobin levels were assessed again after a period of 7 days and the rats were considered anemic when levels fell by 30% of initial hemoglobin values.

Following this, the rats were divided into 8 groups as follows:

Categories	Classification of Groups	Group Specification as per diet and anemic status	Number Required
I	Control	Normal Rat + Basal Diet	3
II	Control	Anemic Rat + Basal Diet	3
I A	Treatment Group	Normal Rats + Basal Diet + High Dose Iron Supplement	5
I B	Treatment Group	Normal Rats + Basal Diet + Medium Dose Iron Supplement	5
I C	Treatment Group	Normal Rats + Basal Diet + Low Dose Iron Supplement	5

II A	Treatment Group	Anemic Rats + Basal Diet + High Dose Iron Supplement	5
II B	Treatment Group	Anemic Rats + Basal Diet + Medium Dose Iron Supplement	5
II C	Treatment Group	Anemic Rats + Basal Diet + Low Dose Iron Supplement	5

The control group contained rats with normal Hemoglobin levels and anemic who were fed the basal diet. The treatment group contained rats who were fed the supplement and effect of supplement was monitored on the hemoglobin levels.

Photo 3:
Female Wistar Rats used for the study

Method of feeding

The rats were fed the standard basal diet in pellet form. Along with this, the supplement was prepared using dried powder of the green leafy vegetable selected along with powdered form of standard rat feed to ensure consumption. Small pellets were prepared and fed along with the basal diet. The supplement was prepared in three doses monitoring the content of iron, High, medium and low doses – 150mg/kg, 100mg/kg and 50mg/kg of iron respectively.

Figure 3.4
Composition of diet

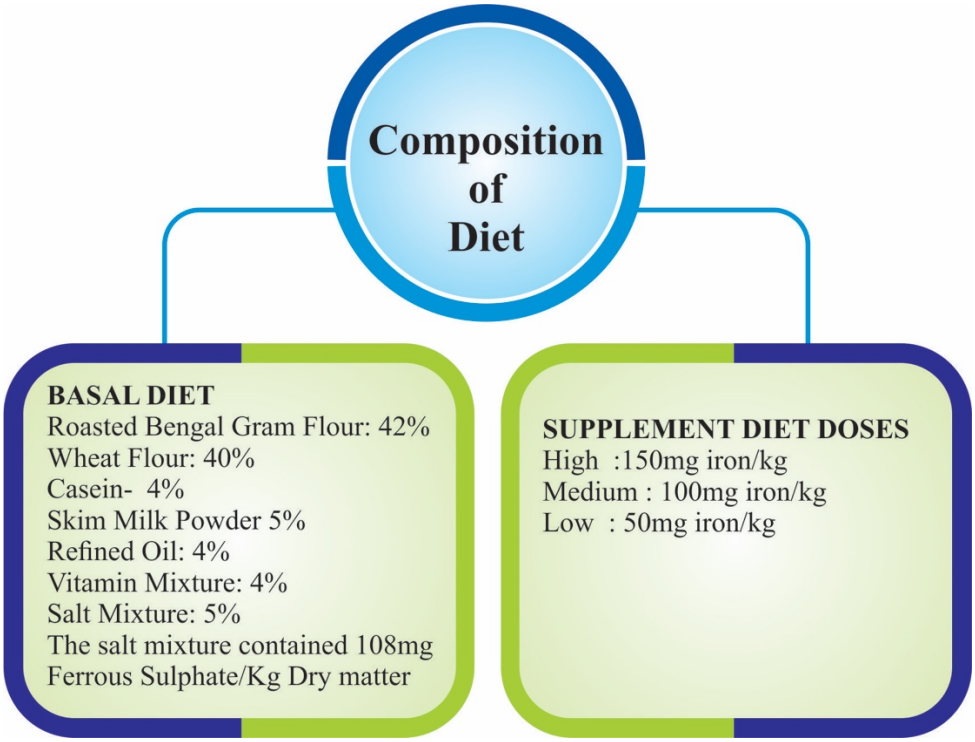


Photo 4:

Supplement Feed prepared

Blood collection

Blood was withdrawn from the retro orbital vein after slight anesthesia using diethyl ether on the day 7, day 15, day 30, day 45 and day 60, after inducing anemia and introducing supplement in the diet. The blood was collected in test tubes containing EDTA to prevent coagulation of blood. Plasma was separated by centrifugation. Then plasma was used for the estimation of red blood cell count and hemoglobin estimation.

Biochemical tests

Hemoglobin

To 5 ml of Drabkins solution, 0.02ml of blood was poured with the help of a pipette. It was mixed well after standing for 5 minutes. The absorbance was measured using a spectrophotometer at 540nm and the hemoglobin concentration was calculated with the help of a standard curve.

$$\text{Hb concentration in test (g\%)} \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} x \frac{\text{Hb concentration of standard (mg/dl) X 251Absorbance of test}}{100\text{mg/g}}$$

Absorbance of standard 100mg/g

Where 251 is the dilution factor (Young, 2001)

Red Blood Cell

The electronic method was used for the estimation of the red blood cells. The method involves diluting anticoagulated blood with a diluting fluid wich is free from particles such as saline or phosphate buffer saline (Johnson et al. 2002).

Testing of feces for iron

The feces from each cage were collected for a period of 7 days and assessed for their iron content to detect basal loss of iron through feces using the iron spectrophotometer method as mentioned earlier. The feces were dried in an oven and stored for further testing for iron content.

Calculation:

$$\text{Fe Absorption (\%)} \frac{\text{Fe intake} - \text{Fe excreted} \quad \text{x100}}{\text{Fe Intake}}$$

(Joshi &Mathur, 2010)

Loco motor Activity levels

With the aid of an acto photometer, it is simple to study locomotor behaviour. The basal activity score of all the animals was registered after each group of animals was placed individually. The activity on each rat was tested for 5 min. The difference in the activity amongst all the groups was recorded for variations and were tested. (Dews, 1953).

Photo 5:

Locomotor estimation with acto-photometer

3.4 Optimization and evaluation of product

One of the most important stages in the production of a successful and cost-effective bioprocess is the optimization of process conditions. RSM was used which is a mathematical statistical tool which uses techniques to calculate interactions between the variables used in the process using as few trials experimentally as possible. The software used in this study was Design Expert version 7.1.1, used to study the effect of different variables on the quality and stability of the beverage.

Optimization of the factors was conducted using central composite design. A four factor CCD matrix was prepared accordingly accounting for 20 randomized runs. The data analysis was carried out by quadratic model fitting followed by multiple linear regression analysis.

The generalized equation for the same is as follows:

$Y= \beta_0+ \beta_1x_1+ \beta_2x_2+ \beta_3x_1x_2+ \beta_4x_1^2+\beta_5x_2^2$

Where, y is the response, β_{1-2} are the coefficients of linear termsx_1-x_2, β_3 are the coefficients two factor interaction terms andβ_{4-5} are the coefficients of quadratic terms foreach factor.

The experiment was carried out at three levels low, medium and high (-1), (0) and (+1) respectively for each factor. For sake of simplicity, all the computations and representations have been done in coded terms.

To maintain the simplicity of the experiment, the countour plots as well as the response surfaces were formulated for all the interactions separately for any two independent variables by holding the value of the other variable constant at that central value.

Table 3.1:

Range of values for the RSM

Independent Variables	-1	0	+1
CAULIFOWER A	10	20	35
SUGAR B	2	8.5	15.5
LEMON JUICE C	1.5	3.25	5

Table 3.2:

Experimental plan as per the design

Run	**Factor 1 Cauliflower (gm)**	**Factor 2 Sugar (%)**	**Factor 3 Lemon (%)**
1	30.0	2.0	5.0
2	20	8.5	3.25
3	20	8.5	5
4	10.0	15.5	5.0

5	20	8.5	3.25
6	20	8.5	3.25
7	30	8.5	3.25
8	20	8.5	5
9	20	8.5	3.25
10	10	15	1.5
11	20	8.5	3.25
12	20	10	3.25
13	20	5	3.25
14	30	15	1.5
15	10	2	1.5
16	30	15	5
17	35	8.5	3.25
18	10	2.0	5.0
19	30.0	2.0	1.5
20	20	8.5	3.25

Product development using the dosage of green showing maximum iron absorption. Further the product was tested for biochemical parameters, sensory evaluation and shelf life.

Product optimization was done on the following basis:

- RDA range for vulnerable human populations – 15 -21mg (ICMR 2020)
- Iron rich beverage must provide 15% RDA /100ml (FSSAI, 2020)
- Bioavailability of cauliflower leaves in the present study average mean – 40.77% (In Vivo study)
- Minimum quantity of cauliflower powder needed to fulfill minimum RDA for human population on the basis of bioavailability – 10-30gms
- 100gms cauliflower powder – 38mg iron
- 15% of RDA range – 2.25mg – 3.15mg

Figure 3.5:
Steps in RTS Beverage preparation

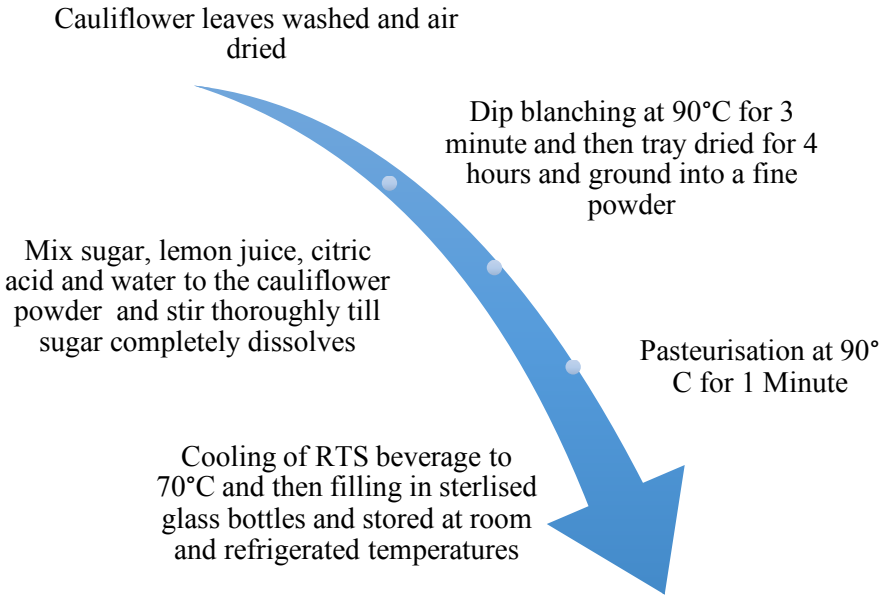


Photo 6:

Beverage prepared as per treatments designed by RSM

Sensory evaluation of product

Sensory evaluation is based on the responses of various sense organs. The following parameters were undertaken to assess the sensory parameters such as colour, flavor, taste, bitterness and overall acceptability by a semi-trained panel using a 9-point hedonic scale, with scores in the range of 9 which is extremely like to 1 which implies extremely dislike.

Biochemical Analysis

pH

pH is the measurement of H+ ion activity which was analysed using a pH meter. The standardized tip of the electrode of pH meter was kept in the solution and the sample was stirred gently to record a constant pH value. (National Bureau of Standards, 1987).

Total soluble solids

TSS was measured with the help of a refractometer which was hand held and expressed as °Brix. On the prism plate of the refractometer, drops of juice were placed and the reading was recording keeping in mind upto one deciamal place. (AOAC,2005).

Titratable Acidity

The pH of the clear juice was measured using a pH meter. To 6 gms of juice, 50 ml water was added in a beaker. Titration was carried out using 0.1N NaOH taking the end point as 8.2 as recorded by th pH meter. The volume of NaOH used was noted. The following formula was used to calculate the acidity:

$$\% \text{ acid} = \frac{[\text{mls NaOH used}] \text{ x } [0.1 \text{ N NaOH}] \text{ x } [\text{milliequivalent factor}] \text{ x } [100]}{\text{Grams of sample}}$$

(Sadasivam & Manickam, 2004)

Shelf Life Study

The product was stored under ambient temperatures (22 ± 2°C) and refrigerated conditions (4 °C). The product was assessed after every 7 days to check for its stability. The following tests were conducted.

Standard Plate Count

9ml of blank (distilled water) was pipetted from the blank sample. To 1ml of the sample, 9ml of blank was added. It became 10^{-1} dilutions.1 ml solution to 10^{-1}petridish was pipetted out and then 0.1ml solution to second petri dish, it became 10^{-2} dilution. Nutrient agar was cooled and poured in both petri dishes. It was swirled gently so that the diluted sample gets spread easily. Incubation was done at 37 °C for a period of 24 hours to see the bacterial count. (APHA, 1992)

Yeast and Mold Count

9ml of blank was pipetted from the blank sample. To 1 ml sample, 9ml blank was added. It became 10^{-1} dilutions.1 ml solution was pipetted to 10^{-1}petridish and pipetted out and then 0.1ml solution to second petri dish, it becomes 10^{-2} dilution. Glucose yeast extract was cooled and poured in both petri dishes. Swirled gently so that the diluted sample gets spread easily. Incubation was done at 25°C for a period of 3-5 days to see the yeast and mold count.(APHA, 1992)

CHAPTER – 4

RESULTS AND DISCUSSION

The current study focused on utilizing underutilized green leafy vegetables with maximum iron content to combat the high prevalence of anaemia.The selected greens were further assessed to check the bioavailability of iron on rats with an aim to prepare a functional iron-rich beverage. This chapter includes a detailed description of the survey, analysis of an*in vivo* study conducted, and product development. The results have been systematically presented under the following heads:

4.1 Survey on the prevalence of anemia

4.2 Screening of different low cost green leafy vegetables for iron content using various processing techniques

4.3 To assess the functional bioavailability of the selected green (in-vivo study)

4.4 Product optimization using response surface methodology and shelf-lifeof the product.

4.1 Survey on prevalence of anemia

The high prevalence across the world has been a significant public health issue amongst children and also pregnant women and lactating mothers across the globe. The main focus of the study was to understand the rate of prevalence in a vulnerable section of the Indian population. For this, a survey on 242 pregnant women of Chandigarh from rural as well as urban areas was conducted. The tool used for the survey was a self-structured questionnaire where different parameters depicting iron deficiency and prevalence of anemia were analyzed.

Prevalence of anemia in rural and urban population

From the filled questionnaire, normal population and cases of different categories of anemia namely severe anemia, moderate and mild anemia were observed. Out of the total 123 and 119 samples of pregnant women from both rural areas and urban areas,50.4% and 44.5% suffered from anemia. Furthermore, it was observed that 19.5%

and 19.3% had mild anemia. 30.9% and 25.2% had moderate anemia (Table 4.1.1). The results showed no severe cases of anemia and no significant difference between different categories of anemia and demographic location.

Table 4.1.1:

Average prevalence rate of anemia for rural and urban population

		Rural	**Urban**	**Total**	**Chi-square**	**p-value**
	Normal	61 (49.6%)	66 (55.5%)	127(52.5%)		
Anemia	Mild	24 (19.5%)	23 (19.3%)	47 (19.4%)	1.09	0.57
	Moderate	38(30.9%)	30 (25.2%)	68 (28.1%)		
	Total	123(100%)	119 (100%)	242(100%)		

No significance found at $p < 0.05$

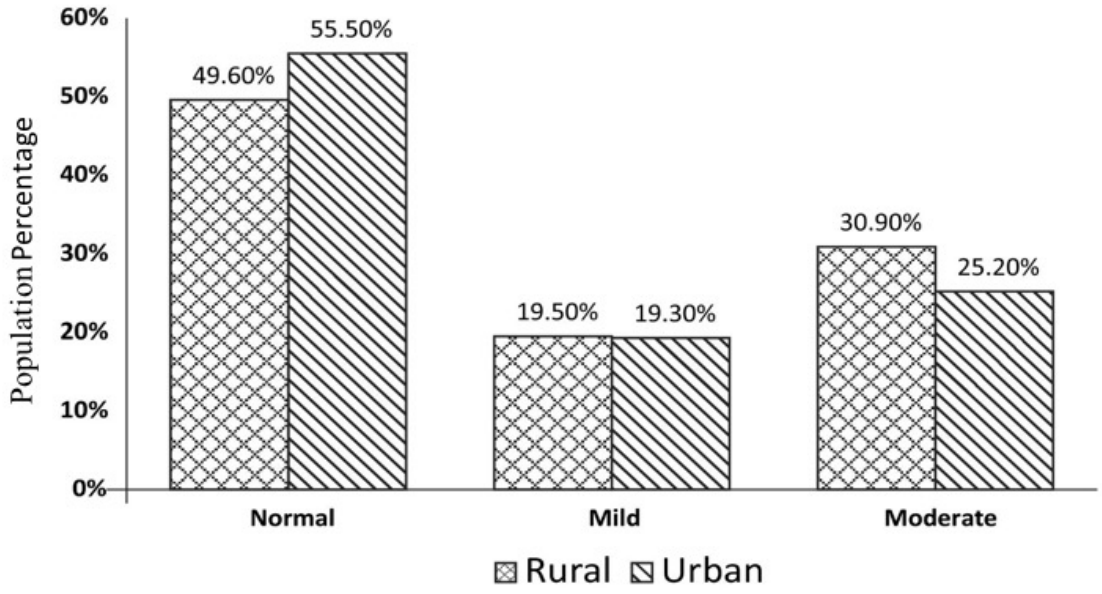
*Note:*All values are expressed as % of total population. Mild and Moderate denote the categories of anemia.

Although not much difference was observed, but these levels depict that rural areas have more moderately anemic women as compared to urban areas, the reason can be attributed to lack of hygienic facilities and ignorance or poor nutritional knowledge and practices (Mahroof et al. 2019). The main causes of anemia apart from hookworm infestation and malaria in developing areas also include lack of nutrition education and availability of resources. Despite large variations in different areas of Chandigarh, the prevalence is found high amongst both as the diets in urban areas lack micronutrients due to excessive processed food being consumed. This result can further be proved by higher prevalence of moderate anemia in India as compared to severe and mild (Didzun et al.2019). Furthermore, these results also signify that geographical location has little or no impact on the severity of the disease. In a longitudinal study conducted by Mangla & Singla (2016), reported that 98% of pregnant women in rural setting of Sonipat suffered from anemia. In another study, 57.2% women and 39.3% men suffer from anemia in a rural setting in South India, indicating that the speculation of anemia being high in rural area is true (Little et al. 2018). A recent study demonstrated that the rate of anemia in the past decade has declined, nevertheless it remains on the higher side in females and children across all age groups (Nguyen et al. 2018). Vulnerable populations

have a higher tendency to develop anemia as compared to others. Need of the hour is to promote nutritional interventions like nutrition education to mitigate the effects of deficiency (Ayensu et al. 2020).

Figure 4.1.1

Average prevalence rate of anemia for rural and urban population



Prevalence of anemia based on socio economic status

The division of the respondents was done on the basis of their socio-economic status as per the scale given by Kupuswamy (2017). The results were tabulated and analyzed to assess the difference between prevalence rates as per the socio-economic status (Table 4.1.2).

Table 4.1. 2

Prevalence of anemia based on socio economic status

		Lower	Lower middle	Upper Lower	Upper Middle	Upper	Total	Chi-square	p-value
Rural	Normal	2 (3.3%)	40 (65.6%)	11 (18%)	8 (13.1%)	0 (0%)	61 (100%)		
	Mild	0 (0%)	12 (50%)	7 (29.2%)	5 (20.8%)	0 (0%)	24 (100%)	17.90	.006**
	Moderate	0 (0%)	13 (34.2%)	21 (55.3%)	4 (10.5%)	0 (0%)	38 (100%)		
Urban	Normal	0 (0%)	24 (36.4%)	5 (7.6%)	35 (53.0%)	2 (3.0%)	66 (100%)		
	Mild	0 (0%)	12 (52.2%)	1 (4.3%)	9 (39.1%)	1 (4.3%)	23 (100%)	7.50	.227
	Moderate	0 (0%)	19 (63.3%)	2 (6.7%)	9 (30%)	0 (0%)	30 (100%)		

Significant at *p<0.01*** *level*

Note: Columns indicate the different categories of socio economic status. The rows represent normal population as well as the categories of anemia and prevalence expressed as % of the total population belonging to rural and urban areas

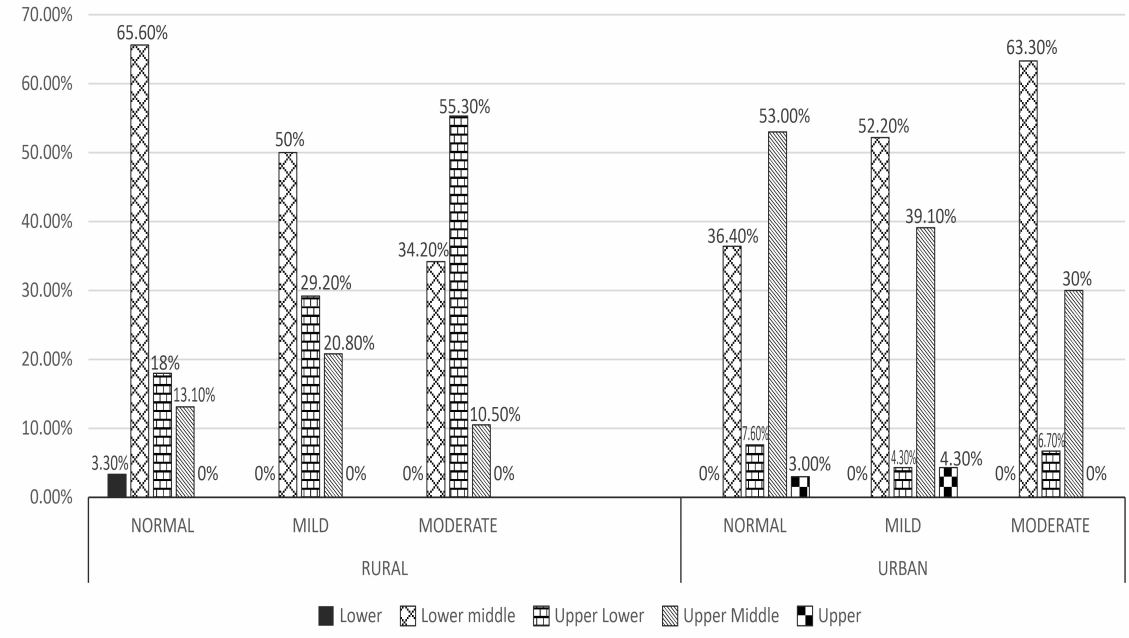
The above table shows the categorization of anemia on the basis of socio economic status and its prevalence in rural and urban areas. It was observed that mild anemia was prevalent amongst 50% of the rural population belonging to lower middle class and 34.2% suffered from moderate anemia. High prevalence of mild (29.2%) and moderate (55.3%) anemia was also observed amongst participants belonging to upper lower class. The association between the socio economic paramters and prevalence of anemia was found to be statistically significant in rural areas. This might be due to poor hygiene and lack of availability of resources and minimal dietary intake of iron rich sources. Similary in the case of urban population, 52.2% of the population belonging to lower middle class and 63.3% suffered from mild and moderate anemia respectively. Lesser prevalence was seen amongst the subjects from upper lower class from the urban population. No significant correlation was found amongst socio economic status and urban population.

Similar results were recorded by Chandrakumari et al. (2019) wherein they observed a significant correlation between the above parameters and anemic conditions.

Prevalence of anemia was high (52.4%) amongst adolescents belonging to low socio economic status. Lower middle and upper lower class having highest anemia prevalence rate can be further testified by Khan et al. (2017) stating that anemia and lower socioeconomic status were found to be significantly related. The results reported in a study conducted by Vindhya et al. (2019) were found to be alike, where 61.1% of the target group belonging to upper lower socio economic statues suffered from anemia. The factors that contribute to higher prevalence in lower class include diet, presence of infections such as malaria, hookworm and consumption of predominantly cereal based vegetarian diets. In another study conducted by Rani & Bandrapalli (2017) it was observed that anemias prevalence was higher in women, vegetarians, children with lower socio economic status. Sarala & Gopalan (2019) also reported that upper socio economic class has relatively less cases of anemia (3.625%). Maximum prevalence was seen in participants belonging to low income group (63.75%) followed by middle income group (32.63%).

Figure 4.1.2

Anemia prevalence based on socio-economic status of the population



Categorization and prevalence of anemia on the basis of age

On the basis of age group it was observed that women belonging to 26-35 years of age were more prone to developing anemia. More than 50% (56%) women residing in rural community falling in the age group of 26-35 years suffered from mild and moderate (43.2%) anemia. In the rural setting, moderate anemia was more prevalent amongst women belonging to the age group 16-25. Wheres in the urban setting, higher prevalence (60.9% and 53.3%) was seen for mild and moderately anemic women belonging to the age group of 26 -35. No statistical difference was inferred amongst the groups.

Table 4.1.3:

Prevalence of anemia on the basis of age of the participants

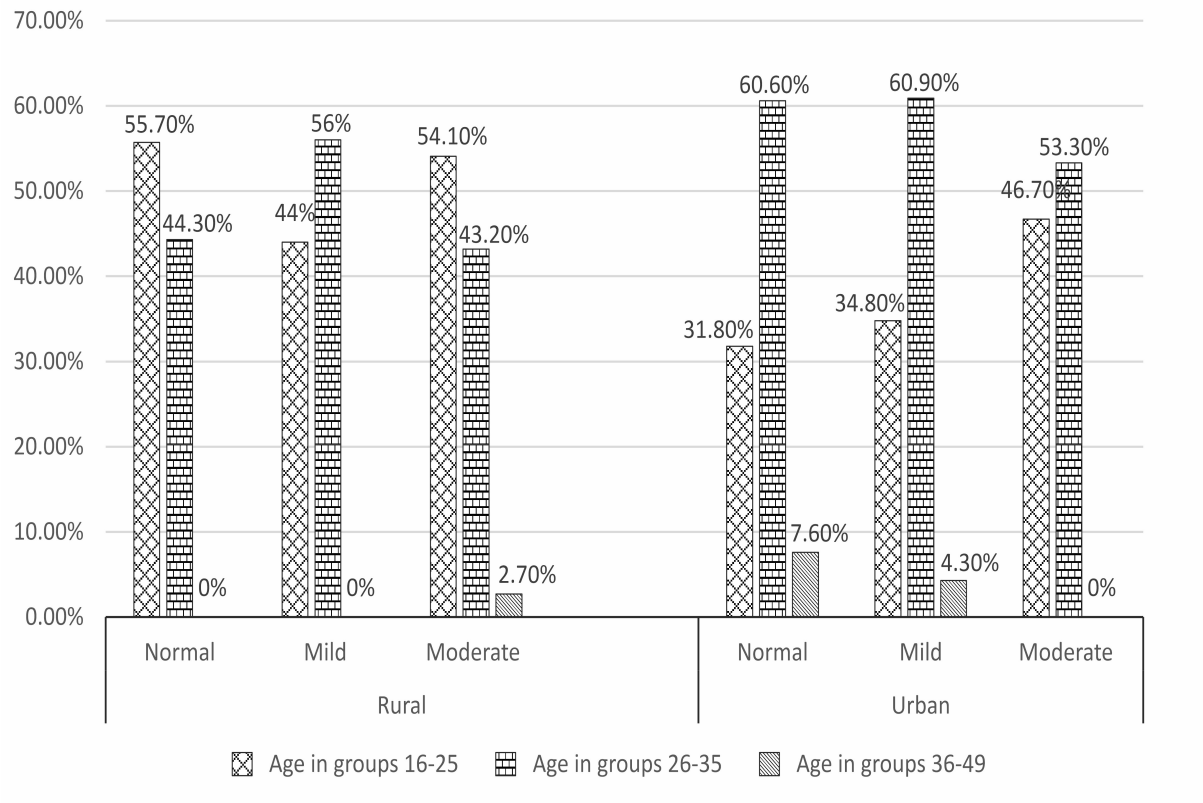
		Age in groups			Total	Chi-square	p-value
		16-25	26-35	36-49			
Rural	Normal	34(55.7%)	27(44.3%)	0(0%)	61(100%)		
	Mild	11(44%)	14(56%)	0(0%)	25(100%)	3.44	0.48
	Moderate	20(54.1%)	16(43.2%)	1(2.7%)	37(100%)		
Urban	Normal	21(31.8%)	40(60.6%)	5(7.6%)	66(100%)		
	Mild	8(34.8%)	14(60.9%)	1(4.3%)	23(100%)	3.85	0.43
	Moderate	14(46.7%)	16(53.3%)	0(0%)	30(100%)		

No significance found at $p<0.05$
Note: Different age groups are represented in columns. The categories of anemia in rows show prevalence as % of total population.

In a similar study conducted by Sarala & Gopalan (2019)**,** it was reported thatmaximum women (61.75%) 20 – 24 years of age suffered from anemia. Kishore et al. in 2020further signified our conjure that no difference were seen in given average age group but significant changes were observed in broader age variations, 5 to 14 years (51.1%) and 15 to 49 years (57.1%).

Figure 4.1.3

Anemia prevalence based on age of the participants



Categorization of anemia on the basis of gravidity

Table 4.1.4shows that with 3rd child anemic patients rose up to 57.6% as compared to 48.3% of 1st child and 42.9% of 2nd child.

Table 4.1.4

Effect of gravidity (birth order of pregnancy) on category of anemia

		I[st]child	II[nd]child	III[rd]child	Total	Chi –square	p-Value
Rural	Normal	24(39.3%)	30(49.2%)	7(11.5%)	61(100%)	10.81	0.03*
	Mild	11(45.8%)	6(25%)	7(29.2%)	24(100%)		
	Moderate	23(60.5%)	9(23.7%)	6(15.8%)	38(100%)		
Urban	Normal	37(56.1%)	22(33.3%)	7(10.6%)	66(100%)	4.83	0.30
	Mild	13(56.5%)	8(34.8%)	2(8.7%)	23(100%)		
	Moderate	10(33.3%)	16(53.3%)	4(13.3%)	30(100%)		

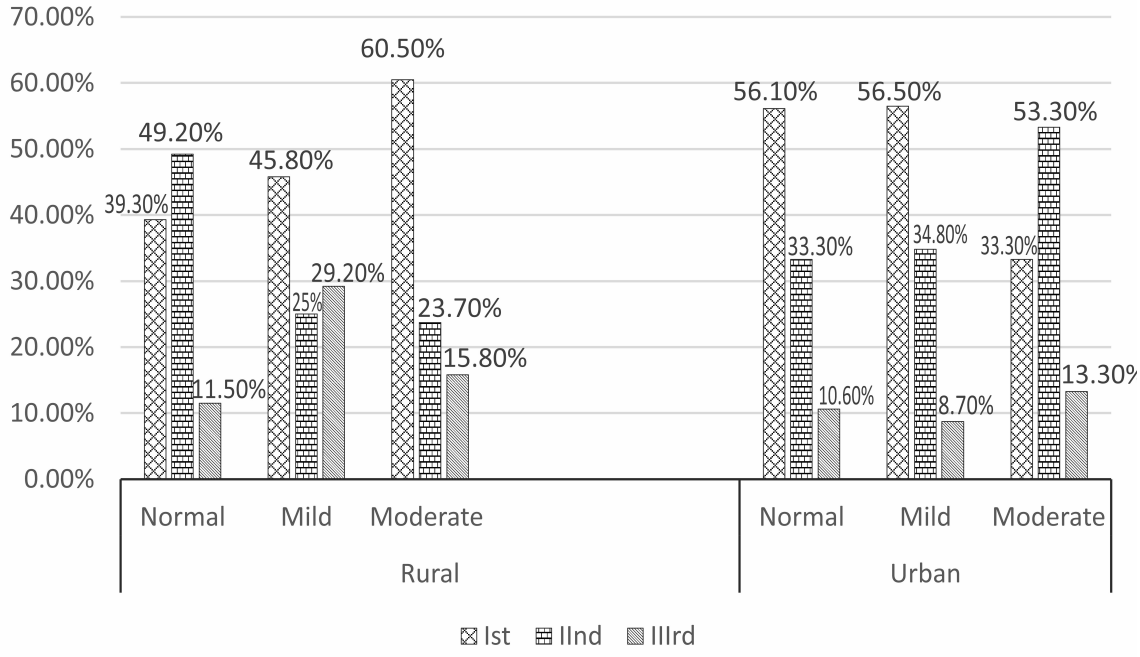
*Significant at $p<0.05$

Note: Column represent the order of pregnancy. The prevalence of anemia category wise is depicted in rows as % of total population.

It was observed that birth order of pregnancy and anemic status of the pregnant women were significantly associated. A correlation was found between the birth order of pregnancy in the rural area. No significant association was found amongst the urban population. A slight difference in percentage (30.3%) was observed amongst women bearing their third child and suffering from moderate anemia. Suryanarayan et al. (2017) observed that anemia is more prevalent in women having frequent pregnancies within 1 year (40.2%). Close spacing of pregnancies had an effect on the anemia status as compared to order of pregnancy.

Figure 4.1.4

Effect of gravidity (birth order of pregnancy) on category of anemia



4.2 Screening of different low cost green leafy vegetables for iron content using various processing technique

Table 4.2.1

Effect of different time – temperature combination of dip blanching on peroxidase enzyme activity inhibition

Sample	Temperature (°C)	Peroxidase Activity			
		Blanching Time (min.)			
		1 min	3 min	5 min	7 min
S_1	80	+	+	-	-
	85	+	-	-	-
	90	+	-	-	-
S_2	80	+	+	+	-
	85	+	+	-	-
	90	+	-	-	-
S_3	80	+	+	+	-
	85	+	+	+	-
	90	+	+	-	-
S_4	80	+	+	-	-
	85	+	-	-	-
	90	+	-	-	-
S_5	80	+	+	-	-
	85	+	-	-	-
	90	+	-	-	-

Note: Where S_1 = Cauliflower Leaves, S_2 = Spinach Leaves, S_3 = Amaranth Leaves, S_4 = Lettuce Leaves, S_5= Radish Leaves. + and – indicate the activation and inactivationof peroxidase enzyme

Blanching was carried out with the objective of inhibiting enzymatic action to prevent contamination within food stuffs that may lead to discolouration, loss of taste and texture, degradation of odour and flavor and nutrient losses. The results depicted the complete inactivation of peroxidase enzyme after 5 miutes of dip blanching indicating an inactivation in all enzymatic activity at this particular time and temperature combination. In a study by Malik et al. (2018) it was reported that after 5 minutes of blanching of beans, carrot and cauliflower, no peroxidase enzyme activity was

observed. FSSAI, (2010) also reported that in green leafy vegetables the inactivation of peroxidase enzyme takes place between 3 to 5 minutes when heated to a temperature of 90^0 C. Xiao et al. (2017) reported that out of all the enzymes found in GLV's, peroxidase enzyme exhibits maximum thermal stability, thus its destruction indicates the destruction of the other enzymes as well. Nurhuda et al. (2013), in their study observed that when blanched for 2.5 minutes, a significant reduction was found in the peroxidase activity in rambutan peel. However, blanching time between 2.5 and 5 min showed no statistical significance in their findings.

Table 4.2.2:

Effect of different times of microwave blanching on peroxidase enzyme inactivation

Sample	Power (W)	Peroxidase Activity Blanching Time (min.)		
		1 min.	**3 min.**	**5 min.**
S_1	900 W	+	-	-
S_2		+	+	-
S_3		+	+	-
S_4		+	-	-
S_5		+	-	-

Note: Where S_1 = Cauliflower Leaves, S_2 = Spinach Leaves, S_3 = Amaranth Leaves, S_4 = Lettuce Leaves, S_5= Radish Leaves. + and – indicate the activation and inactivation of peroxidase enzyme

From the results it was concluded that microwave blanching of 1 minute was effective in inhibiting peroxidase enzyme activity of cauliflower, lettuce and radish leaves whereas it took 3 minutes for enzyme inhibition in the case of amaranth and spinach leaves. The reason for variation in timings might be because of the type of vegetables and size of vegetable pieces. Severini et al. (2016), assessed microwave blanching's effect on enzymatic activity in GLV's. It was reported that maximum (90%) peroxidase activity was inhibited within 90 seconds of blanching in hot water and within 50 seconds after blanching in microwave. For best quality of raw untreated vegetable samples, a reduction in activity by 90% was considered adequate. Xiao et al. (2017) also recorded that when blanching was done using a microwave, enzyme inactivation took place as well as an increase in the anti oxidant capacity was observed due to

degradation of phenolic content. Similar results were reported by Orak. H (2006) who observed the effect of microwave blanching on peroxidase activity. It was seen that 2 minutes at 900W in the microwave caused an inactivation of the peroxidase enzyme.

According to Acho et al. (2015), processing techniques like blanching positively affect raw greens. They help in the bioavailability of various minerals like iron, calcium, magnesium despite causing nutrient losses. In another study by Kawashima and Soares (2005), it was observed that a larger mineral loss was reported at longer duration of blanching i.e. 15 minutes. Many reporters have observed the benefits of microwave blanching over dip blanching. (Acho et al. 2015 and Quenzer & Burns 2006). Keeping this in mind, dip blanching for 3 minutes at 90 ◦C was used for further product development and processing as it is a more economical method.

Table 4.2.3:

Phytochemical profile and iron content of the green leafy vegetables (fresh and dried)

Sample	Treatments (60 °C for 4 hours)	Moisture (%)	Ascorbic acid (mg/100gm)	Beta carotene (mg/100gm)	Iron (mg/100gm)	Total phenol content (mg/100gm)	DPPH (%)
S_1	Raw Fresh Leaves	84.75	44.05± 0.05	2.42± 0.01	39.25± 0.05	53.47± 0.07	72.50± 0.05
	Tray Drying		19.45± 0.04**	1.08± 0.02**	38.95 ± 0.07	210.60± 0.05**	91.65± 0.02**
S_2	Raw Fresh Leaves	90.21	35.45± 0.1	2.78± 0.1	1.10 ± 0.03	10.76± 0.05	52.29± 0.03
	Tray Drying		18.65± 0.05**	1.11± 0.05*	1.20 ± 0.01	71.78± 0.02**	74.71± 0.04**
S_3	Raw Fresh Leaves	85.21	63.46± 0.02	2.82± 0.03	16.91 ± 0.09	34.60± 0.03	56.95± 0.06
	Tray Drying		28.05± 0.01**	0.98± 0.07*	16.79 ± 0.01*	135.72± 0.07**	81.35± 0.07**
S_4	Raw Fresh Leaves	92.75	12.45± 0.07	1.36± 0.01	1.99 ± 0.01	19.17± 0.02	64.05± 0.05
	Tray Drying		7.76± 0.05**	0.61± 0.03*	1.91 ± 0.01*	158.00± 0.04**	85.40± 0.03**
S_5	Raw Fresh Leaves	90.14	65.76± 0.03	2.49± 0.05	16.75 ± 0.07	38.52± 0.07	75.61± 0.02
	Tray Drying		32.61± 0.05**	1.04± 0.02*	16.73 ± 0.04	253.94± 0.05**	93.74± 0.05**

Significant at $p<0.01$** level $p<0.05$*level

Note: All values are expressed as MEAN ±SD (n=2), All values are means of duplicate analysis. The leaves were blanched at 90C for 3 minutes and then tray dried at 60C for 4 hours.

Where S_1 = Cauliflower Leaves, S_2 = Spinach Leaves, S_3 = Amaranth Leaves, S_4 = Lettuce Leaves, S_5= Radish Leaves.

The phytochemical and iron concentration of the fresh greens and blanched and tray dried samples showed variation in the amounts of nutrients present. As drying progressed, the ascorbic acid content decreased fom 63.46mg to 28.05mg in Amaranth, from 44.05mg to 19.45mg in cauliflower leaves, 65.76mg to 32.61mg in radish leaves, 12.45mg to 7.76mg in radish leaves and 35.45mg to 18.65mg in spinach. The leaching of ascorbic acid might be because of its oxidizable, water-soluble and heat liable nature. Parallel results were reported by Naikwade in 2014 where retention of only 28 -35% of ascorbic acid content was observed in green leafy vegetables when dried. Similarly, Chauhan et al. (2016) reported the decrease in ascorbic acid to 65.11% when green leafy vegetables were blanched and then tray dried. In the present study, the percentage ascorbic acid loss ranged from 30 -60%. Like ascorbic acid, significant reduction of beta carotene was observed after blanching and drying and it ranged from 2.42mg to 1.08mg in cauliflower leaves, 2.49mg to 1.04mg in radish leaves, 2.78mg to 1.11mg in spinach, 1.36mg to 0.61mg in lettuce and 2.82mg to 0.98 mg in amaranth. This reduction might be because of the sensitive behavior of beta-carotene towards heat and light. Various other factors that can cause the destruction of beta carotene include exposure to light, temperature, oxygen and presence of catalysts. Gupta et al. (2013) when analyzed the content of beta carotene of fresh and dry leaves, reported that β—carotene retention was only 20–69%, of their initial content. Iron content of the raw and tray dried samples was also analysed. Cauliflower leaves had maximum iron content (39.25mg) followed by amaranth (16.91mg) and radish leaves (16.75mg) respectively. No statistical difference was seen amongst the iron content after drying though a minute change was seen in the mean values for fresh and dried leaves. According to Bhosale & Arya (2010) different drying methods do not significantly affect the iron content in raw greens. This statement can further be proved by the study of Kiremire et al. (2010), swho stated that iron lost is comparatively less on employing various drying techniques as compared to the blanching process. They further reported that variation in iron content can result from differing lengths of exposure to light, heat, and oxygen.

When tested for the total phenol content and anti oxidant activity, a significant increase was observed amongst the samples after tray drying. Cauliflower leaves and radish leaves had the highest total phenol content which significantly increased after drying

from 53.47mg to 210.60mg and 38.52mg to 253.94mg respectively. Spinach reported a total phenol content of 10.76mg which increased to 71.78mg on blanching and drying. Lettuce and amaranth had a phenol content of 19.17mg and 34.60mg which increased to 158mg and 135.72mg respectively on drying. The change in the phenolic compounds is reported because new anti oxidant compounds are formed due to non- enzymatic browning and also as a result of breakdown due to heat which results in degradation of bound phenolic compounds thereby making them more available. During drying, the phytochemical compounds become more bioavailable due to inhibition of enzymes that cause oxidation, breaking of complex bonds and disruption of the outer cell wall. Similar findings were observed by Mongi et al. (2015) wherein they noted that drying methods in bananas, mangoes, pineapple and tomatoes showed statistical significance ($p<0.05$, CI 95%) on total phenol content and antioxidant activity. Meena et al. (2013) also reported a significantly higher total phenol content in green leafy vegetables on drying. Blanching resulted in a decrease in the total phenol content, flavonoids and antioxidant levels in leafy vegetables studied while drying caused increase in concentration of nutrients and also increase in phenol, flavonoid content and antioxidant activity. Oboh (2004) reported that tropical green leafy vegetables have a high phenol content. Turkem et al. (2005) reported the same findings in spinach. When assessed for total phenol content in fresh vegetables it was observed that the values ranged from 183.2 to 1344.7 mg/100 g on dry weight basis. The level of anti oxidant activity was found to be between 12.2% to 78.2%. Raja et al. (2017) reported that on drying, due to maillard reaction, an increase from 6-64% is observed in the phenol content of papaya leaves. The DPPH activity was also on a higher side which has a direct correlation with the total phenol content.

The above mentioned results of the current study are thus advantageous for achieving desired methods in further processing of fruits and vegetables for extraction of useful phytochemicals.

Table 4.2.4

Anti nutritional content of the green leafy vegetables (fresh and dried)

Sample	Treatments	Phytic acid	Oxalate	Nitrate	Tannin	Alkaloid
	(60 °C for 4 hours)	All values are expressed as mg/100gm				
S_1	Raw Fresh Leaves	32.45±0.04	175.45±0.05	28.75±0.02	64.75±0.05	83.45±0.05
	Tray Drying	25.51±0.02	97.78±0.03**	12.44±0.01*	37.80±0.04*	72.81±0.01
S_2	Raw Fresh Leaves	24.25±0.04	154.75±0.04	30.24±0.05	88.65±0.04	72.32±0.04
	Tray Drying	21.67±0.03	84.5±0.03**	9.80±0.03*	42.54±0.03*	68.09±0.03
S_3	Raw Fresh Leaves	54.20±0.05	210.56±0.02	20.65±0.04	69.87±0.05	65.98±0.03
	Tray Drying	22.42±0.03*	72.52±0.02**	7.90±0.28*	39.89±0.03*	53.22±0.01
S_4	Raw Fresh Leaves	43.45±0.04	180.45±0.06	21.35±0.03	72.65±0.04	62.85±0.05
	Tray Drying	25.80±0.02*	65.80±0.02**	11.86±0.01	41.27±0.02*	57.88±0.01
S_5	Raw Fresh Leaves	54.25±0.05	67.65±0.05	44.54±0.06	85.45±0.04	57.45±0.03
	Tray Drying	26.89±0.03*	54.90±0.03	12.76±0.03*	43.70±0.03*	48.80±0.03

*Significant at $p<0.05$

**Significant at $p<0.01$

Note: All values are expressed as MEAN ±SD (n=2). All values are expressed as mg/100gm on fresh weight and dry weight basis. The leaves were blanched at 90C for 3 minutes and then tray dried at 60C for 4 hours. Where S_1 = Cauliflower Leaves, S_2 = Spinach Leaves, S_3 = Amaranth Leaves, S_4 = Lettuce Leaves, S_5= Radish Leaves

Table 4.2.4 shows the anti nutritional content in green leafy vegetables on fresh as well as dry weight basis. It was noted that the phytic acid value was highest in amaranth and radish leaves 54.20 and 54.25mg which reduced to 22.42 and 26.89mg on blanching and drying. The phytic acid content in cauliflower leaves was 34.45 which reduced to 25.51mg on drying. Spinach had relatively lesser content of phytic acid 24.25mg which further reduced to 21.67mg on drying. The reduction in phytic acid during blanching and drying is attributed to the rupture in the cell wall which causes leaching of soluble compounds. According to Natesh et al. (2017) the more phytic acid results in less micronutrient absorption. Similar observations were made by Ogbede et al. (2015), where in phytate was found to be 22.00±0.81mg/100gm in *Brassica olerecea* species. The oxalate content was found high amongst all the vegetables selected with radish recording the least value. On drying the oxalate content of all the greens reduced significantly. Cauliflower leaves showed a reduction of 175.45mg to 97.78mg, spinach from 154.75mg to 84.50mg, amaranth from 210.56 mg to 72.52mg, lettuce from 180.45mg to 65.80mg and radish leaves from 67.65 to 54.90mg. The degradation of oxalate occurs through oxidation, decarboxylation, and acetylation. Heating at high temperatures causes the calcium oxalate cells to breakdown and further on the structure of oxalate collapses. Loss of oxalate takes place due to leaching. According to Sheela et al. (2004) content of oxalic acid can range up to all the way from nil to 121.09mg/100g. Mwanri et al. (2018) in their study proved that oxalate content continues to increase with plant age. The nitrate content of all the vegetables was found to be low which further reduced on drying. Cauliflower leaves had a nitrate content of 28.75mg which reduced to 12.44mg. It further reduced from 30.24 to 9.80mg in the case of spinach, 20.65mg to 7.90mg in the case of amaranth, 21.35mg to 11.86mg in the case of lettuce and 44.54 to 12.76mg in the case of radish leaves. A difference in nitrate can be attributed to various agricultural and environmental factors like intensity of light moisture in soil, variety of crop, temperature and fertilizers (Sinha & Khare, 2017). Further, Gupta et al. (2015) reported that a reduction in the content of oxalic acid and nitrate was observed on boiling the cauliflower leaves. Loss in anti-nutrients by various processing methods like blanching and drying was found significant.

The tannin content ranged from 88.65mg to 64.75mg in all the fresh samples which further reduced to 43.70 to 37.80mg on drying. The analysis shows that average tannin

content in cauliflower is smaller than all the other raw greens. A study by Gupta et al. (2015) depicts that tannin content of green leafy vegetables can vary substantially ranging from 61 to 205 mg/100 g. Average alkaloid content in cauliflower was found to be larger than all the other raw greens 83.45mg which reduced to 72.81mg. Radish leaves reported the least alkaloid content of 57.88 which further reduced to 48.80mg on blanching and drying. Generally alkaloid content is found to be slightly higher in raw green leafy vegetables which can be reduced on boiling (Onyeka & Nwamekwe, 2007). The alkaloid content in cauliflower leaves is beneficial and known to have microbial properties (Gowri & Manimegalai, 2018).

4.3 To assess the functional bioavailability of the selected green (*in-vivo* study)

Table 4.3.1

Average Food Supplement Consumption Pattern of female wistar rats

Groups (n=5)	Supplement provided (g)	Supplement consumption g/animal/day	Average supplement consumption g/animal/day (Mean±SD)	p- Value
Treatment group IA	60gm/day	7.5	7.20±0.52	0.103
Treatment group IB		7.5		
Treatment group IC		6.6		
Treatment group IIA		10	8.87±1.27	
Treatment group IIB		9.1		
Treatment group IIC		7.5		

No significance found at $p<0.05$

Note: All values are expressed as Mean ± SD (n=2). IA- Normal rats on high dose- 150mg/kg, IB- Normal rats on medium dose- 100mg/kg, IC- Normal rats on low dose- 50mg/kg, IIA- Anaemic rats on high dose- 150mg/kg , IIB- Anaemic rats on medium dose- 100mg/kg ,IIC- Anaemic rats on low dose- 50mg/kg

Figure 4.3.1

Average food supplement consumption pattern of female wistar rats

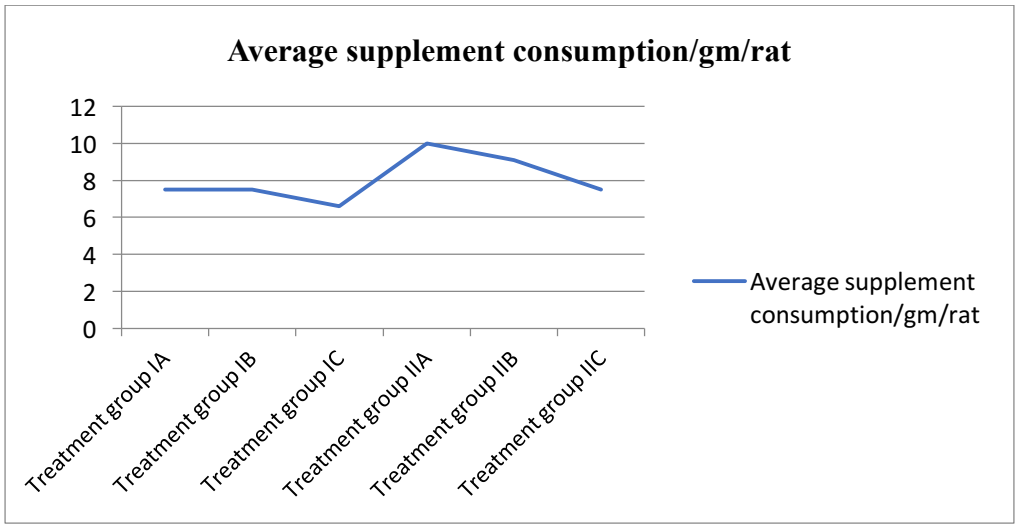


Table 4.3.1 above shows the amount of supplement consumed on an average by each group. 60gms of the supplement was provided to each group once a day along with the basal diet. The supplement was given at 7am in the morning and basal diet at 7pm in the evening. The average consumption was calculated by measuring the amount of supplement left over. The average supplement consumed per rat was divided by the number of rats/ group. This exercise was conducted for a week i.e. 7 days to get an average estimation. A higher average consumption was found in the anemic groups (8.87mg/animal/day) as compared to the normal rats (7.20mg/animal/day) fed the supplement, the difference was statistically insignificant between the consumption patterns of both the groups.

Since the iron content in the diets varied, the iron consumption differed between the classes. Goel et al. (1977) in their study deduced that when cauliflower leaf protein concentrate was fed to rats along with a wheat based diet, a better protein efficiency ratio and growth was observed in rats fed the cauliflower leaf concentrate. In another study conducted by Joshi & Mathur (2010), the feed containing dried cauliflower greens had maximum acceptability amongst all the plant-based supplements.

Table 4.3.2:

Fecal excretion and iron absorption amongst the treatment groups

Group	Iron content of feces (mg/100gm) Day 1-7	Ironintake (mg)	Iron absorption (%)	p – value
Treatment group IA	23.45±0.28			
Treatment group IB	20.65±0.14	30.92	36.48%	
Treatment group IC	10.55±0.14			0.562
Treatment group IIA	26.75±0.14			
Treatment group IIB	21.55±0.00	39.08	45.06%	
Treatment group IIC	10.45±0.04			

No significant difference was found at $p<0.05$

Note: All values are expressed as Mean ± SD (n=2). IA- Normal rats on high dose- 150mg/kg, IB- Normal rats on medium dose- 100mg/kg, IC- Normal rats on low dose- 50mg/kg, IIA- Anaemic rats on high dose- 150mg/kg , IIB- Anaemic rats on medium dose- 100mg/kg ,IIC- Anaemic rats on low dose- 50mg/kg

Table 4.3.2 shows the amount of iron excreted out via feces and iron absorption as per the iron intake. The amount of iron absorbed varied from each group. The highest iron excretion was found in rats consuming high dose supplements of both normal and anemic groups (23.45 and 26.75mg) repestively. The difference was statistically insignificant between the groups in terms of the fecal output. The average iron absorption ranged from 36.48% for normal rats and 45.06% for anemic rats. The high absorption can also be attributed to the presence of Vitamin C in cauliflower greens. In the same study it was also reported that the iron intake ranged from 13-15mg and the fecal output of iron was recorded as 10 – 12%. The higher the consumption of iron, higher is the fecal output as can be seen from this study as well. Iron absorption was found high from the plant based supplements in another study conducted by Joshi & Mathur (2010). Thereafter, Nomkong et al. (2019), reported an absorption as high as 28.09% was from green leafy vegetables. Blanching and oven drying techniques were used for the preparation of the supplement, that may also benefit in increasing the bioavailability of iron by decreasing the anti nutritional components and increasing phytochemical concentrations of the selected vegetables.

Table 4.3.3(a):

Effect of iron supplementation on weight of female wistar rats

Group	Day 1	Day 15	Day 30	Day 45	Day 60
Control I	201.00±3.60^{b}	203.33±5.77^{bc}	205.00±5.00^{c}	213.33±2.88^{c}	215.00±5.00^{d}
Control II	191.66±7.66^{bcd}	195.65±5.00^{c}	201.33±2.88^{c}	205.65±5.00^{cd}	208.33±2.88^{e}
Treatment group IA	187.5±5.24^{cd}	200.00±4.47^{c}	209.67±3.27^{b}	214.67±4.08^{c}	220.83±3.76^{d}
Treatment group IB	209.67±8.52^{a}	219.67±4.55^{a}	226.67±6.95^{a}	229.50±6.57^{a}	235.00±7.85^{a}
Treatment group IC	182.50±4.59^{d}	188.17±3.82^{d}	194.83±4.83^{c}	199.83±4.71^{d}	208.00±5.10^{e}
Treatment group IIA	190.83±7.36^{cd}	205.33±4.97^{bc}	211.17±8.11^{b}	220.33±3.27^{abc}	224.17±3.76^{cd}
Treatment group IIB	195.83±8.61^{bc}	210.00±8.37^{b}	214.17±5.85^{b}	222.00±10.68^{abc}	228.33±10.33^{bcd}
Treatment group IIC	194.67±4.08^{bc}	210.00±6.32^{b}	215.00±4.47^{b}	218.17±1.83^{bc}	225.00±3.69^{bcd}

Significance at $p < 0.05$

Note: All values are expressed as Mean ± SD (n=2). Values with similar superscripts in column do not differ significantly ($p<0.05$) according to Duncan's Multiple Range Test. IA- Normal rats on high dose- 150mg/kg, IB- Normal rats on medium dose- 100mg/kg, IC- Normal rats on low dose- 50mg/kg, IIA- Anaemic rats on high dose- 150mg/kg , IIB- Anaemic rats on medium dose- 100mg/kg ,IIC- Anaemic rats on low dose- 50mg/kg

Table 4.3.3(b):

Effect of iron supplementation on weight of female wistar rats

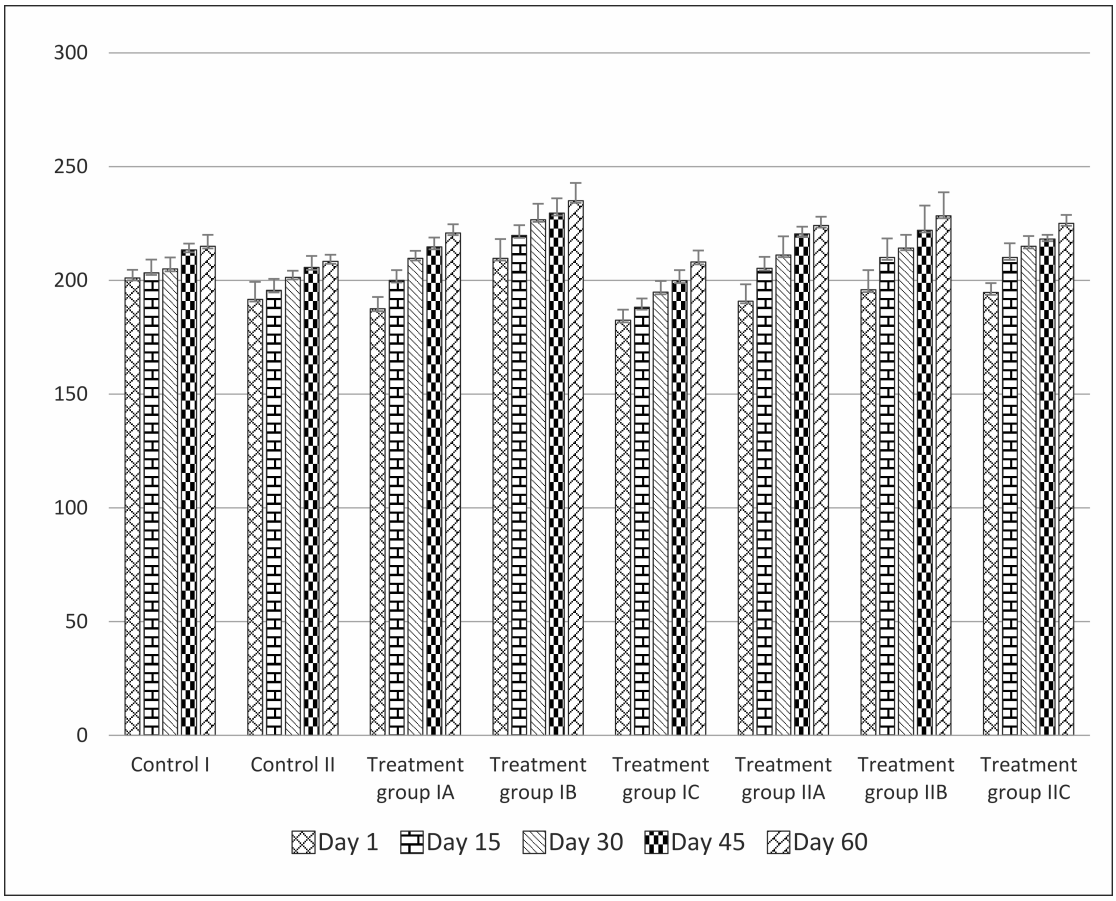
Group	Day 1	Day 15	Day 30	Day 45	Day 60
Control I	201.00±3.60^{c}	203.33±5.77^{c}	205.00±5.00^{b}	213.33±2.88^{a}	215.00±5.00^{a}
Control II	191.66±7.66^{c}	195.65±5.00^{c}	201.33±2.88^{b}	205.65±5.00^{a}	208.33±2.88^{a}
Treatment group IA	187.5±5.24^{d}	200.00±4.47^{c}	209.67±3.27^{b}	214.67±4.08^{b}	220.83±3.76^{a}
Treatment group IB	209.67±8.52^{c}	219.67±4.55^{b}	226.67±6.95^{ab}	229.50±6.57^{a}	235.00±7.85^{a}
Treatment group IC	182.50±4.59^{d}	188.17±3.82^{c}	194.83±4.83^{b}	199.83±4.71^{b}	208.00±5.10^{a}
Treatment group IIA	190.83±7.36^{c}	205.33±4.97^{b}	211.17±8.11^{b}	220.33±3.27^{a}	224.17±3.76^{a}
Treatment group IIB	195.83±8.61^{d}	210.00±8.37^{c}	214.17±5.85^{bc}	222.00±10.68^{ab}	228.33±10.33^{a}
Treatment group IIC	194.67±4.08^{d}	210.00±6.32^{c}	215.00±4.47^{bc}	218.17±1.83^{b}	225.00±3.69^{a}

Significance at $p < 0.05$

Note: All values are expressed as Mean ± SD (n=2). Values with similar superscripts in rows do not differ significantly ($p<0.05$) according to Duncan's Multiple Range Test. IA- Normal rats on high dose- 150mg/kg, IB- Normal rats on medium dose- 100mg/kg, IC- Normal rats on low dose- 50mg/kg, IIA- Anaemic rats on high dose- 150mg/kg , IIB- Anaemic rats on medium dose- 100mg/kg ,IIC- Anaemic rats on low dose- 50mg/kg

Figure 4.3.2

Effect of iron supplementation on weight of female wistar rats



The repeated measures ANOVA was used to compute the results. For post-hoc analysis, Duncan test was used as the variances of the different groups are equal. The results for both the control groups showed that a significant difference in weights of all the groups was observed on all days. An increase of 12.60% weight was observed in control group I fed the basal diet and 18% increase was observed in anemic rats fed the basal diet. Moreover, the graph shows that the mean weight has been increasing as the number of days increase. In the case of all the treatment groups fed the supplement in different doses, it was observed that treatment groups of both normal and anemic rats had an increase in weight up to 15%. A significant difference was observed in both their weights from day 1 to day 15, 30, 45 and 60 respectively. Post hoc analysis further revealed that treatment group IIA showed a significant increase in the weight till day 30, post which the rate of weight again was found similar amongst all the groups.

Similar results were reported when anemic rats had less weight earlier but study indicates that it doesn't differ much from normal rats after a period of 60 days (Nagababu et al. 2008). As each day progressed, weight of rats of each group also increased as proved by our study. Similar results have been reported by Joshi & Mathur (2010) wherein an increase in weight of all rats was observed when diet was supplemented with cauliflower greens. Singh et al. (2019) in their study, reported that in adolescent, when fed a diet supplemented with cauliflower greens, an average increase in weight was observed between 13.45 and 14.66% .

Sufficiency of glucose can be perceived as one of the main reasons for gaining weight when sufficient amount of iron is given to rats (Modupe et al. 2018). In the current study, the rate of weight gain with the supplement in normal and anemic rats was found to be equivalent to the basal diet and no significant difference was observed. Weight was tested as a parameter to ensure that the supplement can support growth as well, the main aim being to increase hematological profile. It can therefore be suggested that a supplement providing high iron is beneficial in supporting growth in normal as well in anemic rats.

Table 4.3.4 (a):

Effect of iron supplementation on the hemoglobin levels of female wistar rats

Group	Day 1	Day 15	Day 30	Day 45	Day 60
Control I	12.17 ± 0.35^{ab}	12.67 ± 0.42^{a}	12.93 ± 0.64^{a}	13.23 ± 0.64^{cde}	13.50 ± 0.61^{bc}
Control II	9.43 ± 0.60^{c}	9.73 ± 0.64^{c}	10.03 ± 0.72^{d}	10.37 ± 0.67^{f}	10.83 ± 0.72^{d}
Treatment group IA	12.35 ± 0.48^{ab}	12.77 ± 0.42^{a}	13.57 ± 0.48^{a}	14.20 ± 0.36^{a}	14.35 ± 0.32^{a}
Treatment group IB	11.93 ± 0.45^{a}	12.57 ± 0.38^{a}	13.40 ± 0.37^{a}	14.03 ± 0.29^{abc}	14.47 ± 0.34^{a}
Treatment group IC	12.78 ± 0.95^{b}	13.22 ± 0.86^{a}	13.53 ± 0.88^{a}	13.88 ± 0.78^{ab}	14.03 ± 0.72^{bc}
Treatment group IIA	8.84 ± 0.46^{c}	11.05 ± 0.37^{b}	12.00 ± 0.35^{b}	12.93 ± 0.21^{bcd}	13.17 ± 0.16^{bc}
Treatment group IIB	9.13 ± 0.64^{c}	11.27 ± 0.55^{b}	12.20 ± 0.42^{b}	13.45 ± 0.34^{de}	13.88 ± 0.37^{c}
Treatment group IIC	8.07 ± 0.22^{d}	10.20 ± 0.45^{c}	11.12 ± 0.26^{c}	12.60 ± 0.63^{e}	13.02 ± 0.43^{c}

Significance at $p < 0.05$

Note: All values are expressed as Mean ± SD (n=2). Values with similar superscripts in column do not differ significantly ($p<0.05$) according to Duncan's Multiple Range Test. IA- Normal rats on high dose- 150mg/kg, IB- Normal rats on medium dose- 100mg/kg, IC- Normal rats on low dose- 50mg/kg, IIA- Anaemic rats on high dose- 150mg/kg , IIB- Anaemic rats on medium dose- 100mg/kg ,IIC- Anaemic rats on low dose- 50mg/kg

Table 4.3.4 (b):

Effect of iron supplementation on the hemoglobin levels of female wistar rats

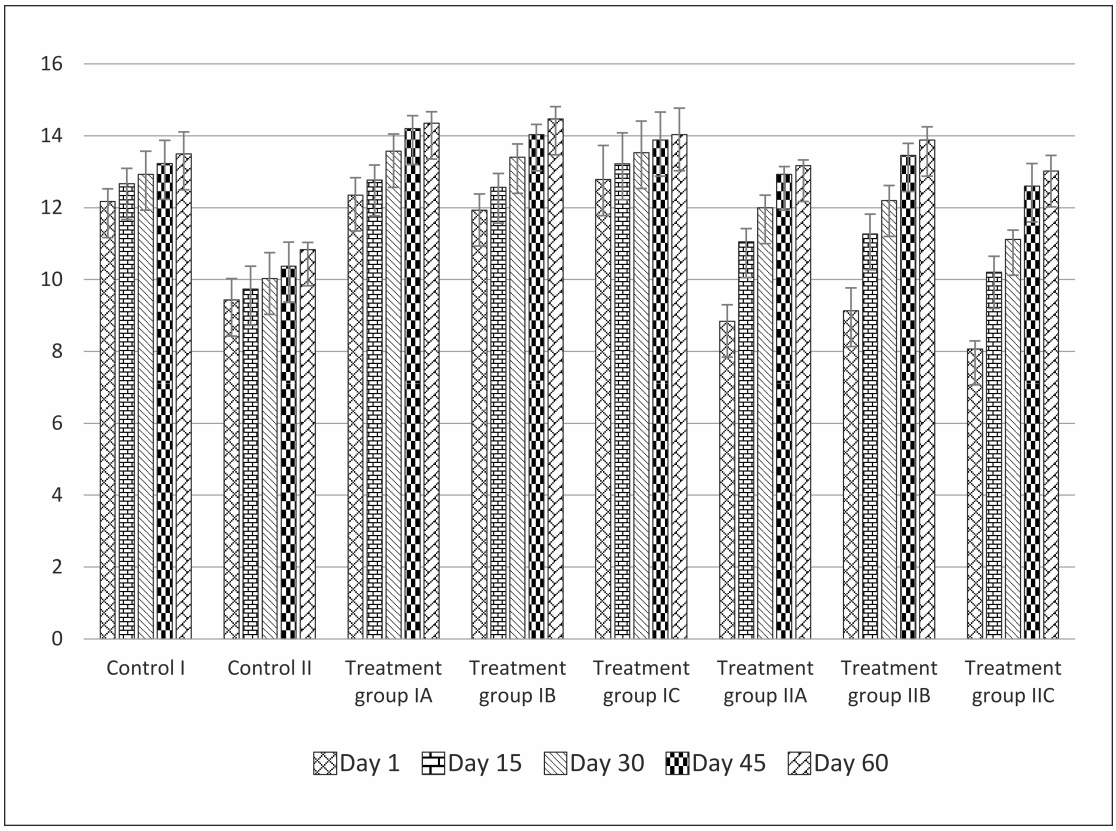
Group	Day 1	Day 15	Day 30	Day 45	Day 60
Control I	12.17 ± 0.35^{b}	12.67 ± 0.42^{ab}	12.93 ± 0.64^{ab}	13.23 ± 0.64^{a}	13.50 ± 0.61^{a}
Control II	9.43 ± 0.60^{b}	9.73 ± 0.64^{ab}	10.03 ± 0.72^{ab}	10.37 ± 0.67^{ab}	10.83 ± 0.72^{a}
Treatment group IA	12.35 ± 0.48^{c}	12.77 ± 0.42^{c}	13.57 ± 0.48^{b}	14.20 ± 0.36^{a}	14.35 ± 0.32^{a}
Treatment group IB	11.93 ± 0.45^{d}	12.57 ± 0.38^{c}	13.40 ± 0.37^{b}	14.03 ± 0.29^{a}	14.47 ± 0.34^{a}
Treatment group IC	12.78 ± 0.95^{b}	13.22 ± 0.86^{ab}	13.53 ± 0.88^{ab}	13.88 ± 0.78^{a}	14.03 ± 0.72^{a}
Treatment group IIA	8.84 ± 0.46^{d}	11.05 ± 0.37^{b}	12.00 ± 0.35^{c}	12.93 ± 0.21^{a}	13.17 ± 0.16^{a}
Treatment group IIB	9.13 ± 0.64^{d}	11.27 ± 0.55^{b}	12.20 ± 0.42^{c}	13.45 ± 0.34^{a}	13.88 ± 0.37^{a}
Treatment group IIC	8.07 ± 0.22^{d}	10.20 ± 0.45^{b}	11.12 ± 0.26^{c}	12.60 ± 0.63^{a}	13.02 ± 0.43^{a}

Significance at $p < 0.05$

Note: All values are expressed as Mean ± SD (n=2). Values with similar superscripts in rows do not differ significantly ($p<0.05$) according to Duncan's Multiple Range Test. IA- Normal rats on high dose- 150mg/kg, IB- Normal rats on medium dose- 100mg/kg, IC- Normal rats on low dose- 50mg/kg, IIA- Anaemic rats on high dose- 150mg/kg , IIB- Anaemic rats on medium dose- 100mg/kg ,IIC- Anaemic rats on low dose- 50mg/kg

Figure 4.3.3 :

Effect of iron supplementation on the hemoglobin levels of female wistar rats



A difference was seen amongst the hemoglobin levels of normal and anemic rats which was statistically significant. In case of control groups for the normal rats fed the basal diet, an increase of 10% was observed in the hemoglobin levels. In contrast, almost 14 % increase in hemoglobin levels was observed for normal rats fed supplements.

Similarly, the increase in hemoglobin levels of anemic rats fed basal diet was 13% whereas anemic rats fed high, medium and low dose supplement showed an increase by 33, 34 and 38% respectively. This increase could also be attributed to higher supplement consumption by the anemic rats in comparison to the normal rats. Significant difference was recorded in the hemoglobin levels between day 1, 15, 30, 45 and 60 for all the groups. On further post hoc analysis it was observed that all anemic groups showed significant difference in their hemoglobin levels as compared to the normal rats. Better absorption of iron is observed in the anemic groups due to the low body stores of iron and physiological status.

In our study half group of rats selected were made anemic where it is found that hematologic indexes of anemic rats was lower in accordance to normal ones as observed by Mondupe et al. (2018) as well. This result is also in consonance with the study of Nagababu et al. (2009) where hemoglobin levels of rat were less when kept on iron deficient diet for around 5 to 9 weeks. In a study it was seen that mean hemoglobin levels were even lower that 10g/dl when fed iron deficient diet for 10 to 15 days (Susanti et al. 2018). According to Hamlin & Dada (2011), when rats are given iron rich, ferrous sulphide, an increase of 38.9% hemoglobin can be seen compared to anemic ones who were given iron deficient diet. Significant elevation in hemoglobin levels can be seen in normal and anemic rats when provided with raw greens for 60 days (Akindele et al. 2018) . This finding is also consistent with our research. Within one month of beginning iron supplementation, the haemoglobin concentration normally rises by at least 1 g/dL. (Morris et al. 2010). Increase in hemoglobin through feeding is not only limited to rat study, but studies also proved that on using cauliflower greens, adolescent girls body show elevation in hemoglobin levels as the leaf concentrate shows maximum bio availability and acceptance amongst the subjects. (Kumar & Bhavani 2004). It can thus be concluded that supplementation with cauliflower greens showed a significant increase in hemoglobin levels of anemic rats (33-38%) and normal rats (10%) and can be further consumed as a supplement for all age groups and physiological conditions.

Table 4.3.5 (a):

Effect of iron supplementation on Red Blood Cell Count of female wistar rats

Group	Red blood cell count x10^6 μl^{-1} Day 20	Red blood cell count x10^6 μl^{-1} Day 40	Red blood cell count x10^6 μl^{-1} Day 60
Control I	7.07±0.31^{ab}	7.17±0.35^{b}	7.50±0.10^{b}
Control II	5.67±0.23^{c}	5.93±0.31^{c}	6.30±0.26^{c}
Treatment group IA	6.75±0.30^{b}	7.42±0.21^{ab}	7.82±0.21^{ab}
Treatment group IB	7.12±0.22^{a}	7.65±0.30^{a}	8.05±0.27^{a}
Treatment group IC	6.75±0.38^{b}	7.45±0.29^{ab}	7.83±0.35^{ab}
Treatment group IIA	4.17±0.16^{de}	5.03±0.25^{d}	5.55±0.22^{d}
Treatment group IIB	3.91±0.23^{e}	5.17±0.20^{d}	5.67±0.27^{d}
Treatment group IIC	4.43±0.16^{d}	5.18±0.23^{d}	5.60±0.17^{d}

Significance at $p < 0.05$

Note: All values are expressed as Mean ± SD (n=2). Values with similar superscripts in column do not differ significantly ($p<0.05$) according to Duncan's Multiple Range Test. IA- Normal rats on high dose- 150mg/kg, IB- Normal rats on medium dose- 100mg/kg, IC- Normal rats on low dose- 50mg/kg, IIA- Anaemic rats on high dose- 150mg/kg , IIB- Anaemic rats on medium dose- 100mg/kg ,IIC- Anaemic rats on low dose- 50mg/kg

Table 4.3.5 (b):

Effect of iron supplementation on Red Blood Cell Count of female wistar rats

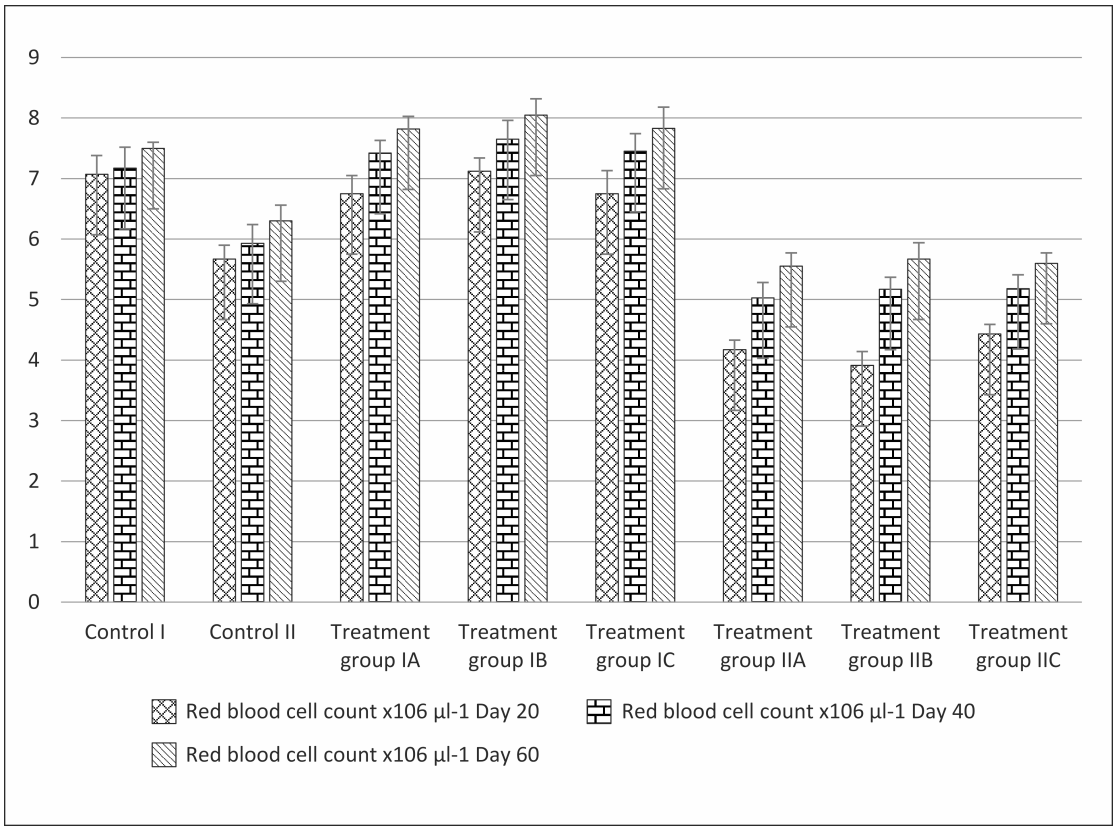
Group	Red blood cell count $x10^6$ μl^{-1} Day 20	Red blood cell count $x10^6$ μl^{-1} Day 40	Red blood cell count $x10^6$ μl^{-1} Day 60
Control I	7.07 ± 0.31^{a}	7.17 ± 0.35^{a}	7.50 ± 0.10^{a}
Control II	5.67 ± 0.23^{b}	5.93 ± 0.31^{ab}	6.30 ± 0.26^{a}
Treatment group IA	6.75 ± 0.30^{c}	7.42 ± 0.21^{b}	7.82 ± 0.21^{a}
Treatment group IB	7.12 ± 0.22^{c}	7.65 ± 0.30^{b}	8.05 ± 0.27^{a}
Treatment group IC	6.75 ± 0.38^{b}	7.45 ± 0.29^{a}	7.83 ± 0.35^{a}
Treatment group IIA	4.17 ± 0.16^{c}	5.03 ± 0.25^{b}	5.55 ± 0.22^{a}
Treatment group IIB	3.91 ± 0.23^{c}	5.17 ± 0.20^{b}	5.67 ± 0.27^{a}
Treatment group IIC	4.43 ± 0.16^{c}	5.18 ± 0.23^{b}	5.60 ± 0.17^{a}

Significance at $p < 0.05$

Note: All values are expressed as Mean ± SD (n=2). Values with similar superscripts in column do not differ significantly ($p<0.05$) according to Duncan's Multiple Range Test. IA- Normal rats on high dose- 150mg/kg, IB- Normal rats on medium dose- 100mg/kg, IC- Normal rats on low dose- 50mg/kg, IIA- Anaemic rats on high dose- 150mg/kg , IIB- Anaemic rats on medium dose- 100mg/kg ,IIC- Anaemic rats on low dose- 50mg/kg

Figure 4.3.4:

Effect of iron supplementation on Red Blood Cell Count of female wistar rats



Similar increase was observed in the red blood cell count of the groups in accordance with the hemoglobin levels. All the treatment groups showed a statistically significant increase in the red blood cell count at the end of day 60. In normal rats fed the high dose supplement, an increase was seen from 6.75 to 7.82 $x10^6$ $\mu l^{-}1$, whereas in anemic rats fed the high dose supplement, the increase was seen to be from 4.17 to 5.55 $x10^6$ $\mu l^{-}1$. The difference between RBC levels amongst the anemic treatment groups was statistically insignificant, but there was a statistically significant difference in the RBC levels found between the normal and anemic groups. We can observe in our study that increase in iron results in increase of RBC. Irrespective of any amount given, supplementation with greens help in increase of RBC, which in our study is cauliflower green and as also proved by using broccoli in study by Avula et al. (2015). In a similar research work by Hong et al. (2019), the results showed that high dose exerted a significant impact on anemic iron by taking RBC level to normal, proving that more the iron content, higher the red blood cell count will be achieved.

Table 4.3.6 :

Effect of iron supplementation on Locomotor Activity of female wistar rats

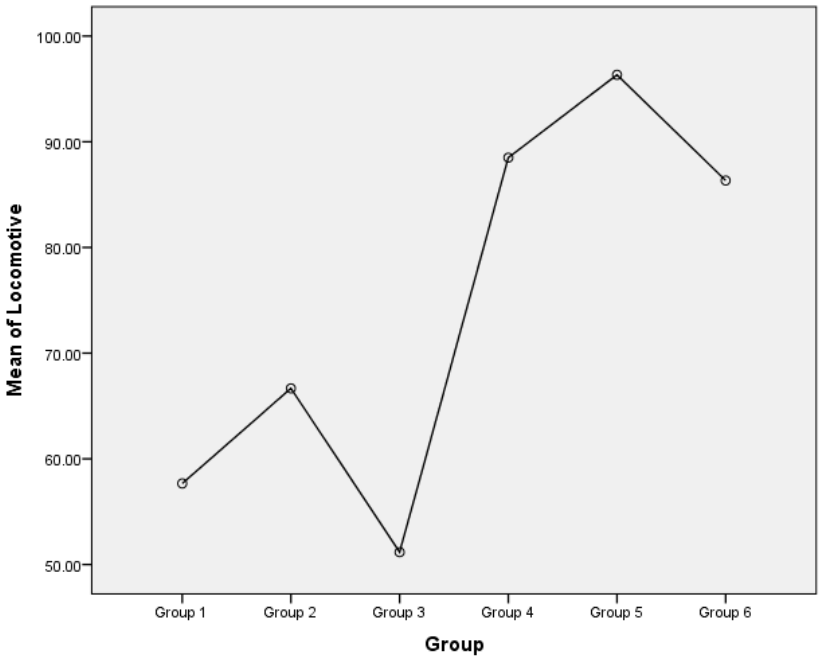
Group	Photocell counts/animal	Average photocell counts (5min)	p-value
Treatment group IA	57.67	58.5±7.78	0.004**
Treatment group IB	67.67		
Treatment group IC	51.17		
Treatment group IIA	88.50	90.39±5.26	
Treatment group IIB	96.33		
Treatment group IIC	86.33		

**Significant at *level p<0.01*

Note: All values are expressed as Mean ± SD (n=2). IA- Normal rats on high dose- 150mg/kg, IB- Normal rats on medium dose- 100mg/kg, IC- Normal rats on low dose- 50mg/kg, IIA- Anaemic rats on high dose- 150mg/kg , IIB- Anaemic rats on medium dose- 100mg/kg ,IIC- Anaemic rats on low dose- 50mg/kg

Figure 4.3.5:

Effect of iron supplementation on Locomotor Activity of female wistar rats



There exists a difference in the mean locomotor activity of all the groups. The treatment groups that were anemic showed a higher level of activity compared to control groups and groups of normal rats fed supplement. There was a significant difference found amongst the normal and anemic rats.

It was observed that treatment group II showed higher locomotor activity when fed the supplement at different doses. Locomotor activity depends on the number of factors one being deprived of iron during perinatal times, this condition leads to reduction in locomotor activity, more rest and less travel distance (Bourque et al. 2008). A slight increase in locomotor activity can be found on consumption of proper amount of iron ($p = 0.033$). Iron deficiency causes poor myelination and monoamine metabolism in the brain. Changes in brain iron status affect glutamate and GABA homeostasis. Memory and learning abilities, as well as motor skills, are harmed as a result of these improvements (Kim & Resnick 2015). Anemic rats showed impairment in running ability compared with normal rats even when their hemoglobin is made equal but when fed with iron for 4 days this disability disappears (Finch et al. 1976). In this study, a higher level of locomotor activity was observed in the treatment groups which were anemic as compared to treatment group fed the supplement but with normal hemoglobin. This could be because of high levels of iron in the diet which is known to reduce brain function (Han & Kim, 2015). Both iron overload and iron depletion decrease motor activity when tested at different time intervals of 14, 17, 20, 27 and 34 days (Pinero et al. 2001).

4.4: Product optimization using response surface methodology and shelf-life study

Process optimization employing the quality by design experimental approach is one of the most successful and versatile approaches to obtain the best possible solution, considering all the factors involved in the study. In this study, response surface methodology (RSM) was used. Throughout the study, design expert version 7 was used to study the factor response relationship as well as for graphical and numerical optimization. Optimization of the factors was done using central composite design. The

data analysis was carried out by quadratic model fitting followed by multiple linear regression analysis.

The generalized equation for the same is as follows:

$$Y1= \beta_0+ \beta_1x_1+ \beta_2x_2+ \beta_3x_3+ \beta_{12}x_{12}+\beta_{13}x_{13}+ \beta_{23}x_{23}+\beta_{13}x_{13}+ \beta_{23}x_{23}+\beta_{11}x_1^2+ \beta_{22}x_2^2 + \beta_{33}x_3^2$$

Where, y is the response, β_{1-2} are the coefficients of linear terms x_1-x_2, β_3 are the coefficients two factor interaction terms and β_{4-5} are the coefficients of quadratic terms for each factor.

The experiment was carried out at three levels low, medium and high. For the sake of simplicity, all the computations and representations have been done in coded terms.

20 different treatments with varying combinations of cauliflower powder, sugar and lemon juice were obtained by using response surface methodology. The combinations were prepared with those 20 treatments were further analyzed for properties which were considered as response variables or dependent variables. The results of analysis of iron content and overall acceptability, the two response variables of 20 treatments are shown. ANOVA was calculated to assess the variance observed between dependant and independant variables. It was observed by analyzing the results that all the model for dependent variables were statistically significant from each other for testing the fitting of the model for different responses like iron content and overall acceptability. The sum of squares of the model was also analyzed by considering the four factors i.e. the linear effect, the regression of the response, the linear effects and the quadratic effects of the responses (X_1, X_2). Model F-value of 21.73 and 24.77 for X_1 and X_2 respectively indicating that the model is significant and this large a value cannot be attributed to noise. The degree of fitness of model was checked by the value of coefficient by determination of r^2. The value of r^2 for all the responses was calculated to be 0.9513(X_1) and 0.9571(X_2), which are satisfactory and showing proper fitting of the model.

Table 4.4.1

Product optimization and overall acceptability of 20 different treatments of RSM

Run	Factor 1 Cauliflower (gm)	Factor 2 Sugar (%)	Factor 3 Lemon (%)	Response 1 Overall acceptability	Response 2 Iron content (mg)
1	30.0	2.0	5.0	6.13	11.62
2	20	8.5	3.25	7.33	9.60
3	20	8.5	5	7.53	8.90
4	10.0	15.5	5.0	7.60	3.62
5	20	8.5	3.25	7.93	8.92
6	20	8.5	3.25	7.86	9.57
7	30	8.5	3.25	7.06	11.05
8	20	8.5	5	6.66	9.20
9	20	8.5	3.25	7.66	8.62
10	10	15	1.5	6.93	3.20
11	20	8.5	3.25	8.20	9.25
12	20	10	3.25	5.73	8.82
13	20	5	3.25	4.60	9.60
14	30	15	1.5	6.60	11.87
15	10	2	1.5	7.13	4.00
16	30	15	5	7.00	11.05
17	35	8.5	3.25	6.13	12.10
18	10	2.0	5.0	6.00	3.60
19	30.0	2.0	1.5	5.66	10.65
20	20	8.5	3.25	8.33	9.15

ANOVA for quadratic model:

Second order polynomial equation and model was fitted to the data under experimentation, and coefficients of variance (r2) was used in second order polynomial equation for all the response variables.

$Y= \beta_0+ \beta_1x_1+ \beta_2x_2+ \beta_3x_1x_2+ \beta_4x_1^2+\beta_5x_2$

Table 4.4.2:

Analysis of variance for response surface quadratic model for factors

	Overall sensory acceptability X1	**Iron content X2**
Intercept	7.25	11.25
A	-0.95	+3.81
B	+0.55	+0.047
C	+0.28	+0.081
AB	-0.019	+0.13
AC	0.14	+0.22
BC	0.28	-0.067
A^2	-0.61	-2.50
B^2	-0.67	-0.38
C^2	-0.094	-0.64
F value	21.73	24.77
R2	0.95	0.95

Effect of independent variables on response variables:

The 3D graphs for response variable indicated that all the independent variables significantly affect all the response variables under study. It was observed that with the increase or decrease in the amount of cauliflower powder, sugar and lemon juice, a significant difference was observed on the responses. This has been represented as follows:

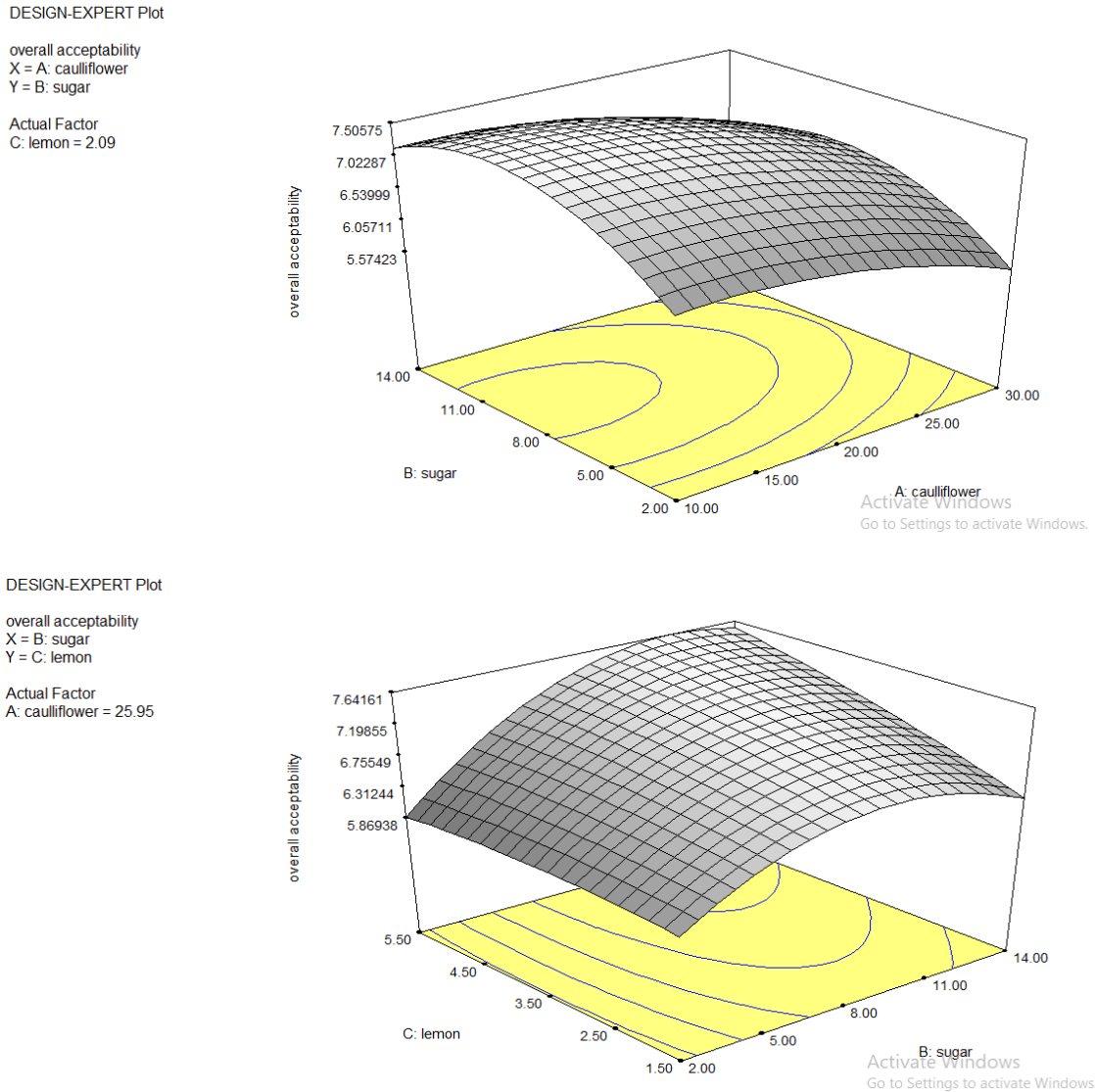
Overall Acceptability:

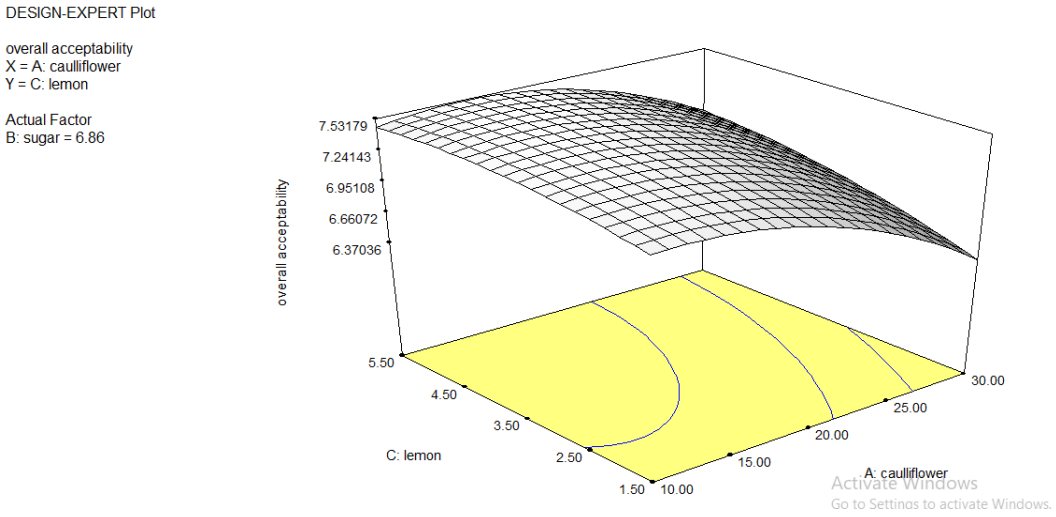
The mathematical expression showing the second order polynomial equation by analyzing the data for all the 20 treatments of RSM for overall acceptability

$7.25-0.95\beta_1 + 0.55\ \beta_2 + 0.28\ \beta_3 - 0.019\ \beta_{12} + 0.14\ \beta_{13} + 0.28\ \beta_{23} - 0.61\ \beta_1^2 - 0.67\ \beta_2^2 - 0.094\ \beta_3^3$

Figure 4.4.1:

Effect of response variables on overall acceptability





The response surface curves were plotted to analyse the optimum level of each variable for highest response and to understand the interaction between the variables. The 3D graphs for overall acceptability were generated from response surface methodology software. It can be clearly seen from 3D graph that sugar concentration is having positive contribution toward the acceptability of the product. In other words, overall suitability increases with increase in sugar concentration up to middle level. The increase in sugar concentration after a level will show a negative effect and the overall acceptability will decrease. While the other factor is cauliflower leaves powder which is also showing the similar effect. This graph indicates that an increase in overall acceptability was significantly achieved with an increase in sugar concentration and cauliflower leaves powder. However, it will give negative effect when these independent variables exceeded certain values. In a research by Mansour et al. (2013), it was observed that increase in sugar concentration had a positive effect on all the sensory parameters of the Roselle beverage. Yang et al. (2014) optimized the sugar content in yogurts and observed that an increase in the overall acceptability was observed with increase in sugar content but after a level it showed a negative effect. Findings on use of cauliflower greens by Chauhan et al (2014) observed that cauliflower leaves increased the sensory profile of the product up to a certain level, beyond which it has a negative effect.

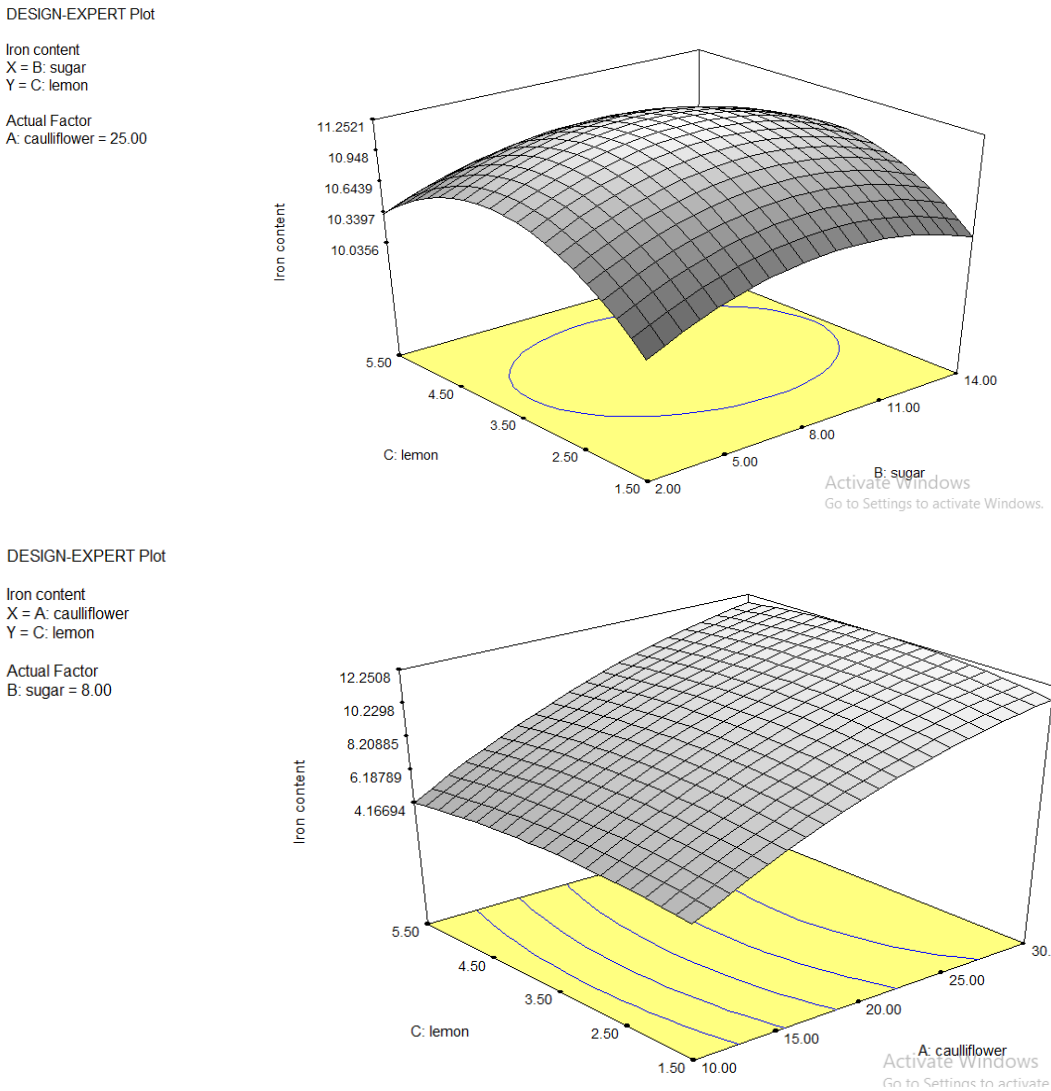
Iron Content

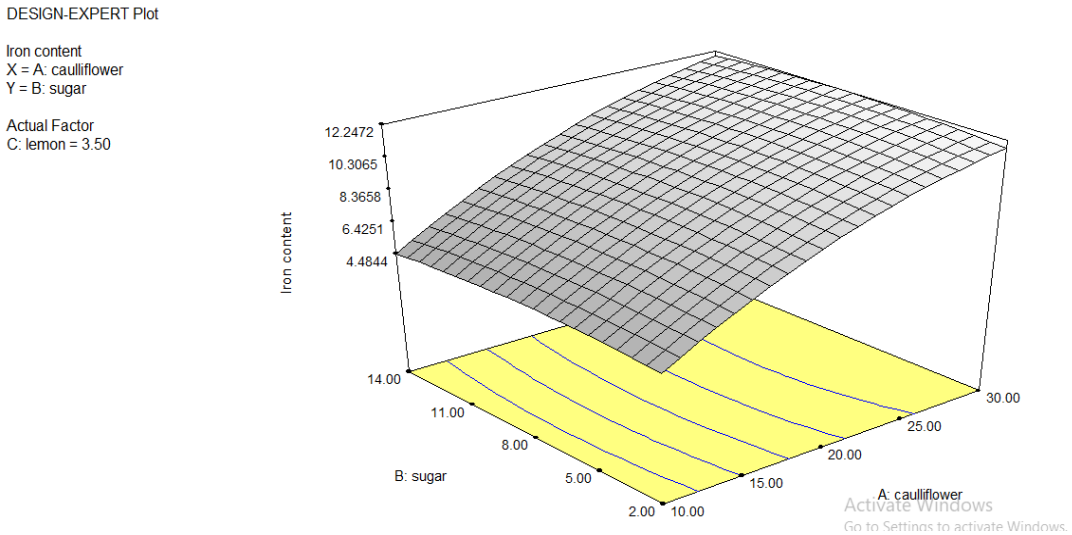
The mathematical expression showing the second order polynomial equation by analyzing the data for all the 20 treatments of RSM for iron content

$$11.25 + 3.81\beta_1 + 0.047\ \beta_2 + 0.081\ \beta_3 + 0.13\ \beta_{12} + 0.22\ \beta_{13} - 0.067\ \beta_{23} - 2.50\beta_1^2 - 0.38\ \beta_2^2 - 0.64\beta_3^3$$

Figure 4.4.2:

Effect of response variables on iron content





The 3D graphs for iron content were generated from response surface methodology software. It can be seen from the 3D graph that cauliflower powder is having a positive contribution towards the iron content of the product. In other words, iron content increases with an increase in cauliflower powder up to middle level. While the other factor is sugar which is also showing the similar effect. This graph indicates that an increase in iron content were significantly achieved with increase in cauliflower leaves powder. However, it will give negative effect when these independent variables exceeded certain values. In similar studies conducted, it was observed that the use of cauliflower greens in food products increases their iron content. Singh et al. (2019) studied the effects of adding cauliflower leaves to the diet and increase in hemoglobin levels was observed. In another study by Kaviyarasi & Abiram (2017) it was observed that adding up to 50g, cauliflower leaves powder in recipes increased the iron content.

Further keeping the above analysis in mind, product development was done with the following formulation:

Cauliflower Leaves Dried – 20gm

Sugar – 8.5gm

Lemon juice – 3.25ml

Table 4.4.3:

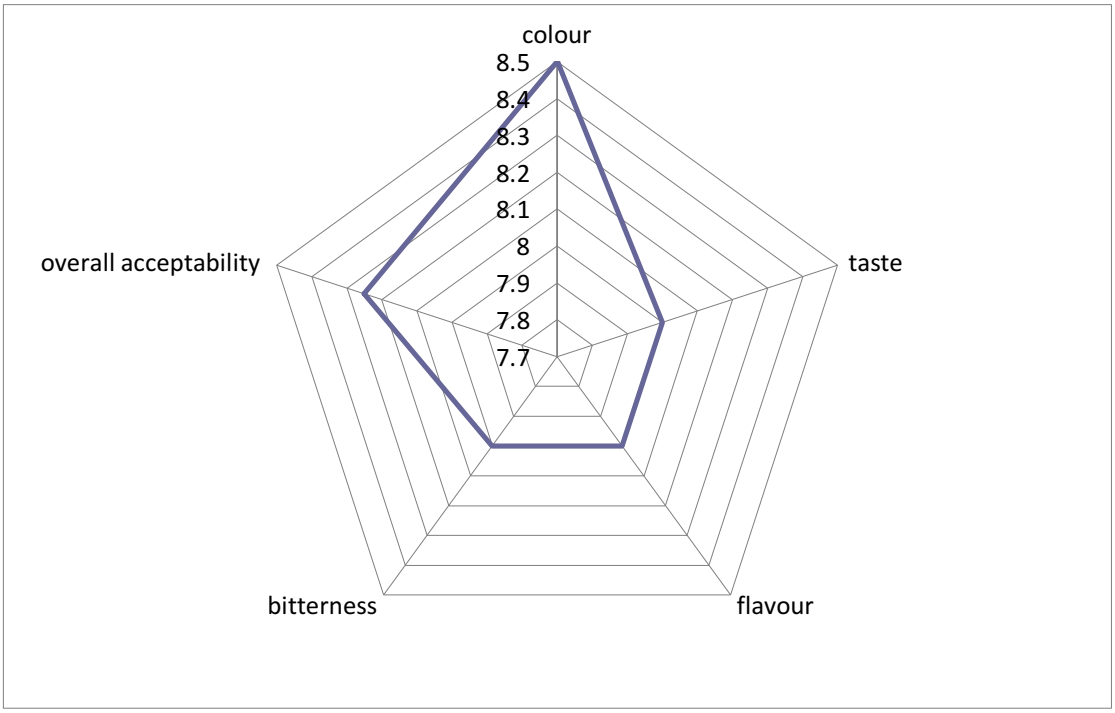
Sensory properties of the freshly prepared beverage from cauliflower leaves

Parameters	**Values**
Colour	8.50±0.04
Flavour	8.00±0.01
Taste	8.00±0.01
Bitterness	8.00±0.03
Overall Acceptability	8.25±0.04

Note: All values are expressed as Mean ± SD (n=2)

Figure 4.4.3:

Sensory properties of the freshly prepared beverage



The beverage was evaluated for sensory evaluation by a semi trained panel of 10. The above table represents the overall acceptability of the beverage which was found to be 8.25 and can be considered highly acceptable. In similar studies by Mann et al. (2015) it was observed that highest overall acceptability of 8 was found amongst all the whey

protein beverages tested, which is in accordance with our study. Kamate & Padghan (2018) conducted a study on organoleptic properties of a beetroot whey beverage and observed a score of 8.51 as highest in terms of overall acceptability.

Table 4.4.4 (a):

Effect of storage on physico chemical and overall acceptability of the prepared beverage under ambient conditions (22±3°c)

Days	Iron (mg)	Vitamin C (mg)	pH	TSS (◦Brix)	Titratable Acidity (%)	Overall Acceptabilty
0	8.93±0.04^{a}	12.40±0.14^{a}	3.58±0.01^{b}	13.68±0.11^{a}	0.40±0.01^{a}	8.25±0.04^{a}
5th	8.92±0.02^{ab}	11.05±0.05^{b}	3.64±0.02^{b}	13.88±0.03^{a}	0.39±0.01^{a}	6.00±0.03^{b}
7th	8.85±0.07^{b}	10.78±0.04^{c}	3.67±0.02^{a}	14.00±0.01^{a}	0.34±0.02^{b}	5.75±0.03^{c}

Significant difference *(p<0.05)*

Note: All values are expressed as MEAN ±SD (n=2). Values with similar superscripts in columns do not differ significantly according to Duncan's Multiple Range Test

Table 4.4.4 (b):

Effect of storage on physico chemical properties and overall acceptability of the prepared beverage under refrigerated conditions (4°c)

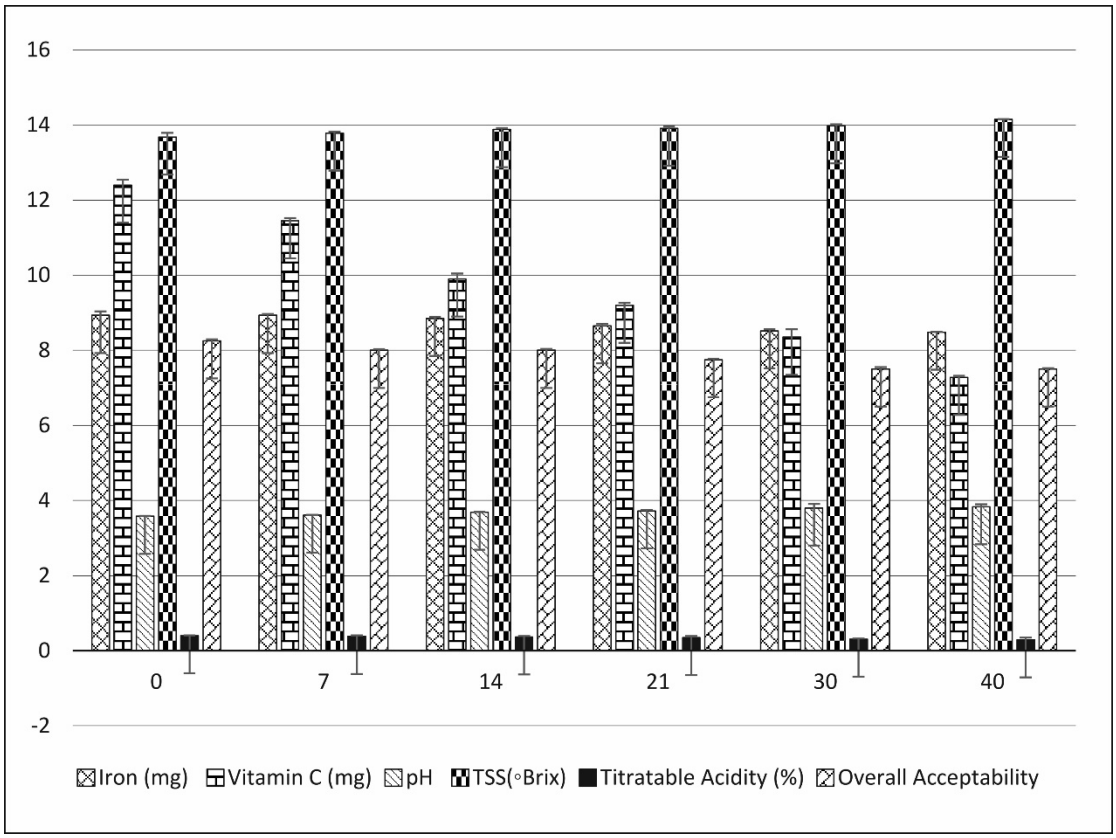
Days	Iron (mg)	Vitamin C (mg)	pH	TSS (◦Brix)	Titratable Acidity (%)	Overall Acceptabilty
0	8.93±0.04^{a}	12.40±0.14^{a}	3.58±0.01^{b}	13.68±0.11^{a}	0.40±0.01^{a}	8.25±0.04^{a}
7th	8.93±0.04^{a}	11.45±0.07^{b}	3.61±0.01^{b}	13.78±0.04^{a}	0.38±0.03^{ab}	8.00±0.03^{b}
14th	8.85±0.07^{a}	9.90±0.14^{c}	3.69±0.01^{ab}	13.88±0.04^{a}	0.37±0.02^{ab}	8.00±0.04^{b}
21st	8.65±0.05^{a}	9.20±0.07^{c}	3.72±0.03^{ab}	13.91±0.05^{a}	0.35±0.04^{ab}	7.75±0.02^{b}
30th	8.52±0.04^{b}	8.35±0.21^{d}	3.80±0.11^{a}	13.98±0.04^{a}	0.31±0.01^{b}	7.50±0.05^{c}
40th	8.48±0.04^{b}	7.28±0.04^{e}	3.83±0.07^{a}	14.15±0.01^{a}	0.29±0.06^{b}	7.50±0.03^{c}

Significant difference *(p<0.05)*

Note: All values are expressed as MEAN ±SD (n=2). Values with similar superscripts in columns do not differ significantly according to Duncan's Multiple Range Test

Figure 4.4.4

Effect of refrigerated storage on physico chemical and sensory properties of the prepared beverage



The beverage provides 8.93mg iron which is in accordance with the minimum requirement of iron for a beverage to be classified as iron rich FSSAI (2000). Similar studies conducted by Thompson & Amoroso (2014), Wani et al. (2011) and Singh et al (2005) showed that adding dried cauliflower greens to a food product increases its iron content and in turn the hemoglobin values. Being rich in lemon juice, the beverage was found to contain sufficient quantity of vitamin C. In studies conducted by Navarro et al. (2020), Marti et al. (2009) and Sinir et al. (2017) the use of lemon in beverages elevated the ascorbic acid content. The pH of the drink was found to be 3.58. All beverages containing lemon juice are considered acidic and have a low pH. Tenuta et al. (2015) developed citrus containing beverages and assesed its pH and acidity which was found to be 3.5 and 0.57 respectively. This is in accordance to our study as well. Where in the pH was found to be 3.58 and titratable acidity 0.41 which can be attributed to use of lemon juice as well as cauliflower greens, both being rich sources of vitamin

C. In a study by Heena et al. (2017) developed a beverage using cucumber reported TSS ranging from 9.00–15.00 °Brix, acidity ranging from 0.22–1.30% and pH from 2.96–5.30.

The above table also represents physico chemical and sensory properties of the beverage on being stored. The iron content of the beverage showed no statistical difference both at ambient and refrigerated temperatures. It decreased from 8.93mg to 8.48mg after a storage of 40 days. Shaukat et al. (2020) analysed the use of iron fortified beverage in preventing anemia. The ascorbic acid content of the beverage significantly reduced after storage at ambient temperature after 7 days from 12.40 to 10.78mg and also in the case of refrigerated sample from 12.40 to 7.28mg. The change was insignificant in the pH of the beverage stored at room as well as refrigerated temperature. It increased from 3.58 to 3.67 and 3.83 respectively. This can be attributed to the conversion of poly saccharides into mono saccharides which causes an increase in sweetness and thereby the sourness decreases. A slight increase in TSS can be attributed to the same reason as increase in pH. The TSS increased from 13.68 to 14 and 14.5 at ambient and refrigerated temperatures respectively. A decrease in acidity (0.4-0.29%) and a decrease in the overall acceptability (8.25 to 7.5) of the beverage was seen after storage of 40 days at 4 degrees celsius. This could be attributed to the change in colour and flavor over a period of time. The change in mouthfeel or bitterness could be due to the increase in pH and decrease in acidity of the beverage. Further Kilima et al. (2014) found an increase in the total soluble solids and pH, and decrease in the titratable acidity with increase in storage time. Ascorbic acid was also found to decrease significantly. This is in alignment with our study where in an increase was observed in the pH and TSS content of the beverage, whereas the acidity and vitamin C content was reduced. Similar changes were also observed by Islam et al. (2013). In another study by Singh et al. (2014) similar physico chemical and sensory parameters were assessed for a ready to serve guava beverage. Results showed that minimum changes were observed in the TSS, whereas the sensory acceptability reduced with increased storage period.

Table 4.4.5 (a):

Effect of storage on microbial properties of the prepared beverage under ambient conditions (22±3°c)

Days	Standard Plate Count (Log CFU/ml)	Yeast &Mould Count (Log CFU/ml)
0	1.68±0.49[b]	1.85±0.14[b]
5th	2.79±0.35[ab]	2.92±0.21[a]
7th	3.02±0.31[a]	3.15±0.04[a]

Significant difference *(p<0.05)*

Note: All values are expressed as MEAN ±SD (n=2). Values with similar superscripts in columns do not differ significantly according to Duncan's Multiple Range Test

Table 4.4.5 (b):

Effect of storage on microbial properties of the prepared beverage under refrigerated conditions (4°c)

Days	Standard Plate Count (Log CFU/ml)	Yeast &Mould Count (Log CFU/ml)
0	1.68±0.49[b]	1.85±0.14 [b]
7th	1.78±0.42[b]	1.90±0.28 [b]
14th	2.92±0.35 [a]	2.06±0.06[b]
21st	2.95±0.21[a]	2.45±0.05[b]
30th	3.06±0.21[a]	3.12±0.04[a]
40th	3.10±0.07[a]	3.19±0.07[a]

Significant difference *(p<0.05)*

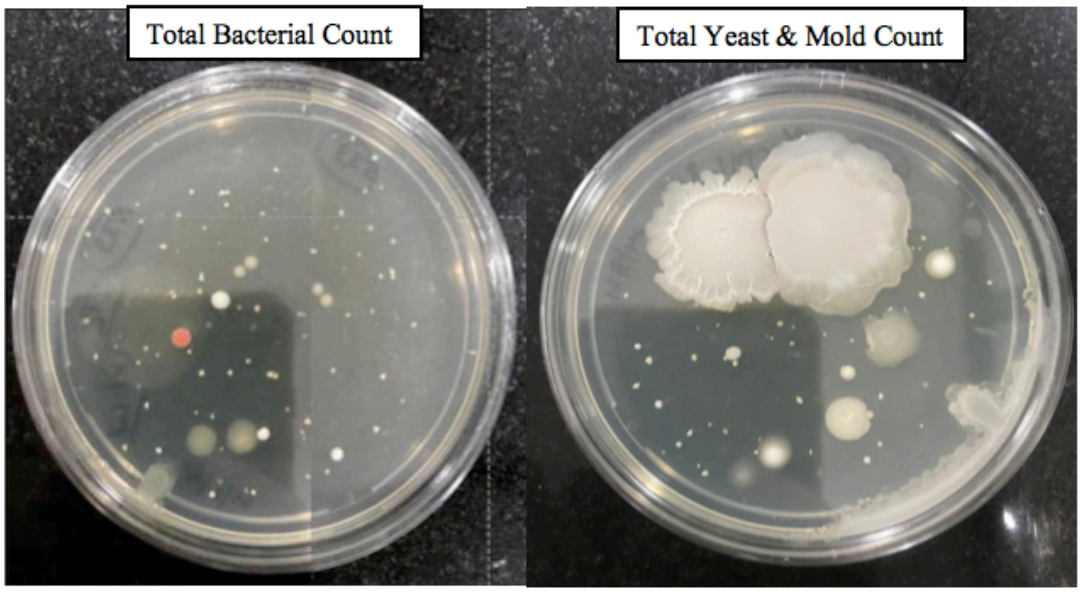
Note: All values are expressed as MEAN ±SD (n=2). Values with similar superscripts in columns do not differ significantly according to Duncan's Multiple Range Test

The microbial estimation of the effect of storage of beverage has been represented in Table 4.4.5. It was observed that the yeast and mold count shows higher growth as compared to the bacterial count. The total bacterial count in the present study was found to be 3.10±0.07Log CFU/mlby the end of storage period. TYMC was found to be a little higher 3.19±0.07Log CFU/mlwhich could be due to presence of sugar, acid and low temperature. Further post hoc analysis shows that a statistically significant difference was observed between the bacterial and yeast count of the sample stored at room temperature between day 0, day 5 and day 7. In case of refrigerated sample, the

total plate count was found to be significant between day 14, day 30 and 40. The counts were still found to be on the lower side. This could be attributed to the low temperature during storage and low pH of the beverage. A study by Shaukat et al. (2020) revealed a small hike in the microbial load, which could be attributed to the use of pulp of fruit. Results on the same line were noted by Alane et al. (2017) wherein they observed the storage stability of a whey based mango beverage. Furthermore, Khan et al. (2015) studied the microbiological quality of some drinks, wherein it was observed that the total viable count ranged from $7.7X10^3$ cfu/ml for the lemon based drink. This is due to the low pH of the drinks. Similarly in our study, a low bacterial count was observed, which could be attributed to the low pH. In another study by Helal et al. (2018) low counts were observed in fruit juices with low pH due to a restriction in the number and type of organisms that can grow in an acidic medium (Prescott et al. 1999). Similar results were observed for yeast and mold count in ambient and refrigerated sample. Significant difference was found between microbial load in storage period of day 0 and day 7 for ambient temperatures and day 30 and 40 for refrigerated sample. Also, Azeredo et al. (2016) found that in acidic beverages yeast grows at a faster rate as compared to bacteria as the latter are inhibited by low pH and presence of preservatives.

Plate 1 & 2:

Total Plate Count and Yeast and Mold Count



CHAPTER 5

SUMMARY AND CONCLUSION

The present study was undertaken to help bridge the gap between the prevalence of anemia which is a public health problem in India and availability of a low- cost iron rich food supplement. Socio economic and demographic factors were analyzed in the survey and their effect on anemia was reported. No significant difference was observed between the prevalence of anemia in rural or urban population. It was found to be 50.4% amongst the rural pregnant women and 44.5% amongst women belonging to urban areas of Chandigarh. Considering the high prevalence, especially amongst the rural population, further screening of low-cost green leafy vegetables rich in iron was done. Cauliflower leaves, radish leaves, amaranth leaves, lettuce were chosen along with spinach as most of these greens are underutilized and are rich in iron. Standardization of blanching time and temperature combination for dip as well as microwave blanching was found to be 90°C for 3 minutes and 1 minute at 900W for dip and microwave blanching respectively for complete inactivation of peroxidase enzyme. Further more, the effect of blanching and tray drying on the phytochemical and iron content of the vegetables showed that as drying progressed the ascorbic acid content of fresh cauliflower decreased fom 63.46mg to 28.05mg in Amaranth, from 44.05mg to 19.45mg in cauliflower leaves, 65.76mg to 32.61mg in radish leaves, 12.45mg to 7.76mg in radish leaves and 35.45mg to 18.65mg in spinach. Like ascorbic acid, significant reduction of beta carotene was observed after blanching and drying and it ranged from 2.42mg to 1.08mg in cauliflower leaves, 2.49mg to 1.04mg in radish leaves, 2.78mg to 1.11mg in spinach, 1.36mg to 0.61mg in lettuce and 2.82mg to 0.98 mg in amaranth. Iron content of the raw and tray dried samples was also analysed. Cauliflower leaves had maximum iron content (39.25mg) followed by amaranth (16.91mg) and radish leaves (16.75mg) respectively. Cauliflower leaves and radish leaves had the maximum total phenol content which significantly increased after drying from 53.47mg to 210.60mg and 38.52mg to 253.94mg respectively. Spinach reported a total phenol content of 10.76mg which increased to 71.78mg on blanching and drying. Lettuce and amaranth had a phenol content of 19.17mg and 34.60mg which increased to 158mg and 135.72mg respectively on drying.

Further, the anti-nutrient content of all the green leafy vegetables was analyzed for raw as well as dried leaves to help complete the screening process. It was observed that though cauliflower leaves had the highest content of alkaloids(72.81mg) and oxalate(97.78mg), which reduced significantly on drying. Thus keeping all the screening methods in mind, cauliflower greens were selected for further study. Considering that green leafy vegetables are limited sources of iron as it is present in the form of heme iron, which has low bioavailability, an in vivo study was conducted to assess the absorption of iron and its effects on the hemoglobin levels in rats. Parameters such as amount of supplement consumed, weight of the animals, hemoglobin levels, red blood cell count, fecal output and locomotor activity was studied over a period of 2 months. At the end of the intervention period, a significant increase was observed in all the treatment groups in terms of their weight and hemoglobin levels. Treatment group II containing anemic rats fed the supplement showed the maximum increase in weight (15%) , hemoglobin (32-38%), and red blood cell count after 60 days. The maximum iron absorption was seen in the high dose groups with as much as 57.53% of iron being absorbed after considering the faecal output. Locomotor activity was also higher in the anemic groups showing that cauliflower greens is able to not just increase the hemoglobin content, but support growth as well.

With a range of iron absorption from 24.64-57.53%, an average mean was estimated and used as a reference for further product development. As per guidelines issued by FSSAI, for a beverage to be classified as iron rich, it must provide 15% of RDA/100ml. Keeping these factors in mind, a minimum level of 10-30gms must be present in the product to ensure minimum iron requirements for all age groups. Response surface methodology was used as a tool to optimize the content of the beverage. Independent variables such as cauliflower powder, lemon juice and sugar were used and their effect on response variables such as iron content and overall sensory acceptability was studied. 20 combinations of treatments were given and concentration of 20gm cauliflower greens powder, 8.5gm sugar and 3.25ml lemon juice was considered optimum. Physicochemical and sensory analysis of the beverage revealed a high iron content (8.93mg/100ml) overall acceptability score of 8.25. Storage study of the product revealed a shelf life of 40 days at refrigerated temperatures.

Thus the aim of the study to prepare a low-costiron-rich beverage to address the growing concerns of anemia was achieved. Further scope of the study can be to assess the efficacy of the developed product through clinical trials as well as further use of other processing techniques to improve the quality and shelf-life of the beverage.

CHAPTER 6
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